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**THE MECHANISMS OF BCR SIGNALING (DE)REGULATION
IN MALIGNANT B CELLS**

Dissertation on Oncology and Hematology

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Abstract

Background: Chronic lymphocytic leukemia (CLL) cell recirculation to and from immune niches plays an important role in CLL biology. However, it remains largely unknown how precisely CLL cells regulate their homing or how they balance survival in peripheral blood and the decision to return to immune niches.

Methods: We performed RNA profiling of CXCR4^{dim}CD5^{bright} versus CXCR4^{bright}CD5^{dim} cells and identified differential expression of dozens of molecules with a putative function in CLL cell migration. By series of *in vivo* and *in vitro* experiments we described the role of GRB2 associated binding protein 1 (GAB1) in the migration process and its positive role in the regulation of tonic signaling. We also tested novel GAB1 inhibitors targeting its PH domain.

Results: CXCR4/CD5 subpopulations have been suggested as a model distinguishing CLL cells ready to re-enter immune niches (CXCR4^{bright}CD5^{dim}) versus recent emigrants from lymph nodes (CXCR4^{dim}CD5^{bright}). Indeed, intravenous injection of sorted CXCR4/CD5 subpopulations into NOD scid gamma (NSG) mice revealed increased migration capacity of CXCR4^{bright}CD5^{dim} cells into the spleen. Subsequent RNAseq of sorted CXCR4/CD5 subpopulations identified 147 migration-related differentially expressed genes, among which we noted upregulation of GAB1 (~2-fold) in CXCR4^{bright}CD5^{dim} cells. GAB1-knockout in MEC1 cells or silencing by siRNA in primary CLL cells largely inhibited their migration capacity towards chemokines SDF1, CXCL13, or conditioned media produced by bone marrow stromal cells (CM^{hMSC}). Importantly, GAB1 knockout or silencing led to impaired migration of malignant B cells to the spleen of NSG mice. Treating cells with SDF1, CXCL13, or CM^{hMSC} led to rapid GAB1 phosphorylation, suggesting that GAB1 is involved in response to various soluble cytokines. We have also identified another GAB1 function in the maintenance of “tonic” AKT phosphorylation via PI3K. Next, we demonstrated that transcription factor FoxO1 transcriptionally activates GAB1 by direct binding to its promotor. Altogether, increased FoxO1/GAB1 levels in CXCR4^{bright}CD5^{dim} CLL cells are contributing to balancing CLL cells’ survival and their decision to migrate into the immune niches. Notably, administration of ibrutinib in CLL patients induces FoxO1-GAB1, which leads to recovery of phospho-AKT within several days or weeks on ibrutinib and this can be targeted by novel GAB1 inhibitors.

Conclusion: Here, we described a novel FoxO1/GAB1-dependent regulatory mechanism of CLL homing and maintenance mechanism for “tonic” AKT signaling activity in an intraclonal CXCR4^{bright}CD5^{dim} CLL subpopulation. We showed for the first time that ibrutinib treatment leads to the accumulation of FoxO1/GAB1 proteins which subsequently leads to AKT phosphorylation and activation. We demonstrated that GAB1 inhibitors can be used to inhibit CLL cell migration, basal AKT activity, and adaptive AKT activation during the ibrutinib therapy.

Keywords: Chronic lymphocytic leukemia, GAB1, FoxO1, AKT, protein kinase B, adaptation, BCR inhibitor, ibrutinib

Abstrakt

Úvod: Recirkulace buněk chronické lymfocytární leukemie (CLL) mezi periferní krví a mikroprostředím imunitních buněk hraje důležitou roli v biologii CLL. Do současnosti však nebylo detailně popsáno, jak přesně CLL buňky regulují tyto migrační procesy, jak kontrolují přežití v periferní krvi, případně své rozhodnutí vrátit se zpět do mikroprostředí lymfatických orgánů.

Metody: Provedli jsme RNA sekvenaci CXCR4^{dim}CD5^{bright} a CXCR4^{bright}CD5^{dim} populací, čímž byly identifikovány desítky rozdílně exprimovaných genů hrajících roli v migraci. Následující *in vivo* a *in vitro* experimenty nám umožnily popsat významnou roli GAB1 (GRB2 associated binding protein 1) proteinu v procesu migrace a regulace takzvané tonické signalizace. Také jsme testovali možnost využití GAB1 inhibitorů cílících na jeho PH doménu.

Výsledky: Již dříve byly CXCR4/CD5 subpopulace navrženy jako model umožňující rozlišit mezi buňkami připravenými migrovat do lymfatických orgánů (CXCR4^{bright}CD5^{dim}) a buňkami, které nedávno vycestovaly z tohoto prostředí do periferní krve (CXCR4^{dim}CD5^{bright}). Intravenózní aplikace těchto dvou subpopulací do NOD scid gamma (NSG) myši potvrdila zvýšený migrační potenciál CXCR4^{bright}CD5^{dim} buněk do myší sleziny. Následná sekvenace CXCR4/CD5 subpopulací identifikovala 147 rozdílně exprimovaných genů zapojených do procesu migrace. Jedním z nich byl i GAB1, který byl v CXCR4^{bright}CD5^{dim} buňkách přítomen v přibližně dvojnásobném množství. Vyřazení GAB1 v MEC1 buňkách, případně jeho utišení pomocí siRNA v primárních CLL buňkách, vedlo k potlačení migračního potenciálu vůči chemoatraktantům jako SDF1, CXCL13 případně kondiciovanému médiu produkovanému stromálními buňkami kostní dřeně (CM^{hMSC}). Podobná data jsme získali i v *in vivo* experimentu, kde vyřazení GAB1 významně redukovalo migraci buněk do myší sleziny. Ovlivnění buněk pomocí SDF1, CXCL13 případně CM^{hMSC} vedlo k rychlé aktivaci GAB1, což naznačuje jeho zapojení do rozdílných chemotaktických signálních drah. Také jsme popsali roli GAB1 v udržování takzvané tonické signalizace, která se projevuje nízkou hladinou aktivního AKT. V dalším kroku jsme popsali mechanismus zvýšení produkce GAB1, který je závislý na aktivaci transkripčního faktoru FoxO1. Zvýšené množství FoxO1/GAB1 proteinů tak v CXCR4^{bright}CD5^{dim} buňkách přispívá k lepšímu přežívání CLL buněk v periferní krvi a významně ovlivňuje jejich následnou migraci do mikroprostředí imunitních orgánů. Podání ibrutinibu pacientům s CLL indukuje FoxO1/GAB1, což následně vede k obnově aktivity AKT během několik dnů až týdnů. Ukázali jsme, že tento proces můžeme blokovat GAB1 inhibitory.

Závěr: Popsali jsme nové mechanismy regulace migrace a tonické AKT signalizace, které jsou založené na modulaci FoxO1/GAB1 proteinů. Role těchto proteinů byla dále zkoumána v kontextu terapie ibrutinibem, která indukuje FoxO1/GAB1 dráhu a využívá k opětovné aktivaci AKT. Demonstrovali jsme také, že tyto mechanismy mohou být inhibovány pomocí nových GAB1 inhibitorů.

Klíčová slova: Chronická lymfocytární leukemie, GAB1, FoxO1, AKT, protein kináza B, adaptace, BCR inhibitor, ibrutinib

I hereby declare that this dissertation thesis is my autonomous work, written under the supervision of doc. MUDr. Mgr. Marek Mráz, Ph.D. with the use of the published sources clearly indicated and quoted in the list of literature.

Author's signature

Dedication

This is for you, Mom. Thanks for everything.

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1. Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is a malignant disease characterized by the clonal expansion and accumulation of small, mature-appearing, CD5+ B cells in the blood and lymphoid tissues. The incidence is 5-6/100 000 and mainly affects the elderly population, with the median age at diagnosis being 65 years.¹ The clinical course of this disease is highly variable. The majority of patients display no symptoms at the time of diagnosis and do not require therapy for years. On the contrary, the aggressive form of the disease, usually accompanied by changes in blood count and symptoms like spleno- and hepatomegaly, night sweating or loss of weight, displays a poor prognosis and patients require specific therapy soon after the diagnosis.²

The diagnosis is based on examination of peripheral blood by microscopy and flow cytometry, when the B cell count is higher than 5×10^9 /liter and cells exhibit clonal origin and characteristic immunophenotype CD19^{positive}, CD5^{positive}, CD23^{positive}, sIg^{dim}, CD79b^{dim}. The severity of the disease is standardly assessed by Rai³ or Binet⁴ score systems created in the 80s of the last century and are mostly based on evaluation of physical symptoms such as high leukocytosis, anemia, thrombocytopenia and enlargement of lymphatic organs. Due to the remarkably variable clinical course of CLL patients, it is currently recommended to evaluate also other prognostic factors such as genetic abnormalities of 11q (*ATM*),⁵ 17p (*TP53*),⁶ 13q (long non-coding RNA *DLEU2* and two micro-RNAs: miR-16-1 and miR-15a),⁷ trisomy of chromosome 12 (more genes involved),⁸ or mutational status of immunoglobulin heavy chain variable region (IGHV),⁹ and ectopic expression of cellular markers such as CD38 and Zeta-chain-associated protein kinase 70 (ZAP70).^{10,11} All these prognostic factors were shown to affect not only the choice of treatment but show the ability to predict the length of the period without treatment, progression-free survival or overall survival.² Current approaches in the treatment of prognostically unfavorable CLL are more and more focused on inhibition of specific signaling pathways rather than conventional chemo-immunotherapy mainly based on DNA-damaging drugs.

2. Migration of CLL cells into microenvironment

Chronic lymphocytic leukemia has been considered for many years as an accumulative disease stemming from defective apoptosis and the prolonged lifespan of malignant B cells. However, recent studies revealed that CLL is quite dynamic malignancy wherein 0.1 – 1% of the entire population multiply each day. It is believed, that the induction of proliferation does not occur in peripheral blood but is located to specific places in the microenvironment of lymphoid tissues called ‘pseudofollicles’.¹² Inside the immune niches, CLL cells obtain several pro-survival and pro-proliferative signals leading to activation of various signaling pathways like B cell receptor (BCR), extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K), protein kinase B (AKT, also known as PKB), or nuclear factor-kappa B (NFκB).¹³⁻¹⁷ Inhibiting CLL cells homing to immune niches or inhibition of microenvironmental interactions represents an important mechanism of action for “BCR inhibitors”, and a possible therapeutic strategy.¹⁸⁻²¹

The recirculation of B cells is a complicated process consisting of several steps including attachment to endothelial cells, rolling, activation by chemokines, firm adhesion, extravasation, and at the end their return back to the peripheral bloodstream.

2.1. Lymphocyte rolling

The initiation of lymphocyte migration into particular tissue depends on their ability to bind to endothelial cells and trigger the process called “rolling”. This occurs only in morphologically specialized veins called high endothelial venules (HEV), which display the characteristic structure of enlarged endothelial cells. These HEVs were found in all secondary lymphoid tissues apart from the spleen, where the blood enters red pulp through open arterioles.²² The adhesion and rolling require rapid association and dissociation of molecular bonds between selectins exhibited on the B cell surface and their ligands produced by endothelial cells.²³

L-selectin (leukocyte-selectin; CD62L) was identified as a key factor controlling the B cell attachment to the HEVs in secondary lymphoid organs. The mice with deficiency in *SELL* gene (L-selectin protein) exhibited strongly impaired attachment of leukocytes to HEV cells

and significantly decreased numbers of lymphocytes inside lymph nodes.²⁴ Similar data were obtained also for CLL cells after functional-blocking of L-selectin by specific antibody.²⁵ Mechanism of L-selectin action relies on its ability to be clustered in structures called microvilli, which increases tethering efficiency during recruitment.^{26,27} In these clusters L-selectins are ready to interact with their ligands and slow down the speed of leukocyte motility across the endothelium. These ligands, collectively called vascular addressins, include glycosylated cell adhesion molecule-1 (GlyCAM-1), CD34, and podocalyxin.²⁸ Another type of ligand is called mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) and is expressed on endothelium in mucosae.²⁸ The interaction of L-selectin with ligands exhibited on endothelium can be terminated by a process called “ectodomain shedding”. This process is based on the proteolytic-dependent mechanism that cleaves L-selectin at the site near to the membrane and is triggered upon cell activation,^{29,30} L-selectin crosslinking³¹ or due to mechanical shedding.³⁰ Ectodomain shedding results not only in faster B cell rolling or even detachment of cell back to the bloodstream,²⁶ but also contributes to the process of transendothelial migration when B cells migrate towards a gradient of chemoattractants and need to be detached from endothelium.³² In humans, the expression of L-selectin is mainly driven by forkhead box protein O1 (FoxO1),^{33,34} the transcriptional factor which we currently identified as upregulated in CLL cell subgroup exhibiting higher migration capacity towards immune niches.³⁵

Altogether, the interactions between selectins and their ligands are responsible for attaching and rolling of leukocytes, which finally results in specific homing. However, these processes are just the first step in the transendothelial migration process and further actions are required.

2.2. Lymphocyte attachment to endothelium by integrins

The next step in the migration cascade is integrins' activation, which allows B cells to adhere more firmly to the endothelium. The general structure of integrins is formed by the noncovalent association of two structurally different glycoproteins - α and β subunit. So far, seventeen α subunits and eight β subunits have been identified, these further form more than 20 different types of integrins (Figure 1), however only a few of them play a key role in B cell migration.³⁶ Other members of the integrin family participate in various processes like organ development, wound healing, and also autoimmune diseases or cancer.^{36,37}

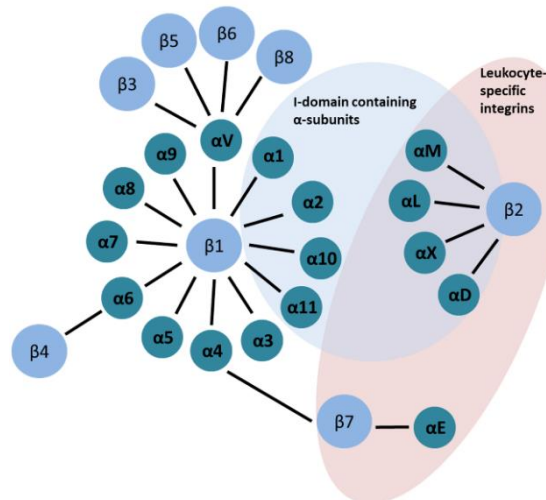


Figure 1. The integrin family. Integrins are formed by noncovalent association of α and β subunits. Their combinations can create more than twenty types of integrins with specific functions. (Adapted from Harzschel *et al.*, 2020.)³⁸

A integrin heterodimer forms a globular ligand-binding headpiece, which is connected to the membrane by two “legs”, followed by the transmembrane part and cytosolic tail.³⁹ Interestingly, all types of β cytoplasmic tails except β_4 exhibit a relatively high level of homology in comparison to α tail that is conserved only in relatively short region (GFFKR) ensuring their association with β tails. This association is required to maintain integrins in an inactive state, where the “legs” are folded in knee-like globular domains and the headpiece is unapproachable for ligands.⁴⁰⁻⁴² Once the cell reaches the place with an increased concentration of chemokines or BCR ligands, several signaling kinases are recruited to the proximity of activated receptors. This triggers various signaling pathways like PI3K, Bruton’s tyrosine kinase (BTK) or phospholipase $C\gamma$ (PLC γ) (see Chapter 2, BCR signaling) leading to cytoskeleton rearrangement, integrin’s β tail motive (NPxY) phosphorylation, detachment from inhibitory proteins like filamin and binding to integrin-activating proteins like talin or kindlin-3. The binding of talin results in α/β tail dissociation and conformational changes in the extracellular part, resulting in integrin “legs” unfolding and activation of integrin headpiece (Figure 2).^{37-39,43-47} In addition, talin also interacts with filamentous actin (F-actin) and actin-binding proteins, which provides a link between the cell cytoskeleton and extracellular matrix.⁴⁸ The negative regulation of integrin activation can be caused by inhibitory proteins such as docking protein 1 (DOK1) or integrin cytoplasmic domain-associated protein 1 (ICAP1), which bind to

the β subunit tail competitively and block binding of activation proteins like talin.^{49,50} Other mechanism of integrin inhibition rely on activation of ERK signaling (see Chapter 2.2.1., Focal adhesions).⁵¹

One of the two key integrins expressed by B cells is very late antigen 4 (VLA4), which is composed of $\alpha 4$ and $\beta 1$ subunits (also known as CD49d and CD29, respectively).^{52,53} The studies with chimeric mice deficient for $\alpha 4$ subunit displayed a great reduction of B cells in bone marrow accompanied by the inhibition of pro-B cell differentiation.⁵⁴ Major VLA4 ligands are extracellular matrix protein fibronectin and vascular cell adhesion molecule 1 (VCAM1), which is constitutively expressed on the surface of endothelial and stromal cells and can be expressed by other leukocytes during inflammatory response in NF κ B-dependent manner.^{55,56} In addition, the expression of VLA4 was detected in approximately 40 % of CLL cases and stands for one of the most relevant predictors of overall survival and progression-free survival.⁵⁷

Other key integrin produced by B cells is composed of αL and $\beta 2$ subunits (CD11a/CD18, respectively) and is known as leukocyte-function-associated antigen 1 (LFA1). Similar to VLA4, LFA1 also participates in the trafficking of B cells and its knock-down in mice results in impaired retention of B cells in lymph nodes. Interestingly, the homing capacity of FLA1^{-/-} and LFA1^{+/+} in bone marrow or spleen does not significantly differ,⁵⁸ which supports the hypothesis that a specific combination of adhesion molecules is required for migration into specific niches. The major LFA1 ligands are proteins from the intercellular adhesion molecule family (ICAM) expressed by endothelial cells.⁵⁹ Interestingly, it has been shown that CLL cells in comparison to healthy B cells exhibit significantly lower LFA1 and VLA4 expression, which leads to impaired migration into lymph nodes and bone marrow. The migration towards the spleen is LFA1 independent so there is no markable difference in homing.⁶⁰

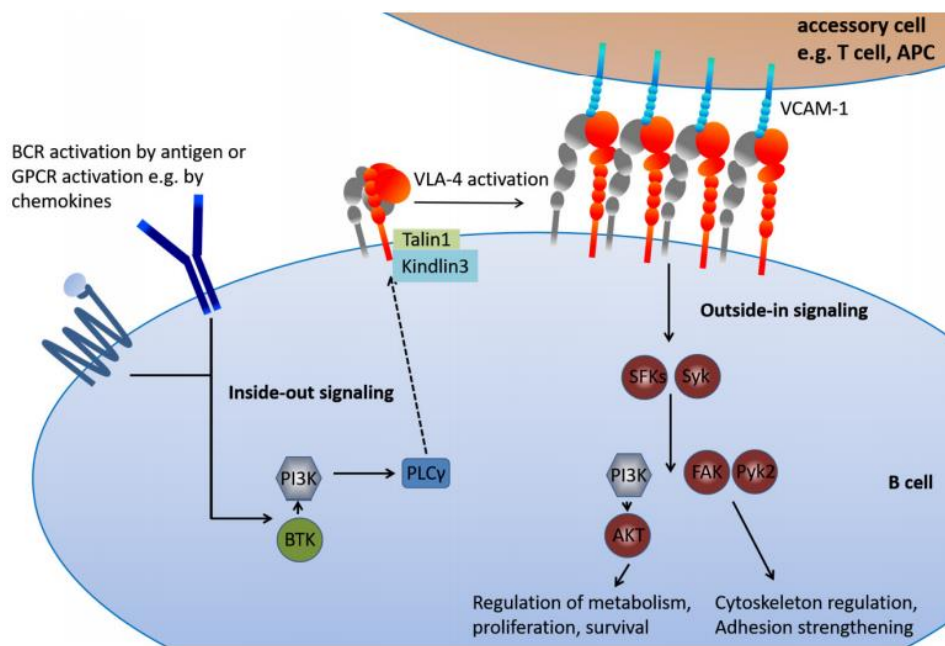


Figure 2. Integrin signaling in B cells. The activation of integrins is initiated by various signaling receptors, mostly chemokine receptors or BCR receptor. Their activation triggers downstream events resulting in activation of effector proteins like PI3K, BTK, PLC γ , and subsequent β -tail phosphorylation. This enables the binding of talin or kindlin-3, which enables α/β subdomains dissociation and proper integrin activation. The binding of integrins to multivalent substrate results in clustering, recruiting of other proteins, and finally to the creation of focal adhesions which regulates cytoskeletal reorganization and cell motility. (Adapted from Harzschel *et al.*, 2020.)³⁸

2.2.1. Focal adhesions

One of the features of integrin ligands is their multivalent potential ensuring integrin clustering. Intracellularly, this leads to the creation of multiprotein structures called focal adhesions. These structures are constantly formed at the leading edge of migrating cells and disassembled at the opposite side. Their role is to provide a mechanical link between the actin cytoskeleton and extracellular matrix.⁶¹ During migration, focal adhesions prevent membrane contraction and promote protrusions at the leading edge thus enabling cellular movement.⁶² In addition, focal adhesions may contain dozens of proteins, creating hot spots with increased amounts of various kinases promoting integrin signaling (as demonstrated in Figure 3).⁶³

Focal adhesion kinase (FAK) and cellular Src (c-Src) belong to the first kinases recruited into newly created focal adhesions, where they intensively interact with each other.⁶⁴ Importantly, the studies in human embryonic kidney cells showed that the precise localization of FAK and

other proteins in focal adhesions is regulated by activated GRB2-associated-binding protein 1 (GAB1) protein, given that the GAB1 mutant cells failed to properly localize not only the FAK protein but also paxillin and other migration-related proteins.⁶⁵ Upon recruitment, FAK is promptly autophosphorylated on Y397, which creates a high-affinity binding site for the SH2 domain of c-Src.^{64,66} This allows autophosphorylation of c-Src on Y416, which subsequently phosphorylates FAK on additional residues (Y576, Y577, Y861, and Y925).⁶⁴ This initial phosphorylation enables recruitment of other proteins like GAB1, growth factor receptor-bound protein 2 (GRB2), or paxillin which creates together a docking site for other downstream molecules like p85 subunit of PI3K, mitogen-activated protein kinases (MAPK), or Ras kinase.^{64,65,67-70} Their activation generates both pro-proliferative and pro-survival signals,⁷¹ but also triggers the negative feedback leading to focal adhesions dissociation. This occurs directly after FAK/GRB2 mediated ERK2 activation. Activated ERK2 phosphorylates FAK on Ser910, which could enhance the release of FAK from focal adhesions.⁷² Other inhibitory mechanism is dependent on other FAK/c-Src substrate named p38-regulated and activated kinase (PRAK). This kinase colocalizes with FAK in focal adhesions and exhibits the ability to disrupt the phosphorylation of FAK on Y861 and thus inhibits cell adhesion and motility.⁶⁴

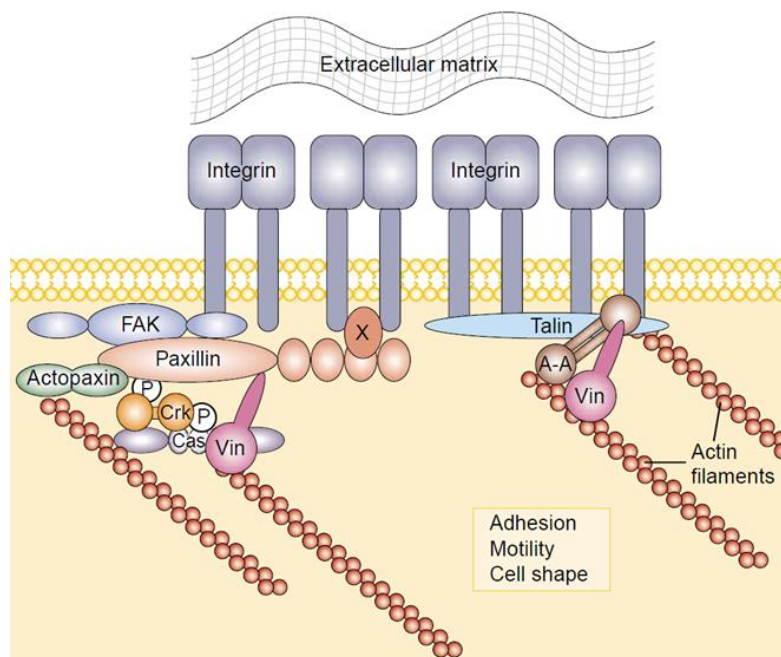


Figure 3. Focal adhesions. The integrin engagement to the extracellular matrix leads to reciprocal activation of FAK adaptor protein and c-Src kinase. The newly created binding sites, especially on FAK, bind other proteins like paxillin, which together create a site for the recruitment of various kinases and downstream effector proteins that contribute to actin cytoskeleton rearrangement. In this figure two proteins are depicted, vinculin (Vin)

and actopaxin, which physically link actin filaments to the paxillin. In addition, integrins are linked to the actin cytoskeleton via talin, α -actinin (A-A) and vinculin. Together these places are called focal adhesions. (Adapted from Turner, 2000).⁷³

2.3. Activation of cells by chemokines

Chemokines are chemotactic cytokines that control the migration of all immune cells.⁷⁴ It is a group of small (7-16 kDa), secreted proteins with conserved sequences and structural features, that can be used to classify them into four families based on the position of the first two cysteines – CC, XC, CXC, and CX3C. Until this time we recognize more than 50 chemokines and 20 chemokine receptors.^{74,75} Another chemokine classification is based on their physiological features and recognizes the group of inflammatory chemokines (highly produced during inflammation) and a group of homeostatic chemokines (expressed continually and maintain the homeostasis of immune cells). However, some chemokines like CCL21 and stromal cell-derived factor 1 (SDF1 also known as CXCL12) take a role in both of these processes.^{74,76} The repertoire of chemokine receptors is specific for each type of immune cell. This ensures precise cell navigation along the gradients of their ligands since the binding of ligand establishes cell polarity, cytoskeletal rearrangement, increased adhesiveness, and finally the cell locomotion.⁷⁶

2.3.1. CXCR4 and SDF1

One of the best-described chemokine receptors is CXCR4, which was intensively studied due to its role as a co-receptor for human immunodeficiency virus (HIV) entry⁷⁷ and its ability to mediate metastasis of several cancers.⁷⁸ Despite the general promiscuity of other chemokine receptors, CXCR4 has been described to interact exclusively with SDF1 chemokine ligand.⁷⁹ This monogamous relationship was demonstrated in knock-out mouse models, where the inactivation of either *CXCL12* or *CXCR4* led to identical lethal phenotype accompanied with defects in B cell lymphopoiesis, bone marrow colonization, and malfunctions in the cardiovascular system.^{80,81} SDF1 ligand is highly produced by perivascular stromal cells and is present at the luminal side of the endothelium, which contributes to guiding of CXCR4 positive cells into the microenvironment.⁸² Interestingly, the gradient of SDF1 in the subendothelial matrix can be formed by interaction and further immobilization of SDF1 to glycosaminoglycans

like heparan sulfate, the important component of the extracellular matrix. This association was described as beneficial for cell migration when compared to SDF1 only, since the SDF1 oligomerization enables CXCR4 clustering, translocation into lipid rafts and robust activation of further effects.⁸³⁻⁸⁵ CXCR4 activation triggers various signaling kinases like FAK, PI3K, Ras/MAPK, Janus kinase (JAK) proteins, or various Src-family members tyrosine kinases.^{86,87} The activation of downstream signaling is a crucial step in the initiation of cytoskeleton rearrangement, activation of integrins, and induction of cell motility.

CLL cells in peripheral blood were shown to express relatively high levels of CXCR4, which increases their responsiveness to SDF1 and may positively affect integrin activation and cell motility.⁸⁸ On contrary, CLL cells in bone marrow exhibit low levels of surface CXCR4, which is likely an outcome of receptor endocytosis after SDF1 binding.^{88,89}

2.3.2. CXCR5 and CXCL13

Another key player in CLL cell migration is the CXCR5 receptor and its ligand CXCL13 (also known as B cell-attracting chemokine 1; BCA-1).⁹⁰ The CXCR5 receptor expression was originally described in Burkitt's lymphoma and shortly after was its role described also in CD4 and CD8 positive T cells or dendritic cells. Recent evidence also described CXCR5/CXCL13 involvement in various solid tumors like breast cancer, gastrointestinal tumors, prostate cancer, or osteosarcoma, where it may contribute to metastatic potential.⁹¹⁻⁹⁴ In the case of CLL cells, the *in vivo* studies demonstrated that CXCR5/CXCL13 pathway contributes more to CLL cells' precise navigation within the lymph node rather than to their homing capacity from peripheral blood. In this study, CXCR5 deficient cells were localized mostly in the marginal zone of B-cell follicle whereas CXCR5 wild-type cells were gathered in proximity to follicular dendritic cells.⁹⁵ This is in line with the observation that CXCL13 is constitutively expressed by nurse-like cells and stromal cells (mainly follicular dendritic cells) in the germinal centers of secondary lymphoid organs.^{90,96} In addition, the CXCR5 knock-out mice displayed not only the defective formation of primary follicles but also a lack of inguinal lymph nodes and impaired leukemogenesis.⁹⁵

Similarly to CXCR4, the binding of ligand results in rapid CXCR5 endocytosis and triggering of various downstream effects like Ca^{2+} influx, PI3K/AKT, MAPK/ERK activation, actin polymerization, increased adhesion via LFA1, and cellular chemotaxis.⁹⁷⁻⁹⁹

2.3.3. CCR7 and CCL19, CCL21

The importance of the CCR7 receptor in lymphocyte homing has been demonstrated by gene targeting. The CCR7 knock-out mice exhibit impaired migration of lymphocytes into lymph nodes and Peyer's patches accompanied by their accumulation in peripheral blood, spleen, and bone marrow.^{89,100,101} This surprising result of the CCR7 dichotomy in the migration process was further explained by McHeik *et al.*, 2019,¹⁰¹ and relies on CCR7 ability to inactivate CXCR4 by direct binding. Their coupling blocks the signal transmission from activated CXCR4 to the downstream kinase Gαi2 impairing thus the migration towards SDF1. Deletion of CCR7 therefore increases the responsiveness of CXCR4 positive cells to SDF1.¹⁰¹ In addition, CCR7 was demonstrated to guide B cells to the T cell areas, whereas CXCR5 directs cells to the B cell follicles. Here, the B cells in response to high levels of CXCL13 down-regulate the CXCR5 and further upregulate the CCR7. This mechanism allows transient migration of B cells towards the T cell zone, where activated B cells may receive further stimulation from T helper cells.^{102,103}

CCL19 and CCL21 are the only known ligands for CCR7. Northern blot analysis revealed abundant expression of *CCL19* in the thymus, lymph nodes, or appendix, and low or very low levels in bone marrow and spleen,¹⁰⁴ indicating that CCR7/CCL19 chemokines are guiding cells only in specific niches. In addition, CCL19 is secreted by dendritic cells or by stromal cells localized in the vicinity of the vasculature.¹⁰⁵

Despite the fact that CCL21 is exposed on the surface of HEV cells, previous studies identified that human HEV cells do not express *CCL21* mRNA.¹⁰⁶⁻¹⁰⁸ Instead, the CCL21 chemokine is produced in T cell areas and further translocated into vessels' lumen to be presented by endothelial cells.¹⁰⁸ This process is ensured by the unique structure of the C-terminal tail of CCL21 that allows binding to glycosaminoglycans produced by endothelial cells.^{109,110}

The overview of leukocyte adhesion is summarized in following Figure 4.

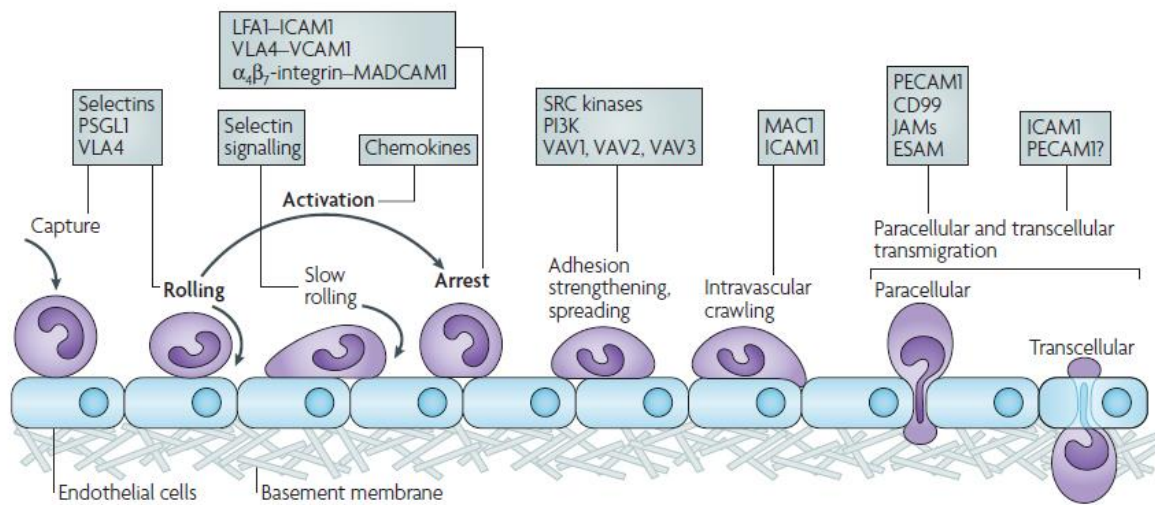


Figure 4. Leukocyte adhesion and migration. The initial binding and subsequent rolling of lymphocyte across the endothelial surface is ensured by selectins. The activation of lymphocytes by various chemokines (SDF1, CXCL13, CCL19, or CCL21) leads to integrin activation and firm adhesion to endothelium. These are the crucial steps in paracellular or transcellular transmigration in lymphoid organs. Key molecules involved in each step are indicated in boxes. (Adapted from Ley, *et al.*, 2007.)²³

2.4. Lymphocyte egress from lymph niches

Healthy B cells usually spend approximately 24 hours in secondary lymphoid organs trying to interact with microenvironmental cells, and if they are not stimulated, they will exit them. In contrary to the entry, where several factors play a role, only one requirement for egress has been identified so far – the expression of sphingosine-1 phosphate receptor (S1PR1).^{111,112} Initial attempts to generate mice lacking the expression of S1PR1 in all lineages failed due to significant vascular defects resulting in embryonic lethality.¹¹³ For this reason the further approaches were focused on the conditional deletion of S1PR1 from lymphocytes. This kind of experiment proved that T cells lacking the expression of S1PR1 developed normally, but failed to exit the thymus.^{114,115} Similarly for B cells, the expression of S1PR1 was not required for exiting the bone marrow, however, it was crucial for exiting the secondary lymphoid organs.¹¹⁵

The only known physiological ligand of S1PR1 is a signaling sphingolipid sphingosine-1-phosphate (S1P).^{111,116} This molecule is produced by various cell types like macrophages, thrombocytes, mast cells, or endothelial cells, however, the major reservoir of S1P are erythrocytes.¹¹⁷ The highest S1P concentration throughout the body was found in blood and

was significantly less abundant in tissue fluids.¹¹⁸ This concentration gradient is utilized by lymphocytes to egress from tissues back to peripheral blood. Interestingly, this sharp concentration interface is maintained not only by physical barriers but mostly by the S1P-degradation enzyme S1P lyase. Its inhibition or knockdown results in the rapid increase of S1P concentration in organs and disruption of S1P gradient (Figure 5).¹¹⁹

Interestingly, the impaired expression of S1PR1 was observed in CLL patients with unfavorable prognosis. It was suggested that defective egress of malignant B cells from the pro-survival niche of lymph nodes extends their survival. Interestingly, the administration of idelalisib (PI3K inhibitor) increases the expression of S1PR1 on CLL cells, reducing the lymphadenopathy and triggering the redistribution of CLL cells into the bloodstream.^{19,120,121}

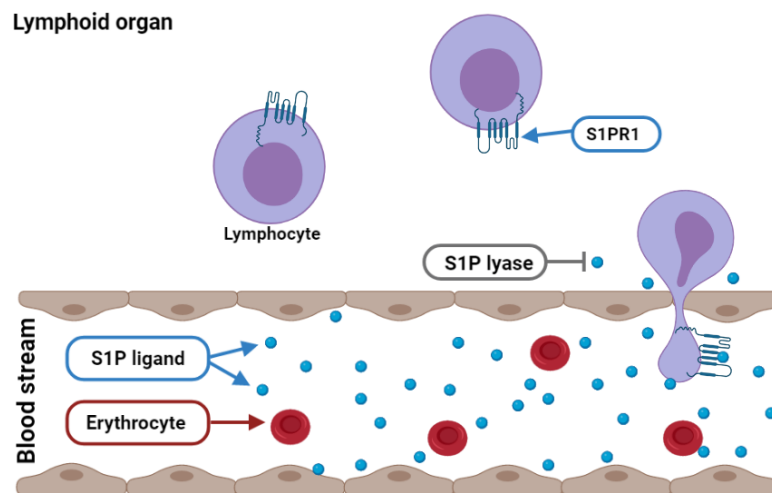


Figure 5. Lymphocyte egress from lymphoid organs. The lymphocyte egress from lymphoid organs is guided by S1PR1 and the concentration gradient of the S1P ligand. S1P is produced mostly by erythrocytes and is highly abundant in the bloodstream. The sharp concentration interface of S1P is maintained by a physical barrier and by S1P lyase expressed in lymphoid organ, which degrades all S1P ligand passing the endothelium. (According to Schwab *et Cyster*, 2007.)¹¹¹

2.5. CXCR4/CD5 as a model for CLL cell recirculation

The recirculation of CLL cells to and from immune niches has been proposed as a feature of CLL biology,^{13,122-127} however this has not been entirely experimentally proven. Inside the immune niches, CLL cells obtain various pro-survival and pro-proliferative signals leading to BCR, PI3K/AKT, and NFκB pathway activation.^{13-17,35} Currently, it is hypothesized that migration of CLL cells is not a completely random process, but depends on cell ‘specific setting’ in form of over-expression of migration-related genes.

One proposed model to study CLL cell recirculation is to use CXCR4/CD5 intraclonal subpopulations. The original experiments with blood samples from patients who consumed heavy water ($^2\text{H}_2\text{O}$) identified that CXCR4^{dim}CD5^{bright} cells represent a proliferative fraction of cells since this population exhibited the highest proportion of ^2H . As the proliferation of B cells and CLL cells occurs predominantly inside the lymphoid organs, it is believed that CXCR4^{dim}CD5^{bright} cells represent the recent emigrants from the microenvironment. On the contrary, the CXCR4^{bright}CD5^{dim} subpopulation corresponds to resting cells circulating in peripheral blood for an extended time before their return back to immune niches (Figure 6).^{13,122-127} The hypothesis about increased migration potential of CXCR4^{bright}CD5^{dim} subpopulation is based mostly on increased expression of chemokine receptor CXCR4, which is known to regulate CLL cell migration (see Chapter 2.3., Activation of cells by chemokines).⁸⁸ However, further experimental validation of this migration model is required.

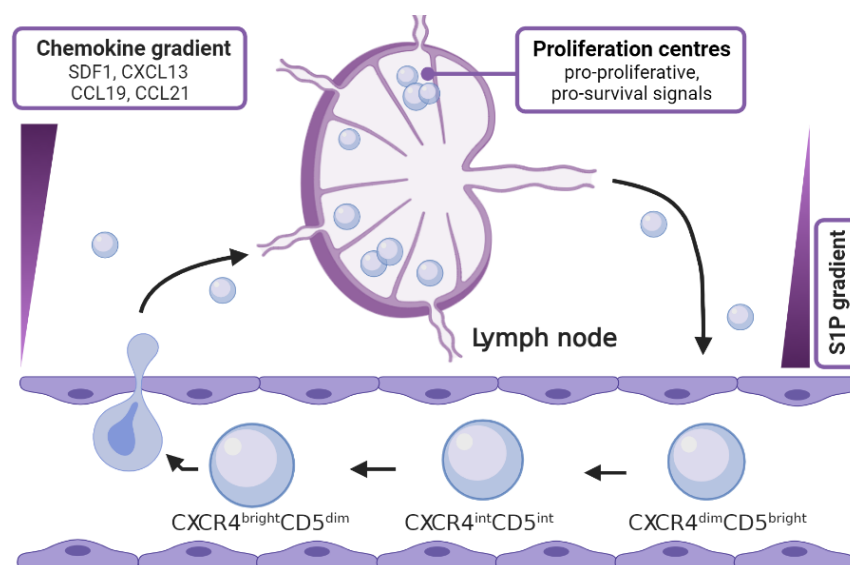


Figure 6. Proposed mechanism of CLL cell recirculation. Currently, the only known model for studying recirculation of CLL cell subpopulations is based on CXCR4/CD5

levels. Cells which are circulating for a prolonged period of time in peripheral blood exhibit higher levels of CXCR4 chemokine receptors, which makes these cells more responsive to SDF1 ligand. After the signal integration, integrin activation, and transendothelial migration CLL cells enter their microenvironment in lymphoid organs. Here they are stimulated with various supportive signals triggering pro-survival and pro-proliferative events. In the case of healthy B cells, proliferation centers are the place where B cells can encounter the antigen structures exhibited by antigen-presenting cells. After some time CLL cells are guided by the S1P concentration gradient back to the bloodstream.

3. B cell receptor signaling pathway

To effectively fight the wide range of pathogens, the lymphocytes have evolved a mechanism that is capable to recognize a broad variety of antigens. This is ensured by the immunoglobulins (Ig), glycoproteins produced by B cells. Each B cell produces immunoglobulin of a single specificity, which can bind a limited repertoire of antigens. Only the broad amount of structurally and thus specifically different immunoglobulins ensures that adaptive immunity can recognize any possible antigen structure and target it. The specificity of immunoglobulins against antigen is increased during a process called somatic hypermutation. This process, accompanied by increased B cell proliferation, introduces mutations in the antigen-binding part of immunoglobulin, generating the B cell subclones with adjusted immunoglobulin affinity.¹²⁸ This process in CLL cells is, however, limited or fully disrupted. The percentage of mutated CLL cells stratifies the patients into two categories – those with mutated subclones and favorable prognosis, and those having the uniform population of CLL cells and exhibiting unfavorable prognosis.¹²⁹ In addition, another process of B cell maturation is disrupted as well – the class switching. The first types of immunoglobulins that are expressed in mature naive B cells are IgM and IgD. The antigen recognition initiates the process called class switch recombination, which switches the immunoglobulin production to one of the three structurally and functionally different types of immunoglobulins – IgG, IgA, or IgE. CLL cells express preferentially IgM and IgD and to a lesser extent IgG.¹³⁰

Immunoglobulins can be produced in two forms. The secreted form enables the pathogen opsonization and triggering of immune response in a short period of time. Another form of immunoglobulin is anchored in the cell membrane by the C-terminal part, and together with other signaling proteins creates a receptor complex called BCR, which triggers the signaling after antigen binding. This BCR complex affects the B cell life from the beginning of its expression and controls various events like cell maturation, survival, clonal expansion, production of antibodies, anergy, and even apoptosis.^{131,132}

3.1. Signal initiation

The membrane-bound immunoglobulin does not have any kinase activity. The signaling initiation after antigen binding is ensured by non-covalently coupled signaling subunits CD79 α /CD79 β and their tyrosine residues in immunoreceptor tyrosine-based motives (ITAMs). Together they create multiprotein complex BCR.^{133,134} In non-activated conditions, BCRs are distributed more or less equally on the plasma membrane in form of monomers which do not have the ability to trigger signaling.^{135,136} Growing evidence, however, indicates that even the non-stimulated B cells can exhibit some level of BCR clustering, which can generate relatively weak, basal signaling (see Chapter 3.5., Tonic signaling).^{137,138} The robust BCR signaling is triggered after the binding of multivalent antigen, this enables the creation of ‘larger’ BCR microclusters accompanied by their translocation into lipid rafts (Figure 7).^{133,134,139} These lipid rafts were shown to contain high levels of Src-family members kinases like LYN or FYN, BLK, and LCK, which ensure the initial phosphorylation of tyrosine residues in the ITAM network.^{136,139} Phosphorylated ITAMs create docking sites for downstream SH2 domain-containing proteins such as kinase spleen tyrosine kinase (SYK), whose activation can be considered as a first and crucial step in the propagation of BCR signaling to more distant effector proteins.¹⁴⁰⁻¹⁴³

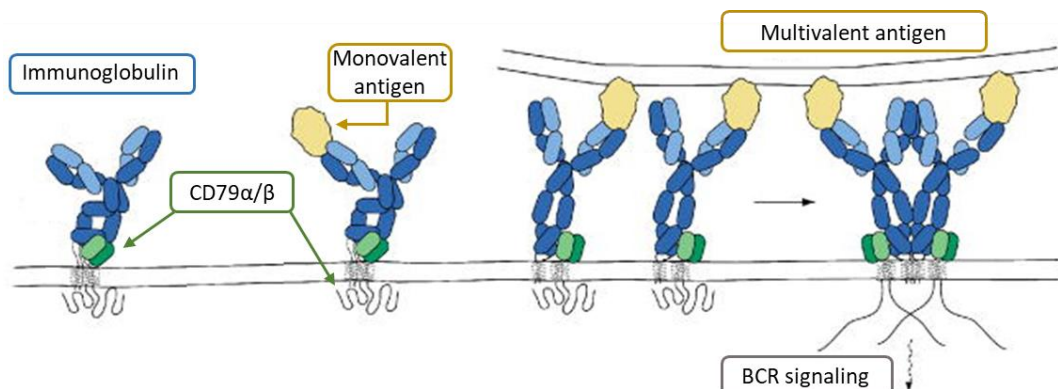


Figure 7. Schematic illustration of BCR clustering. The BCRs are usually present as monomers with no signaling activity in unstimulated cells and a similar situation occurs after binding of monovalent antigen since this kind of binding does not induce BCR clustering. Binding to the multivalent antigen leads to BCR aggregation, translocation into lipid rafts, and further phosphorylation of ITAM motives by various Src kinases. This creates a docking site for downstream messengers like SYK kinase. (Adapted from Tolar *et al* Pierce, 2010.)¹⁴⁴

3.1.1. Regulation of BCR signaling initiation

The proper initiation of BCR signaling is regulated by at least three other transmembrane molecules – CD19 (also known as B-Lymphocyte Surface Antigen, B4), CSK (C-terminal Src tyrosine kinase), or CD45 (also known as the pan-B cell marker, B220).

CD19, the specific marker of B cells, is a transmembrane glycoprotein that was shown to participate in the initiation of the whole BCR signaling by acting as an adaptor protein recruiting cytoplasmatic proteins like LYN to the proximity of ITAMs.¹⁴⁵ It has been proposed that in resting cells CD19 is coupled preferentially with IgD-BCR, whereas upon antigen-induced BCR signaling CD19 is translocated to the lipid rafts where it transiently associates with IgM-BCR microclusters and enables the proper interaction with LYN kinase.¹⁴⁶⁻¹⁴⁸ This function was reflected in the studies with B cells deficient for CD19, which exhibited significantly defective initiation of BCR signaling and also reduced accumulation of downstream effector proteins and reduced formation of microclusters.¹⁴⁸ In addition, CD19 also participates in BCR signaling amplification by recruiting downstream kinases such as SYK or PI3K.^{148,149}

CSK is an endogenous non-receptor tyrosine kinase protein that suppresses the activity of the Src proteins by adding the phosphate at their conserved C-terminal tail site. This phosphorylation locks the Src proteins in an inactive conformation even upon their phosphorylation by activator proteins.¹⁵⁰ In addition, the deletion of CSK leads to hypophosphorylation of LYN on the inhibitory site Y508 and thus to the easier activation of B cells.¹⁵¹

The receptor-like tyrosine phosphatase CD45 serves as a critical regulator of BCR signaling which is able to remove inhibitory phosphorylation on LYN (Y508).¹⁵² This ensures the sensitivity of the BCR to stimulation by maintaining and balancing the amount of ‘ready to act’ LYN kinase.¹⁵³ To give an example, the expression of an over-activated CD45 mutant variant leads to B cell hyperactivity and tumor formation¹⁵⁴ and *vice versa*, the down-regulation of CD45 leads to hyperphosphorylation of LYN in C-terminal inhibitory tyrosine (Y508) and lower activity of B cells.¹⁵³

3.2. Signal propagation and amplification

The propagation of BCR signaling is tightly connected to the plasma cell membrane, given that the most important secondary messengers are generated from phospholipids in the cell membrane. Their generation is ensured by the activation of BCR downstream proteins exhibiting enzymatic activity.¹³¹

3.2.1. PI3K

PI3K is a group of cytosolic membrane-associated lipid kinases, consisting of three subunits: p85 or p55 regulatory subunit, and p110 catalytic subunit.¹⁵⁵ In normal conditions PI3K is present in cytosol in form of a heterodimer containing one regulatory and one catalytic subunit; in B cells predominantly p85/p110. The role of the catalytic subunit is to add phosphate to the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to create one of the most important secondary messengers phosphatidylinositol-3,4,5-triphosphate (PIP3). In unstimulated cells, this function is blocked by the p85 regulatory subunit. Upon BCR activation p85 is phosphorylated by SYK, or with lower affinity by LYN, resulting in conformational changes and PI3K activation.¹⁵⁶⁻¹⁵⁸ Since the substrates for the p110 catalytic subunit are present in the phospholipid bilayer, the PI3K complex needs to be translocated to the proximity of the cell membrane. This is usually ensured by pleckstrin homology (PH) domain exhibiting high affinity to PIP3 and with the lesser extent to PIP2.^{159,160} However, PI3K does not contain any PH domain and thus the mechanism of translocation is dependent on the coupling to various membrane-associated docking proteins, namely B cell adaptor for phosphoinositide-3-kinase (BCAP), CD19 and GAB1 via SH2 and SH3 domains in p85.^{161,162}

In addition, the generation of PIP3 is an essential step in the recruitment and activation of PH-domain containing proteins like BTK, AKT, protein kinase C (PKC), or PLC γ , the main downstream effectors of BCR signaling.¹⁶³

3.2.2. Adaptor protein GAB1

This protein was first identified and described in 1996 as GRB2-binder protein, which exhibits a structure typical for adaptor proteins. It contains an N-terminal PH domain (binding to PIP2

and more selectively to PIP3), a central proline-rich domain (binding site for SH3 domain-containing proteins), and multiple tyrosines (creating the binding motives for SH2 domain-containing proteins).¹⁶⁴ Due to these features GAB1 protein can connect various proteins and facilitate their interactions.

Original studies identified GAB1 as a protein activated after epidermal growth factor (EGF) receptor engagement.¹⁶⁵ Since then, its role was described in various pathways including insulin,¹⁶⁵ cytokines,¹⁶⁶ adhesion,¹⁶⁷ toll-like receptor,¹⁶⁸ or BCR signaling.¹⁶⁹ The receptor engagement leads to GAB1 anchoring in cell membrane followed/accompanied by Src-family kinases dependent phosphorylation. The classical model of GAB1 anchoring in the cell membrane is based on the PH domain binding the PIP3, and enabling further GAB1 activation.^{170,171} The importance of this process was emphasized by PH-mutants, which failed to become fully tyrosine phosphorylated upon BCR engagement.^{169,172} Another possible mechanism of GAB1 translocation to the membrane is PIP2/3-independent, based on direct binding of GAB1 to the cytoplasmatic domain of the activated c-Met receptor.¹⁷³ Last possible mechanism of GAB1-recruitment is indirect via coupling with other docking proteins e.g. GRB2, serving as a bridge between GAB1 and targeted protein.^{165,173}

The phosphorylation of GAB1 creates several docking sites for proteins containing the SH2 domain. One well-defined binding partner is the p85 domain of PI3K. The mutation of the site surrounding the Y472 of GAB1 leads to disruption of their physical association and remarkable inhibition of PI3K/AKT activation.^{170,174-177} Another described interaction partner of GAB1 is the Src homology region 2 domain-containing phosphatase-2 (SHP2 phosphatase), binding to motives surrounding Y627 and Y659. The functional significance of their interaction has been intensively studied for several years bringing the evidence that GAB1-dependent activation of SHP2 results in MAPK signaling activation. This process is dependent on the NH₂-terminal PH domain of GAB1,^{178,179} and inhibition of this domain by novel GAB1 inhibitors¹⁸⁰ may impair the MAPK/ERK activity.³⁵ The recruitment of SHP2 also contributes to attenuation of PI3K signaling by dephosphorylating GAB1 at the p85 binding site.¹⁸¹ In addition, the phosphorylation of GAB1^{Y307} is required for binding PLC γ ,¹⁸² and recruitment of Crk, FAK, or paxillin to focal adhesions.⁶⁵ The overview of GAB1 signaling is summarized in following Figure 8.

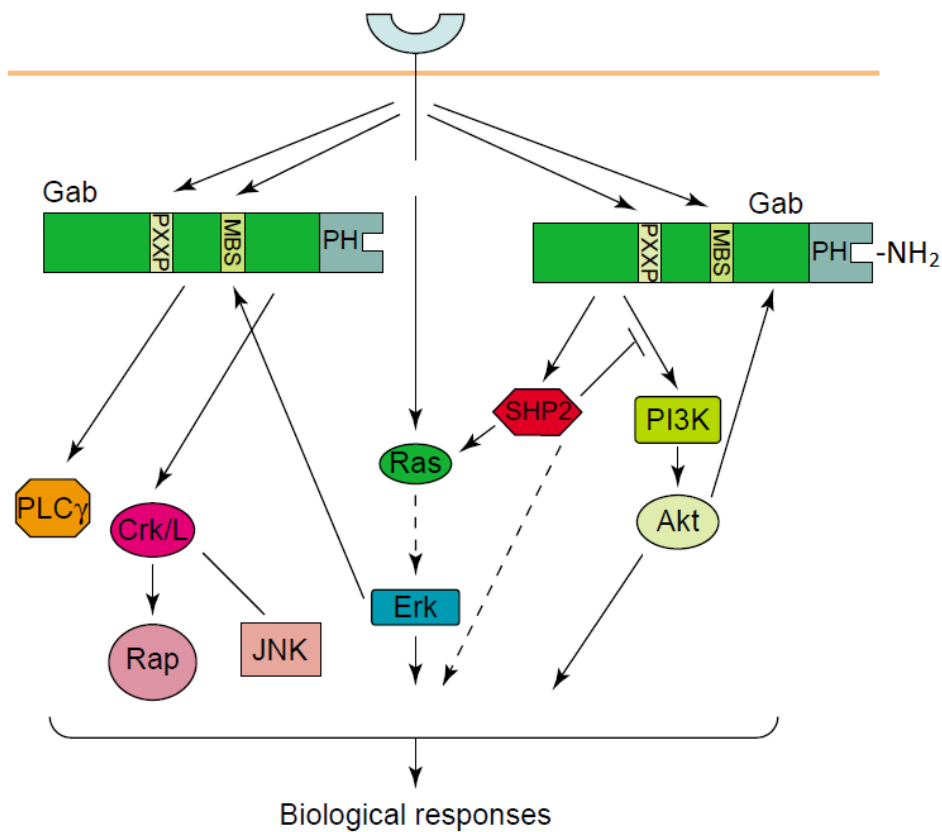


Figure 8. Signal transduction from GAB1 protein. The activation of surface receptors like BCR or cytokine receptors leads to GAB1 recruitment to the cytoplasmic membrane and further phosphorylation by kinases from Src-family. GAB1 can subsequently recruit various signaling proteins like p85, SHP2, or PLCγ and amplify the whole signaling process. (Adapted from Gu *et Neel*, 2003.)¹⁸³

3.3. Signal propagation and integration

The generation of second messenger PIP3 helps to trigger various signaling events leading to the activation of almost all signaling events – to the modulation of transcription factors activity or effector proteins activity. During signal propagation, the initial stimulation diverges in a labyrinth of multiple pathways, which in final steps can be again partially converged at the level of a transcription factor or other effector proteins. The importance of this signal propagation process is the modulation of the transmitted signal. Some streams of signaling axis can be enhanced and some attenuated, which finally results in a desired physiological outcome like gene expression, but also cell motility and others.

3.3.1. PI3K / AKT

One of the first event after PIP3 generation is the activation of AKT, the serine/threonine protein kinase regulating multiple cellular processes such as metabolism, apoptosis, cell proliferation, transcription, or cell migration.^{184,185} The proper AKT activation is ensured by phosphorylation of two various residues; initial phosphorylation at T308 is ensured by phosphoinositide-dependent protein kinase 1 (PDK1) and second phosphorylation at S473 by mammalian target of rapamycin complex 2 (mTORC2 complex).^{186,187} In non-stimulated cells AKT is present in an inactive conformation, where the PH domain blocks interaction with any of these activators. This is however changed after coupling of AKT-PH domain with PIP3 generated by PI3K. This binding induces conformational changes providing accessing AKT for PDK1-dependent T308 phosphorylation.^{188,189} The maximal AKT activation is further ensured by the mTORC2 complex providing the phosphorylation on S473 which stabilizes the initial T308 phosphorylation (Figure 9). The lack of S473 was shown to greatly diminish the final AKT activity.¹⁸⁷ PIP3 generation is required also in second, mTORC2-dependent AKT phosphorylation. The coupling of PIP3 and PH-domain of Stress-activated map kinase-interacting protein 1 (SIN1) protein (member of mTORC2 family) leads to mTORC2 translocation to the proximity of the cell membrane, conformational changes, and further AKT phosphorylation.¹⁹⁰ In addition, the regulation of the mTORC2 complex is a highly complex process that is currently under intensive investigation.

The lifetime of activated AKT is relatively short, up to two hours. In this time frame, the fully activated AKT can be detached from PIP₃, relocalized into cytoplasm or nucleus, where it can interact and phosphorylate substrates (reviewed in Manning and Toker, 2017).¹⁹¹ In addition, the signal is terminated by two phosphatases – protein phosphatase 2A (PP2A) dephosphorylating T308, and the PH domain leucine-rich repeat protein phosphatase (PHLPP) removing the phosphate from S473.^{192,193}

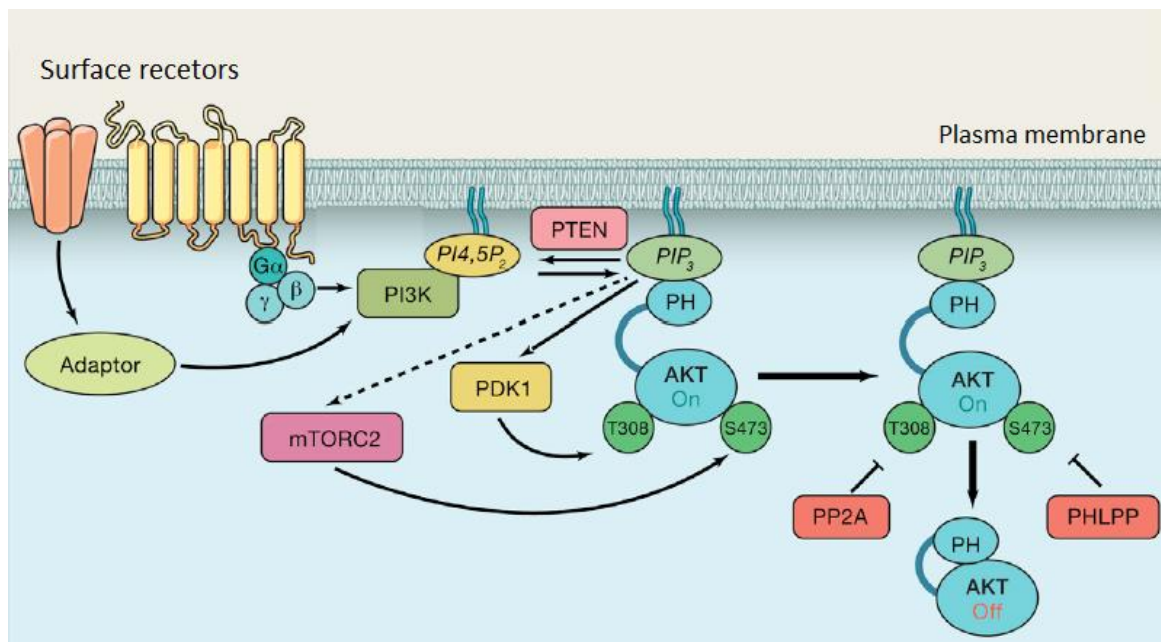


Figure 9. Mechanism of AKT regulation. The activation of various surface receptors (BCR, chemokine receptors) leads to PI3K activation and PIP₃ production. This second messenger molecule is bind by the PH domain of AKT enabling thus the PDK1-dependent T308 phosphorylation and mTORC2-dependent S473 phosphorylation. Signal termination is mediated by PP2A and PHLPP phosphatases. (Adapted from Manning *et* Toker, 2017.)¹⁹¹

AKT / FoxO1 pathway

One of the direct targets of activated AKT is FoxO1, which is localized in the nucleus of unstimulated cells serving as a transcriptional factor. Activated AKT directly phosphorylates FoxO1 on S256, which leads to changes in the DNA-binding domain charge from positive to negative and further disconnection of FoxO1 from DNA.¹⁹⁴ Additional phosphorylation of

FoxO1 on T24 creates a recognition motif for the 14-3-3 proteins, which facilitates the export from the nucleus and further degradation.^{195,196}

FoxO1 has been considered as a tumor suppressor gene for a long time, given that it positively regulates the expression of pro-apoptotic proteins (e.g. BIM, PUMA) or proteins participating in cell-cycle arrest (overexpression of p21 and p27; suppression of cyclin D1 and cyclin D2). Recent studies, however, showed that FoxO1 is overexpressed in proliferative compartments of lymph nodes,¹⁹⁷ and also aberrantly transcriptionally active in B cell lymphomas.¹⁹⁸ In some cases, this can be accompanied by activating mutations, preventing FoxO1 inactivation by AKT.¹⁹⁹ FoxO1's role in influencing genes (e.g. *SELL*, *CXCR4*, *ITGB2*) involved in cell migration corresponds to observations describing its involvement in dendritic cell homing,²⁰⁰ T cell homing,³⁴ and normal B cell migration within the germinal centers.^{197,201} Notably, the Fox-proteins have been suggested as one of the three protein-families responsible for CLL cell identity.²⁰²

AKT / GSK3

GSK3 (Glycogen Synthase Kinase 3) is a serine/threonine protein kinase that provides inhibitory phosphorylation to substrates.²⁰³ GSK3 is involved in regulation of cell survival (phosphatase and tensin homolog [PTEN], induced myeloid leukemia cell differentiation protein [MCL1], rapamycin-insensitive companion of mTOR [RICTOR]),²⁰⁴⁻²⁰⁶ proliferation (c-MYC, c-JUN),^{207,208} or metabolism (hypoxia-inducible factor 1 [HIF1 α], CCAAT enhancer-binding protein [CEBP]).^{209,210} GSK3 is generally active in the absence of stimulatory signals. Upon cell activation, the AKT exerts inhibitory phosphorylation on the GSK3 amino-terminal motive, which inhibits the substrate accessibility to GSK3. AKT activation thus may lead to stabilization of some GSK3 substrates.²⁰³ In addition, active GSK3 positively regulates NF κ B signaling pathway, by providing phosphorylation to the p65 subunit. Phosphorylation of p65 triggers the conformational changes, that lead to disruption of inhibitory complex and increase in p65 transcriptional activity. Interestingly, GSK3 inhibition or genetic ablation has no effect on I κ B degradation or nuclear p65 translocation.^{209,211} In addition, the GSK3 inhibition leads to epigenetic silencing of NF κ B target genes and induction of apoptosis.²¹²

3.3.2. BTK and PLC γ

BTK is another non-receptor kinase that is involved in various signaling pathways, that control migration (e.g., CXCR4, CXCR5), cytokine signaling (interleukin 6 receptor [IL6R]), immune response (toll-like receptor [TLR]), or BCR signaling itself. BTK is predominantly cytoplasmic protein, which can be transiently translocated to the cytoplasmic membrane through its PH domain and PIP3.²¹³ Subsequent BTK activation is dependent on binding to B-cell linker (BLNK) adaptor molecule, which mediates the SYK-dependent BTK phosphorylation at Y551.²¹⁴⁻²¹⁶ It is thought that active conformation of BTK is ensured by autophosphorylation at Y223.²¹⁷ This, however, remains controversial and unresolved, since another study showed that mutated form of BTK (BTK^{Y223F}) did not significantly affect the BTK function in mice.²¹⁸ Nevertheless, phosphorylated BTK can further activate its' substrate PLC γ , which is recruited to the BTK proximity by BLNK. Activated PLC γ cleaves PIP2 just before the phosphate group, generating thus the inositol triphosphate (IP3) and diacylglycerol (DAG).^{219,220} (Demonstrated in Figure 10.) In addition, previous studies showed that BCR-mediated AKT activation is just partially dependent on BTK, and activation of PI3K was not affected by BTK deletion.²²¹

IP3

Whereas the DAG remains bound to the membrane, IP3 is directly released into the cytoplasm. Here it diffuses through the cytosol to find IP3 receptors, mostly expressed on the smooth endoplasmic reticulum. Their coupling promptly releases the calcium ions from endoplasmic storage into the cytoplasm (Figure 10). This event is usually followed by a second wave of calcium influx, this time released by calcium channels anchored in the cytoplasmic membrane.²²² This process is known as capacitive calcium entry and the mechanism behind the activation of membrane channels relies on two protein families. The family of stromal interaction molecules (STIM) serves in the endoplasmic reticulum as a calcium level sensor, which is translocated to the proximity of the cell membrane upon the calcium release. Here the STIM proteins can activate the proteins from Orais' protein family, which act as pore-forming subunits of calcium channels.²²³ The increased concentration of calcium ions (from ~100nM to ~1 μ M) activates downstream signaling pathways and transcriptional factors.²²⁴

Calmodulin is one of the best described binding partners of calcium ions. This complex activates calcium pumps, which promptly remove calcium from the cytosol or store it in the endoplasmic reticulum. This complex also binds to calcineurin, the calmodulin-dependent

serine/threonine phosphatase, which can activate the nuclear factor of activated T-cells (NFAT) transcription factors by removing the inhibitory phosphorylation.²²⁴

DAG

Due to the hydrophobic properties, DAG remains anchored in the cell membrane, where it serves as a docking site for PKC. Interestingly, unlike other kinases, PKC does not require acute phosphorylation for activation. It is fully activated upon the conformational changes caused by binding to DAG. It is believed that PKC requires the presence of calcium ions for its proper function (Figure 10),²²⁵ however, the experiments with IP₃ receptors deletion (calcium channels) showed no effect on PKC activation.²²⁶ Once the PKC is activated, it can positively regulate the activity of various signaling cascades like NFκB, JNK, or MAPK/ERK.^{227,228} Apart from this, active PKC induces negative feedback by phosphorylating BTK on S180 leading to its dissociation from membrane and termination of its function.^{228,229}

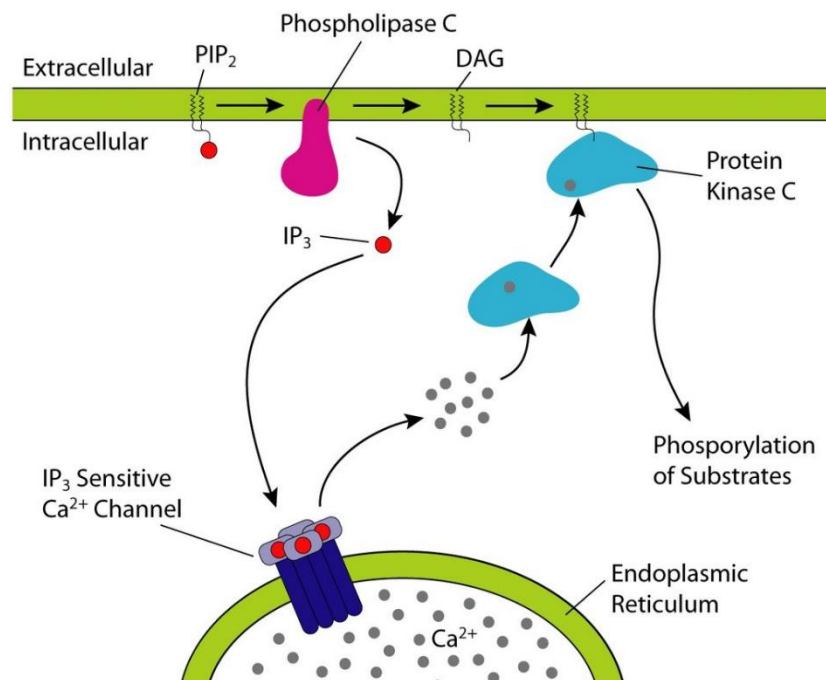


Figure 10. PIP₂ cleavage and downstream events. PIP₂ represents less than 1 % of all membrane phospholipids, however, it triggers a remarkable number of cellular processes. Its cleavage by PLCγ generates two different kinds of second messengers: IP₃ and DAG, which can further activate various positive and also negative signaling events. Adapted from URL 1.

3.4. Signaling termination - negative regulators of BCR signaling

Negative regulation of B cell receptor signaling is an essential mechanism controlling abnormal B cell activity. The downregulation or the complete loss of negative regulators has been described in several leukemias and lymphomas.²³⁰⁻²³²

One of the most important negative regulators of BCR signaling is LYN kinase. Besides its role in BCR signaling initiation (see Chapter 3.1. Signal initiation), it also triggers the main negative regulatory feedbacks mediated by phosphatases.²³³ Its role as a negative regulator was demonstrated in LYN-deficient mice, where the peripheral B cells exhibited enhanced BCR signaling activity²³⁴ and increased proliferative responses.²³⁵ These counterintuitive results indicated the indispensable role of LYN in negative regulatory actions, and its redundancy with other Src-family members during ITAM activation.^{151,236} To suppress the BCR signaling, LYN kinase phosphorylates the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in adaptor molecules like Fc receptor for IgG type IIB (FC γ RIIB), CD22 or CD5. Subsequently, these adaptor molecules recruit various phosphatases which further remove the activation phosphate/s from BCR-downstream proteins.^{237,238}

The activation of FC γ RIIB or CD5 leads to the creation of a docking site for PTEN and Src homology 2 domain-containing inositol polyphosphate 5-phosphatase 1 (SHIP1). These phosphatases can remove the 3' phosphate of PIP3 and thus attenuate the PI3K/AKT signaling or BTK/PLC γ downstream signaling.²³⁹⁻²⁴¹ The most prominent binding partner of CD22 is the Src homology region 2 domain-containing phosphatase-1 (SHP1),²⁴² which directly inactivates substrates like SYK, BLNK, Vav, CD19, or CD22 itself.²⁴³⁻²⁴⁵ In addition, CD22 was recently identified as an important BCR signaling inhibitor, which is upregulated in response to therapy with DNA-damaging drugs.²⁴⁶

Recently, another type of negative regulation of BCR signaling has been described. It is based on the ability of the IgM Fc receptor (Fc μ R) to interact with BCR.²⁴⁷ Their interaction leads to the complex internalization and significant increase in stimulatory threshold required for B cell activation. In addition, the absence of Fc μ R leads to increased BCR expression and enhanced BCR signaling.²⁴⁸

The complexity of BCR signaling pathway is summarized in the following Figure 11.

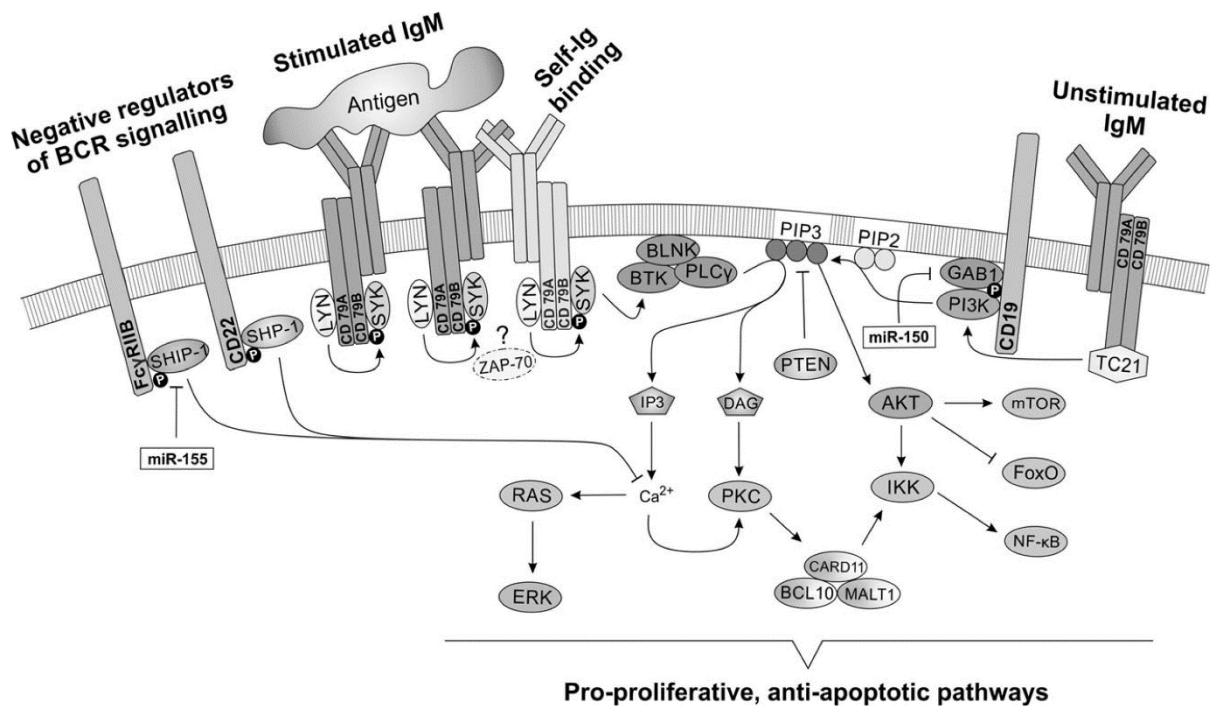


Figure 11. BCR signaling pathway. Triggering of BCR pathway by antigen or self-binding leads to activation of various downstream pathways. The intensity of BCR signaling and levels of active kinases (LYN, SYK, BTK, PI3K) is precisely regulated by the balance between pro-activator molecules like (GAB1, BLNK, GRB2) and phosphatases (SHIP1, SHP1) together with their adaptor molecules (FcγRIIB, CD22). Activation of AKT, ERK, or NFκB contributes to the survival and proliferation of CLL cells. (Adapted from Seda *et Mraz*, 2015.)²⁴⁹

3.5. Tonic signaling

It has been noticed several years ago that even in the absence of antigen, the resting lymphocytes exhibit low-level phosphorylation of signaling intermediates like LYN, SYK, AKT, or NFκB.²⁵⁰⁻²⁵² This antigen-independent activity was termed as “tonic” signaling and was shown to play an essential role in B cell survival, given that the conditional deletion of BCR in mice resulted in lymphocyte clearing with a half-life of 3-6 days.²⁵⁰ Interestingly, the constitutive activation of different pro-survival signaling mediators like NFκB, ERK, or upregulation of B-cell lymphoma 2 (BCL2) was not sufficient to sustain the survival of BCR-negative cells. Only the low level of constitutively active PI3K/AKT signaling exhibits the ability to rescue survival

of mature B cells with a lack of BCR. This implies that tonic signaling and B cell survival rely on BCR/PI3K/AKT pathway.^{250,253}

The attempts to identify the origin of this signaling were divided into two major streams – activation mutations in BCR-related proteins or the existence of endogenous antigen. Since the sequencing of CLL B cells failed to identify universally shared, signaling-relevant mutations in BCR or downstream mediators,²⁵⁴⁻²⁵⁷ the further attempts focused more on the self-antigen hypothesis. Recently, Ziegler *et al.*, 2019 described that CLL cells bind autoepitopes in the same surface immunoglobulins leading to antigen-independent oligomerization of BCRs, creating microclusters and triggering basal signaling.¹³⁸ Similarly, the serum immunoglobulins isolated from CLL patients were able to bind B cells from healthy donors, whereas immunoglobulins from healthy donors were not.²⁵⁸ This self-binding ability is localized in the conserved epitope in the second framework of the VH domain of CLL BCR. The simple point mutation in this region suppressed oligomerization of BCRs and autonomous signaling, however, the antigen-induced signaling was not affected supporting the idea of antigen-independence.^{138,258,259}

In addition, growing evidence indicates that so-called “tonic” signaling plays an important role in CLL cell survival and thus represents an interesting therapeutic target. However, this will require further investigation.

4. CLL therapy

Chronic lymphocytic leukemia is still considered to be an incurable disease with highly variable prognosis. The indication to initiate therapy is very specific and usually includes disease-related changes in the blood count like anemia or thrombocytopenia, or physical symptoms like lymphadenopathy, splenomegaly, night sweats, fatigue, weight loss, fever, and others.² The treatment of CLL can be based on chemotherapy, chemotherapy combined with immunotherapy, or inhibitors targeting specific proteins from the BCR signaling pathway or BCL2 protein.¹

The chemotherapy has been used for the treatment of CLL for more than 50 years. The most commonly used agent is purine analog fludarabine, which inhibits DNA synthesis by interfering with ribonucleotide reductase and DNA polymerase. Another chemotherapy is based on alkylating agents such as chlorambucil, cyclophosphamide, or bendamustine, which inhibits the cell proliferation by crosslinking guanine in DNA double-helix strands making them unable to uncoil and separate during DNA replication.^{2,260} Currently, the most common combination of drugs for CLL treatment is FCR regimen consisting of fludarabine, cyclophosphamide, and anti-CD20 monoclonal antibody rituximab. Almost 90% of patients respond to this treatment and approximately 40% of them achieve complete remission.²⁶¹ However, FCR therapy is not suitable for patients after relapse or for those diagnosed with specific mutations like del(17p) or del(11q), given that these patients exhibit resistance to this kind of treatment. Moreover, the use of these conventional drugs usually leads to severe side effects like myelosuppression, nausea, loss of hair, but may also lead to the development of secondary cancer such as acute myeloid leukemia.¹ Current approaches in the drug development are focused on inhibition of specific proteins involved in pathways important for cell survival and/or proliferation. This enables to induce remission in patients exhibiting abnormalities in the *TP53* gene or after FCR relapse. The most widely used inhibitors are targeting BTK (ibrutinib), PI3K (idelalisib) or BCL2 (venetoclax).^{18,121,262}

Ibrutinib is an orally administered small-molecule drug that covalently and selectively binds to BTK and inhibits its enzymatic activity. Inhibition of BTK function impairs not only the BCR-related downstream signaling but also signaling from various chemokine receptors such as

CXCR4 or CXCR5, or integrin activity,^{20,263} leading to reduced lymphadenopathy and transient leukocytosis lasting for approximately 8 months or even more than 12 months.^{264,265} This increased survival of CLL cells in the periphery was not associated with a worse prognosis or potential to relapse,²⁶⁵ however their slow apoptosis during the ibrutinib therapy suggests the possible existence of an adaptation mechanism that prolongs CLL cell survival. Indeed, recent studies are pointing more and more at compensatory activation of the PI3K/AKT pathway, which is known for its ability to rescue B cells from apoptosis.^{21,250,253} For example, the studies in diffuse large B cell lymphoma revealed that overexpression of CD79 β had been sufficient to induce AKT activation and resistance to ibrutinib.²⁶⁶ Similarly in mantle cell lymphoma, the activation of the mTORC2 complex resulted in increased phosphorylation of AKT.²⁶⁷ However, the information about the mechanism triggering this adaptation process is still very limited.

Idelalisib is another type of BCR inhibitor commonly used in CLL treatment. The drug acts as a competitive inhibitor of the ATP binding site of the PI3K catalytic subunit 110 δ , which impairs not only the BCR downstream signaling but also the migration towards chemokines and adhesion to stromal cells. This is further reflected in reduced lymphadenopathy and increased numbers of lymphocytes circulating in peripheral blood. The final physiological outcome of idelalisib administration is thus very similar to ibrutinib.^{121,268} Recently, a new generation of PI3K inhibitors targeting also PI3K subunit p110 γ was approved for CLL treatment. This is of note since the p100 γ subunit was shown to increase CLL cell migration towards CXCL12 or strengthen the response to CD40L and IL4.¹⁸⁵ Despite the good initial response to this treatment, a fraction of CLL patients develop resistance. However, unlike in ibrutinib treatment, the mechanism of PI3K resistance remains even more unclear.²¹

BCL2, the venetoclax target, is often upregulated in CLL cells, which contributes to their prolonged survival and accumulation.²⁶⁹ The mechanism of BCL2 upregulation in CLL was a mystery for a long time, until 2002 when Calin *et al.* discovered that direct inhibitors of *BCL2* translation, miRNA-15 and miRNA-16, are down-regulated in the majority of CLL.⁷ Moreover, the subsequent studies showed that loss of miRNA-15 and miRNA-16 expression induces BCL2 expression and promotes proliferation of mature B cells.²⁷⁰ Administration of venetoclax and inhibition of BCL2 protein thus sensitize the CLL cells to apoptosis and inhibits the cell proliferation.²⁷¹

5. Aims of the thesis

The main aim of the presented dissertation is the identification and further functional validation of novel regulators of CLL cell migration into supportive microenvironmental niches. This thesis is also focused on the regulation of so-called “tonic” signaling, the signaling which is utilized by malignant B cells to survive in peripheral blood without supportive microenvironmental stimuli before their return to immune niches.

- 1) Identification and validation of migration-related genes
 - *Chapters: 7.1. – 7.3.*
- 2) Mechanism of upregulation of selected migration-related gene
 - *Chapter: 7.4.*
- 3) Contribution of GAB1 to tonic BCR signaling in CLL cells without therapy, and in the context of targeted therapy
 - *Chapters: 7.5 and 7.6*
- 4) Investigation of the effect of novel GAB1 inhibitors on migration, tonic signaling, and survival of malignant CLL cells.
 - *Chapter: 7.7.*

6. Material and Methods

6.1. CLL samples

Primary CLL samples were collected with written informed consent, and the Institutional Review Board approved the study. Peripheral blood samples were obtained from patients who did not receive any therapy for at least six months (except the ibrutinib/idelalisib treated patients). The samples from ibrutinib/idelalisib-treated patients were collected at a day before the drug administration and during therapy at the time points indicated in the figure legend. All primary CLL samples were purified by negative selection with RosetteSep Human B Cell Enrichment Cocktail (Stemcell Technologies) and RosetteSep Human CD3 Depletion Cocktail (Stemcell Technologies) and Ficoll-Paque PLUS (Sigma Aldrich) according to manufacturer's protocol and the final purity was >95% of CD5+19+ cells. Purified primary CLL cells were cultivated in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Biosera) and 100 U·ml⁻¹ / 100 µg·ml⁻¹ of penicillin/streptomycin (Sigma Aldrich) and in 5% CO₂ at 37°C. For patients characteristics see Supplementary material 3 (page 194).

6.2. Cell culture

MEC1 cell line was obtained from the German Collection of Microorganisms and was cultivated in Iscove's Modified Dulbecco's Medium (IMDM, Biosera) supplemented with 10% fetal bovine serum (Biosera) and 100 U·ml⁻¹ / 100 µg·ml⁻¹ penicillin/streptomycin (Sigma Aldrich) and further cultivated in 5% CO₂ at 37°C. OSU-CLL cell line was a kind gift from Dr. Byrd (The Ohio State University) and was cultivated in RPMI-1640 media (Biosera) supplemented with 10% fetal bovine serum (Biosera) and 100 U·ml⁻¹ / 100 µg·ml⁻¹ penicillin/streptomycin (Sigma Aldrich) and further cultivated in 5% CO₂ at 37°C. HS5 cell line as well as HEK293-FT cell line were obtained from the German Collection of Microorganisms and both were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Biosera) supplemented with 10% fetal bovine serum (Biosera) and 100 U·ml⁻¹ / 100 µg·ml⁻¹ penicillin/streptomycin (Sigma Aldrich) and further cultivated in 5% CO₂ at 37°C.

6.3. Gene expression analysis

Total RNA was isolated by TRIzol (Molecular Research Center) as described previously.²⁷² Protein-coding gene expression analyses was performed using TaqMan Gene Expression Assays (Thermo Fisher Scientific) and normalized to an endogenous control (*GAPDH*, *HPRT*), as described previously.²⁷³ Probes used in this study: *GAB1* (Hs00157646_m1), *FoxO1* (Hs00231106_m1), *NCK2* (Hs02561903_s1), *MYLK* (Hs00364926_m1), *GPR183* (Hs00270639_s1), *FLNA* (Hs00924645_m1), *SERPINF1* (Hs01106937_m1), *SEMA4F* (Hs01586592_m1), *HPRT1* (Hs02800695_m1), *GAPDH* (4333764F), mouse *CXCL12* (Mm00445553_m1), mouse *CXCL13* (Mm00444534_m1), mouse *HPRT* (Mm03024075_m1).

6.4. mRNA-seq and data analysis

The sequencing of poly(A) transcriptome of CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} CLL intracлонаl subpopulations was performed as described previously.¹²³ Briefly, libraries were prepared using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) according to the manufacturer instructions. The final libraries were diluted to the desired concentration of 2 pM and were used to prepare a paired-end flow cell using a cBot System (Illumina) and then sequenced with Illumina NextSeq 500/550 High Output v2.5 kit (Illumina) generating 75-bp paired-end sequences.

The quality of the sequencing data was checked using the FastQC²⁷⁴ (v0.11.3) and Kraken package²⁷⁵ (v13-274). The presence of adapters was scanned using Minion and Swan (Kraken package,²⁷⁵ v13-274). Pre-processing of the raw reads was done using Trimmomatic²⁷⁶ (v0.36) in the following steps (i) *N* and very low-quality bases (Phred < 3) from both 5' and 3' ends were removed, (ii) Poly(A) and poly(T) tails were removed, (iii) Sequencing adapters were removed, (iv) Reads were trimmed to a maximal length of 75 bp, (v) Low quality ends with average Phred score < 5 of 4 consecutive bases were trimmed using the sliding window approach, (vi) Reads shorter than 15 bp and without a proper pairing after the pre-processing were removed. The pre-processed reads were then mapped to the *Homo sapiens* reference genome (Ensembl²⁷⁷ release 84, primary assembly) together with corresponding Ensembl genome annotation (Ensembl release 84) using STAR²⁷⁸ (v2.5.2a) in 2-pass mapping approach. Exon-mapped reads were summarized to genes using STAR (a similar approach to the HTSeq-count²⁷⁹ 'union') . Only uniquely mapped reads were used. Strandedness of the sequencing protocol was taken into account and 'reverse' counts were used. Differential gene expression was analyzed using DESeq2²⁸⁰ (v1.13.8) Bioconductor²⁸¹ package for genes with a base mean

count >200 per million. P-values were adjusted for multiple testing using the Benjamini-Hochberg method. The fold change was calculated as CXCR4^{dim}CD5^{bright}/CXCR4^{bright}CD5^{dim} cells. The heatmap visualization was created using the Heatmapper tool.²⁸¹

6.5. siRNA transfection

MEC1 cells or freshly isolated primary CLL cells (5×10^6) were electroporated (Neon Transfection System, Thermo Fisher Scientific (program for MEC1 cells: 1500 V / 20 ms / 2 pulses; program for primary CLL cells: 1900V / 30ms / 1 puls) with a short interfering RNA (siRNA) against *GAB1*, or against *FoxO1* (both On-Target Plus Smartpool siRNAs, 1000 nM, Dharmacon), or by On-Target Plus Smartpool Negative Control (1000 nM, Dharmacon). The cells were harvested 48 hrs after transfection for further analyses.

6.6. Flow cytometry and cell sorting

The primary CLL cells or cell lines were labeled with one or combination of these antibodies: CD5 (FITC, UCHT2; Sony), CD184 [CXCR4] (PE, 12G5; Sony), CD19 (PECy7, JC-119, Beckman Coulter), CD45 (BV510, HI30; SONY), CD105 (A647, 43A3; SONY) and labeled with SYTOX-BLUE (Invitrogen) for viability. For intracellular staining, the cells were further fixed using 4% paraformaldehyde (PFA) and 50% methanol and stained with primary rabbit antibodies against *GAB1* (#3232, 1:100; Cell Signaling) or *FoxO1* (#2880, 1:100; Cell Signaling) and secondary anti-rabbit antibody conjugated with Alexa-647 (#4414, 1:500; Cell Signaling). Data were collected from at least 20,000 events using BD FACS Verse and analyzed using FlowJo software (version 10). Normalized expression of intracellular proteins was calculated as mean fluorescent intensity (MFI) of the protein of interest relative to MFI for secondary antibody only.

Cell viability was investigated by 3,3'-dihexyloxacarbocyanine iodide (DiOC6) together with propidium iodide (PI) staining (Thermo Fisher Scientific). All measurements were performed on FACS Verse (BD Biosciences-US) and cell sorting on BD FACS Aria Fusion (BD Biosciences-US).

6.7. Calcium flux assay

The primary CLL cells (1×10^6 /ml) were incubated in Hanks Balanced Salt Solution (HBSS) containing 2 mM Fluo-4AM (Molecular Probes) for 30 min at 37°C. We established a baseline fluorescence threshold for each sample (by acquiring samples for 60 seconds without any

stimulus). We then calculated the peak Fluo-4AM signal and fluorescence intensity above baseline Fluo-4AM signal following stimulation.

6.8. HS5 coculture and conditioned media

HS5 cells were seeded at 2×10^6 in 75 cm² culture flasks in DMEM with 10% FBS. The next day, DMEM medium was replaced by IMDM or RPMI media containing 10% FBS and 2.5×10^6 /ml of MEC1 cells (in IMDM) or 5×10^6 /ml primary CLL cells (in RPMI). The HS5 coculture was allowed for the specified time and then harvested and stained with Sytox blue and CD105 antibody. The purity of B cells after sorting was validated by anti-CD19 antibody. The conditioned media was harvested after cultivating confluent HS5 cells in DMEM with 10% FBS for 48 hrs.

To prepare conditioned media, 3.5×10^6 of HS5 cells was seeded in T75 cultivation flask (TPP) in 12 ml of DMEM media. The next day, original media was discarded, and the cells attached to the bottom of the flask were washed once with 10 ml of PBS. Subsequently, 25 ml of RPMI media was added to the attached HS5 cells. The conditioned media was collected after 48 hrs, separated from cell-debris by 10 minutes centrifugation at 2000 RCF and diluted with fresh RPMI media containing 10% FBS and $100 \text{ U} \cdot \text{ml}^{-1}$ / $100 \text{ } \mu\text{g} \cdot \text{ml}^{-1}$ penicillin/streptomycin to the final concentration of 50% conditioned media. This media was immediately used for experiments.

6.9. Primary human mesenchymal stromal cells and conditioned media

Human bone marrow was obtained from a healthy male donor with a written consent. Cells were resuspended in osmolysis buffer and spined (300g for 10 min). This step was repeated till all erythrocytes were removed. Cells were subsequently washed with PBS and plated in alphaMEM media (Sigma-Aldrich) containing 10% FBS and $100 \text{ U} \cdot \text{ml}^{-1}$ / $100 \text{ } \mu\text{g} \cdot \text{ml}^{-1}$ of penicillin/streptomycin (Sigma-Aldrich). Cells were cultured at 37°C for 48 hrs and subsequently washed (3x) with PBS in order to remove non-adherent cells. Adherent cells were resupplemented in fresh media, and incubated for another 48 hrs. For all experiments only cells within 2nd-6th passage were used. To prepare conditioned media, 4×10^6 of the primary human bone marrow mesenchymal stromal cells were seeded in T75 cultivation flask (TPP) in 12 ml of full alpha-MEM media. The next day, original media was discarded, and the cells attached to the bottom of the flask were washed once with 10 ml of PBS and supplemented with fresh 12 ml of alpha-MEM media. The conditioned media was collected after 72 hrs, separated from

cell-debris by 10 min centrifugation at 2000 RCF and immediately used for experiment or directly frozen at -80°C.

6.10. Chemokine concentration measurements by ELISA

The levels of chemokines SDF1 (CXCL12) and CXCL13 (BLC/BCA-1) were measured in conditioned media produced by HS5 or by primary human bone marrow mesenchymal stromal cells (for conditioned media preparation see above), or in serum from CLL peripheral blood or paired serum sample from sternal puncture in CLL patients. All measurements were performed according to manufacturer's protocol (Quantikine, R&D Systems, Minneapolis, MN). The absorbance at 450 and 570 nm was measured by TECAN SPARK.

6.11. Migration assays and F-actin polymerization

The competitive migration assay was performed by staining of cells transfected with siRNA against *GAB1* (or control), or *GAB1/ FoxO1* knock-out cells, with CFSE or FarRed CellTrace dye according to manufacturer protocol and incubated overnight. In some cases were the cells traced with a plasmid encoding fluorescent protein (GFP, Azurit). The next day, the stained cells were analyzed for viability (stained with Sytox Blue; Thermo Fisher Scientific). The paired samples with low viability (<80%) were excluded from further experiments as well as samples where the difference in viability between paired samples was higher than 5%. To compare the migration, the stained cells were mixed in ratio 1:1 and injected into the tail vein of NSG mice,²⁸² or loaded to transwell inserts with 5 µm pores (Falcon) according to manufacturer protocol. The migration towards SDF1 (100 ng/ml for CLL cells or 250 ng/ml for MEC1 cells), CXCL13 (250 ng/ml for CLL cells or 500 ng/ml for MEC1 cells), 100% conditioned media produced by primary human bone marrow mesenchymal stromal cells, or 50% conditioned media produced by HS5 cells was allowed for the indicated time and subsequently analyzed by flow cytometry. The F-actin polymerization was performed by staining with Phalloidin-iFLuor488 (Abcam) and visualized by EVOS Cell Imaging Systems (Thermo Fisher Scientific).

6.12. Plasmid transfections, CRISPR/Cas9, transduction of shRNAs

The *GAB1*^{MYC} plasmid was kindly provided by Armelle Yart¹⁷⁶ and empty control plasmid was a gift from Adam Antebi (Addgene plasmid #52535). pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid #48138;).

The gRNA sequences targeting the second exon of the *GAB1* gene or first exon of *FoxO1* gene were designed in Benchling (Biology Software, 2019) online tool. The sequence 5'-CTACTTGGTAGCAGACAGCG-3' for *GAB1*-gRNA or 5'-ATCCACATCGAGGCTCCTCG-3' for *FoxO1*-gRNA was cloned into pSpCas9(BB)-2A-GFP vector (#48138, Addgene). To obtain the mutant variant, the MEC1 wild-type cells (MEC1^{WT}) cells were electroporated as described above for siRNAs. After 48 hrs viable cells were sorted according to GFP-positivity, expanded, and validated for absence of *GAB1* or *FoxO1*.

To perform inducible shRNA expression, the sequence 5'-CCGGAGTTAACACACTCGTAGTATTCTCGAGAATACTACGAGTGTGTTAACTTTTTTG-3' was purchased from Sigma Aldrich and subsequently cloned into the empty plasmid Tet-pLKO-puro for shRNA expression.²⁸³ This plasmid was purchased from Addgene (Addgene plasmid #21915) as well as the envelope plasmid pCMV-VSV-G (Addgene plasmid #8454). The packaging plasmid dR8.91 was kindly provided by M. Smida (Masaryk University). Virus particles were produced in HEK-293-FT (obtained from ATCC) after plasmids transfection by Dharmacon (Horizon) and added to desired B cell lines. The transduced cells were subsequently selected by puromycin (3 µg/ml, Sigma Aldrich) for 5 days. The expression of shRNA in B cell lines was induced by adding tetracycline (1 µg/ml; Sigma Aldrich) into media (tet+) for 48 hrs, and control cells were treated with vehicle (tet-).

For *in vivo* growth assay MEC1^{WT} and MEC1^{GAB1-KO} cell lines were transduced by GFP or Azurit encoding vectors (both obtained from Addgene; #36083 and #36086, respectively) as described above. Cells were subsequently sorted for GFP or Azurit positive cells.

6.13. Immunoblotting

Cells were lysed in lysis buffer (1% SDS, 50 mM TRIS-HC pH 6.8, 10% glycerol) with phosphatase and protease inhibitors (Sigma Aldrich) and protein concentration was determined using DC Protein Assay (BioRad). Equal amounts of protein were separated by SDS-PAGE and transferred to the PVDF membrane (0.45 µm pore size, Millipore). The membranes were incubated with the Cell Signaling antibodies specific for the following immobilized proteins: *GAB1* (#3232, 1:2000), p*GAB1*^{Y307} (#3234, 1:2000), *FoxO1* (#2880, 1:1000), p*FoxO1* (#9461, 1:2000), *AKT* (#2920, 1:3000), p*AKT* (#4060, 1:3000), *BCL_{XL}* (#2764, 1:2000), *SYK* (#12358, 1:2000), *PLCγ* (#3872, 1:2000), *CD79α* (#3351, 1:2000), *ERK* (#4696, 1:3000) p*ERK* (#4377, 1:3000), *B-actin* (#4970, 1:5000), *GAPDH* (#2118, 1:4000), or with antibodies obtained from Abcam - p*GAB1*^{Y627} (ab131458, 1:2000), anti-*CD20* antibody (Ab78237, 1:2000). Secondary

horse-radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibodies (both Cell Signaling) were used to detect primary antibodies. Immunocomplexes were detected using ECL (BioRad), and the chemiluminescent signal was digitally detected with UVItect Alliance 4.7 (UVItect).

6.14. ChIP

Chromatin immunoprecipitation (ChIP) was performed as previously described by Carey *et al.*²⁸⁴ Briefly, 20x10⁶ MEC1 cells were fixed in 1% paraformaldehyde. The fixation was quenched by glycine after 10 min. After washing with cold PBS, the fixed cells were lysed in 10ml of ChIP-lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40) and incubated on ice for another 10 min. The cell nuclei were pelleted by centrifugation and subsequently lysed in 1ml of nuclei-lysis buffer (50 mM TRIS-Cl pH 8.0, 10 mM EDTA, 1% SDS) containing protease inhibitors. Sonication was performed by the Covaris S220 sonicator in original glass tubes. The instrument was set according to manufacturer protocol (PIP:140, DF: 5, CBP: 200, treatment time: 20 min, temperature min/max: 3/9°C). The cell debris was subsequently discarded by centrifugation and DNA concentration was measured by Nanodrop 2000 (Thermo Fisher Scientific). One-hundred µg of chromatin was aliquoted per antibody and diluted with dilution buffer (16.7 mM TRIS-Cl pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% TRITON X-100, containing protease inhibitors) to a final volume of 300 µl. Chromatin was precleared with 50 µl of protein A/G ChIP-grade magnetic beads (Thermo Fisher Scientific). The magnetic beads were discarded after 2 hrs of incubation, and 10 µl of anti-FoxO1A antibody (ab39670; ChIP Grade, Abcam) or 10 µl of control rabbit IgG (#2729; Cell Signaling) was added to chromatin. The samples were rotated and incubated at 4°C overnight. The next day, the immunocomplexes were pulled down by 50 µl of A/G ChIP-grade magnetic beads (Thermo Fisher Scientific) blocked by salmon sperm and BSA. The beads were washed three times in 1ml of High-salt wash buffer (50 mM HEPES pH 7.9, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% TRITON X-100, 0.1% deoxycholate). The elution was performed in 300 µl of Elution buffer (50 mM TRIS-Cl, 10 mM EDTA, 1% SDS) supplemented with 20 µg of proteinase K. Samples were incubated for 2 hrs at 55°C and then overnight at 65°C to reverse crosslinking. The DNA was purified by QIAquick PCR Purification Kit (Qiagen). The qRT-PCR was performed by using Luna Universal qRT-PCR Master Mix (New England Biolabs), and three different sets of primers (see supplemental Table 5), which were designed using Primer3 online tool. The prediction of possible FoxO1-binding sites in the 3.5 kb promotor

region of the *GAB1* gene was performed with ContraV3 online tool. The non-specific primer-set was designed into the 2nd intron of *GAB1* where there is no predicted binding site for FoxO1 (forward: 5'-CAGTCAGTCTAGTAGGAGGGACAA-3', reverse: 5'-CTGAAAAGATCACTGCTGCTGCTA-3'). Two sets of primer were designed for the promotor region with predicted binding sites for FoxO1 (TBS no. 4: forward 5'-TTTGGGGCTTTTTCACAAAT-3', reverse 5'-TGCCTACTTCCTTGTGTGTTTC-3'; TBS no.10: forward 5'-ATGCTGCCTATGGGAGAAGA-3', reverse 5'-AAACCAAAGCACTTCTTTTAAAGT-3'). The results were calculated as % of input.

6.15. Confocal microscopy

The MEC1 cells were plated on poly-L-lysine coated (0.01%) glass for 30 min. Glass-attached MEC1 cells or primary CLL cells in suspension were subsequently fixed with 1% paraformaldehyde for 15 min/room temperature and washed (2x) with TBS-T. The 1 hr long blocking step in 5% BSA was followed by overnight incubation in primary antibody against FoxO1 (#2880, 1:100; Cell Signaling). The next day cells were washed (3x) in TBS-T and incubated in a mixture of secondary antibody (anti-Rabbit, Alexa-647 #4414, 1:250; Cell Signaling) and Phalloidin-iFLuor488 (1:1000; Abcam) for 90 min/room temperature (in dark). Subsequently, three wash steps with TBS-T were followed by staining with DAPI (1:1000; 15 min; Abcam), and cells were mounted to glass slides using Vectashield (Vector laboratories), and visualized on confocal microscope Leica DM5500 Q with TCS SPE confocal laser scanning system.

6.16. Cell motility by holographic microscopy

Cell motility speed and other measurements were performed as described previously.²⁸⁵⁻²⁸⁷ Briefly, tissue culture treated μ -Slide (Ibidi) with 0.8 mm channel height was first filled with the culture medium to coat the substrate with serum proteins. After several minutes, the medium was removed and the μ -Slide channel was filled with 200 μ l of cell suspension containing 10 000 exponentially growing MEC1 cells in a conditioned medium to achieve a subconfluent culture. Cells were incubated for 1 h at 37°C in a humidified atmosphere enriched with 5% CO₂. The μ -Slide with cells was then positioned on the stage of our Multimodal Holographic Microscope Q-PHASE (Tescan/Telight) and subjected to time-lapse recording for 1 h with 10 s lapse-interval using 10 $\times$ objective lenses 0.3 NA using the holographic mode measuring the optical thickness of cells with an accuracy of 2 nm. The recording was repeated 3 times for each

cell type on two different days producing 12 time-lapse sequences which were processed using in-house developed image processing procedure using Mathematica (Wolfram Research), which removes background fluctuations, reconstructs and subtracts the background, converts the optical thickness of cells into their dry mass distributions, segments the cells, calculates centroids of dry cell mass, and morphometric parameters of *Circularity*, *Elongation*, *Mass* and *Spreading* defined as:

$$Circularity = \sqrt{\frac{(4 \pi A)}{P^2}}$$

where A is cell area in μm^2 and P is the perimeter length in μm ;

$$Elongation = 1 - \left(\frac{Width}{Length}\right)$$

where *Width* and *Length* are the smallest and largest axes of the best fit ellipse respectively;

$$Spreading = A/Mass$$

where *Mass* is the total dry mass of a cell expressed in pg (picogram = 10^{-12} g).

The software also tracks the cells in time on the basis of the nearest cell with a similar dry mass in the next frame. Tracked centroids were processed in order to calculate *Displacements* and *Persistences*. *Persistence* was defined as the ratio of direct translocation in 60 s and the wiggly track distance was determined as the sum of six 10 s displacements. *The Persistence* of a cell moving in a straight line is 1, and bending of the trajectory reduces the *Persistence* towards 0.

6.17. Immunohistochemistry of paraffin-embedded tissues

Immediately after the tissue of experimental animals was dissected out, it was fixed in 4% paraformaldehyde pH 7.4 at room temperature overnight. The formalin-fixed paraffin-embedded (FFPE) tissue sections (1 μm) were automatically processed and stained in VENTANA BenchMark Ultra system. Universal DAB Detection Kit (Ventana) was used for staining in combination with UltraView Amplification Kit (Ventana). Counterstaining was achieved by using Hematoxylin II (Ventana) and post-counterstaining by using Bluing reagent (Ventana). Immunostaining was performed using the anti-CD20 (for MEC1 cells; M0701, DAKO) or anti-CD45 (for primary CLL cells; M0755, DAKO) antibody.

6.18. Immunohistochemistry of cryopreserved tissues

Immediately after the tissue of experimental animals was dissected out, it was fixed in 4% paraformaldehyde pH 7.4 at 4 °C for 5 hrs. Subsequently, liver and spleen were cryopreserved in 30% sucrose at 4 °C overnight and embedded in OCT medium (Tissue-Tek, 4583) on dry ice. Femurs were decalcified in 10% EDTA pH 7.4 for 7 days at 4 °C prior to cryopreservation and OCT embedding. Samples were sectioned on a cryostat (Leica CM1850UV) as 14-µm thick slices and collected on SuperFrost Plus Adhesion slides (Thermo Fisher Scientific, J1800BMNZ). Staining with primary antibody anti-mouse collagen IV (COL4, AbD Serotec, 2150-1470; 1:500) was performed at 4 °C overnight, followed by incubation with Alexa-conjugated secondary antibody (Invitrogen; 1:1000) at room temperature for 2 hrs. Cell nuclei counterstaining was performed with DRAQ5 Fluorescent Probe Solution (Thermo Fisher Scientific, 62251), and slides were mounted with Fluoromount Aqueous Mounting Medium (Sigma–Aldrich; F4680). Imaging was performed using Zeiss LSM880 laser scanning confocal microscope. ZEN2.1 (ZEISS) and Imaris (Bitplane) software was used for image processing.

6.19. Statistical analysis

Differences in expression levels between paired samples were compared using paired t-test or Wilcoxon matched matched-pairs test and differences in non-matched groups were compared using unpaired t-test or Mann-Whitney U test. The appropriate tests were chosen based on the results of Shapiro-Wilk test of normality of input data. Statistical analysis of GAB1 inhibitors' effect on cell viability in comparison to their combination with ibrutinib was analyzed by 2-way ANOVA including Tukey test. All analysis were performed in GraphPad v8.0.1.

7. Results

7.1. Validation of CXCR4/CD5 migration model

Seda V, Vojackova E, Ondrisova L, *et al.* FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia. *Blood*. 2021.

Pavlasova G, Borsky M, **Seda V**, *et al.* Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis. *Blood*. 2016;128(12):1609-1613.

CXCR4/CD5 cell subpopulations have been suggested as a model distinguishing cells ready to re-enter immune niches (CXCR4^{bright}CD5^{dim}) versus recent emigrants from lymph nodes (CXCR4^{dim}CD5^{bright}).^{13,122-127} However, this model has not been experimentally proven. To validate this model, we aimed to perform a direct *in vivo* competitive migration assay of sorted subpopulations, however labeling of the cells with an anti-CXCR4 antibody can affect their responsiveness to CXCR4 ligand SDF1. To overcome this, we used only CD5 antibody and forward scatter area for sorting since the lower forward scatter area (FSC-A) is typical for CXCR4^{bright}CD5^{dim} and higher FSC-A for the CXCR4^{dim}CD5^{bright} subpopulation. Small aliquot of sorted subpopulations was used to validate the CXCR4/CD5 positivity (Figure 12 A). Subsequently, the sorted populations were stained by two cell-trace dyes (CFSE or FarRed), mixed in ratio 1:1, and injected into the tail vein of NOD scid gamma (NSG) mice. After four hours mice were euthanized and blood, spleen, bone marrow, and liver were analyzed for the presence of stained cells. In line with the supposed model, the CXCR4^{bright}CD5^{dim} CLL cell subpopulation was significantly more migrating into the spleen from peripheral blood than CXCR4^{dim}CD5^{bright} cells. The migration of stained subpopulations into the bone marrow or liver was not statistically different (Figure 12 B), and their numbers were low (data not shown), which is in line with previously published data.²⁸⁸ We further confirmed the higher migration rate of CXCR4^{bright}CD5^{dim} subpopulation in an *in vitro* transwell migration assay towards microenvironmental cytokines SDF1, CXCL13, and conditioned media produced by primary human mesenchymal stromal cells (CM^{hMSC}) (Figure 12 C).

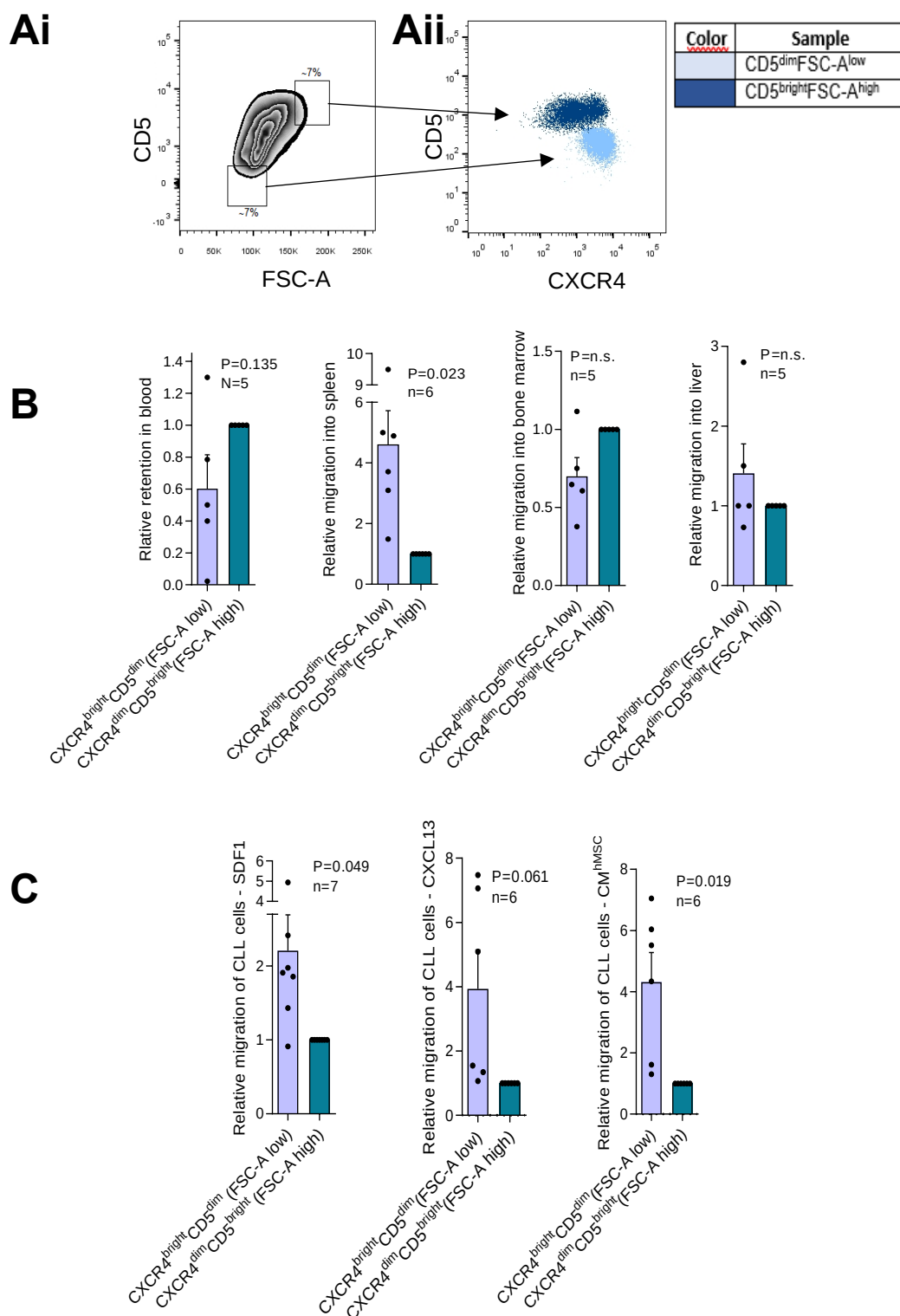


Figure 12. Validation of CXCR4/CD5 migration model. (Ai) Representative example of a gating strategy for sorting of CLL cells according to CD5 and FCS-A (forward scatter area). The CD5/FSC-A was used to prevent labeling by an antibody binding to the CXCR4 receptor, which would interfere with the migration to SDF1. **(Aii)** Evaluation of CXCR4 and CD5 expression in cells sorted according to the gating strategy shown in

[Ai]. Small aliquots of sorted CD5^{dim}FSC-A^{low} and CD5^{bright}FSC-A^{high} cells (correspond to CD5^{dim}CXCR4^{bright} and CD5^{bright}CXCR4^{dim} cells, respectively) were stained for CXCR4 and measured by flow cytometry to validate CXCR4 cell-surface levels and separation of CXCR4/CD5 subpopulations (this validation was performed in all samples used for experiments below). The sorting approach using CD5/FSC-A was successful in separating CXCR4/CD5 subpopulations in approx. 75% of CLL cases and only the samples with an acceptable profile of CXCR4/CD5 after validation by flow cytometry were used in further experiments. **(B)** Competitive migration of CXCR4/CD5 CLL cell subpopulation into the spleen, bone marrow and liver of NSG mice and their relative retention in the blood (migration continued for 4 hours, n=6). Cells were sorted according to FCS-A and CD5 as described in [Ai]. One sample was excluded from the analysis for technical reasons. **(C)** Competitive migration assay of CXCR4/CD5 CLL cell subpopulations towards SDF1, CXCL13, or conditioned media produced by bone marrow mesenchymal stromal cells from a healthy donor (CM^{hMSC}). Cells were sorted as described in [Ai], stained with two different CellTrace dyes, mixed in a 1:1 ratio, and loaded into transwells.

In the next step, we sorted CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} (for sorting strategy see Figure 13 A) subpopulations and used them for transcriptome analysis (RNA seq). This identified 1372 differentially expressed genes (fold-change>1.5, P<0.001), which contained 147 genes (fold-change>1.5, P<0.001) annotated in databases as migration-related (Figure 13 B). Several of these randomly selected genes were validated by qRT-PCR (Figure 13 C). The enrichment of migration/adhesion-related genes was confirmed by Gene Ontology Analysis (Table 1).

These experiments proved that CXCR4^{bright}CD5^{dim} subpopulation exhibits higher migration rate in spleen or towards microenvironmental cytokines, and exhibits increased expression of several migration-related genes.

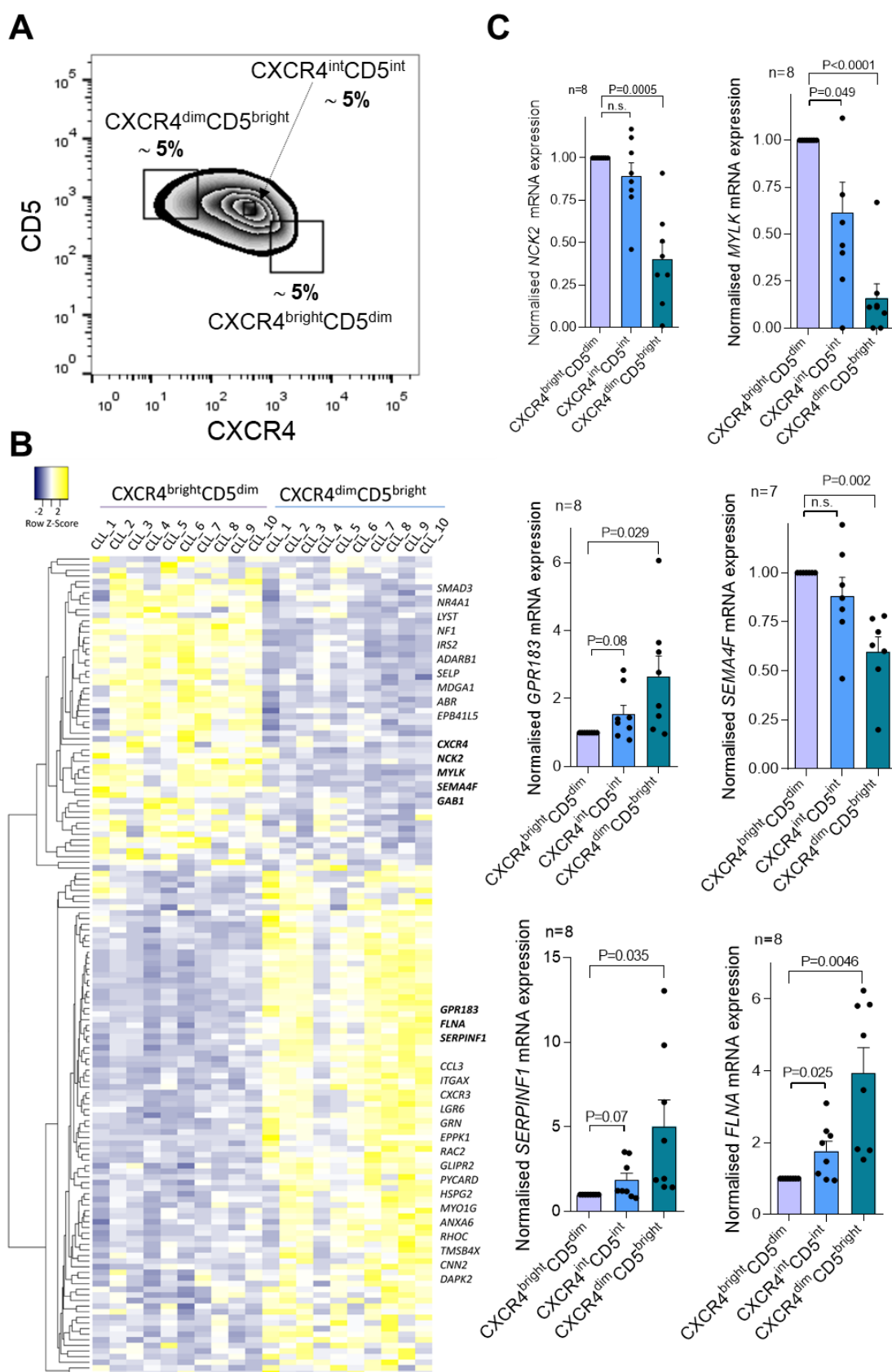


Figure 13. Identification of migration related genes differentially expressed in

CXCR4/CD5 subpopulations. (A) Representative gating strategy for CLL cells sorted according to CXCR4/CD5 expression (each subpopulation gated as ~5% of cells). This sorting, directly based on CXCR4/CD5 labeling, was used for RNAseq profiling and all experiments other than migration assays. **(B)** Heat map of differentially expressed genes in CXCR4/CD5 subpopulations (fold change >1.5; $P < 0.001$; $n = 10$), subsequently overlapped with a database of migration-related genes (GO:0016477).²⁸⁹ Lower expression is indicated in blue, higher expression in yellow. Gene names marked in bold were validated by real-time qRT-PCR these data are presented in [C]. **(C)** qRT-PCR validation of several randomly selected genes from [B]. The names stand for genes coding: NCK adaptor protein 2 (NCK2), myosin light chain kinase (MYLK), G-protein coupled receptor 183 (GPR183), Semaphorin-4F (SEMA4F), Serpin family F member 1 (SERPINF1) and filamin alpha (FLNA).

GO Term	Description	P-value
GO:0110053	regulation of actin filament organization	6.39E-12
GO:0032956	regulation of actin cytoskeleton organization	8.98E-12
GO:0032970	regulation of actin filament-based process	6.71E-11
GO:0030832	regulation of actin filament length	9.03E-11
GO:0008064	regulation of actin polymerization or depolymerization	2.79E-10
GO:0007015	actin filament organization	3.46E-9
GO:0030833	regulation of actin filament polymerization	1.62E-8
GO:0030029	actin filament-based process	1.52E-7
GO:0030036	actin cytoskeleton organization	1.95E-7
GO:0030838	positive regulation of actin filament polymerization	1.63E-6
GO:0032233	positive regulation of actin filament bundle assembly	3.86E-5
GO:0034314	Arp2/3 complex-mediated actin nucleation	7.41E-5
GO:0008154	actin polymerization or depolymerization	1.01E-4
GO:0045010	actin nucleation	3.41E-4
GO:0030834	regulation of actin filament depolymerization	4.3E-4
GO:0032231	regulation of actin filament bundle assembly	7.97E-4
GO:0040012	regulation of locomotion	1.35E-5
GO:0040017	positive regulation of locomotion	1.21E-4
GO:0040011	locomotion	1.65E-4
GO:2000145	regulation of cell motility	3.78E-6
GO:2000147	positive regulation of cell motility	4.5E-5
GO:0048870	cell motility	3.31E-4
GO:0030334	regulation of cell migration	3.58E-6
GO:0030335	positive regulation of cell migration	2.91E-5
GO:1903039	positive regulation of leukocyte cell-cell adhesion	1.77E-6
GO:0030155	regulation of cell adhesion	4.11E-6
GO:0045785	positive regulation of cell adhesion	1.1E-5
GO:0022409	positive regulation of cell-cell adhesion	1.27E-5
GO:1903037	regulation of leukocyte cell-cell adhesion	7.02E-5
GO:0022407	regulation of cell-cell adhesion	2.55E-4
GO:0032956	regulation of actin cytoskeleton organization	8.98E-12
GO:0051495	positive regulation of cytoskeleton organization	1.55E-6

Table 1. GO term analysis of differentially expressed genes in CXCR4/CD5 subpopulations.

7.2. Identification of GAB1 as a regulator of CLL cell migration

Seda V, Vojackova E, Ondrisova L, *et al.* FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia. *Blood*. 2021.

Among these putative migration-related genes we have noticed upregulation of the *GAB1* gene (~1.8-fold) in CXCR4^{bright}CD5^{dim} cells compared to CXCR4^{dim}CD5^{bright} cells and this upregulation was confirmed by immunoblotting, flow cytometry, and qRT-PCR (Figure 14 A-C). This is of note since the *GAB1* was identified as a migration regulator in epithelial, muscle, and breast cancer cells,²⁹⁰⁻²⁹² and its levels associate with shorter overall survival in CLL.²⁹³ For this reason, we hypothesized that *GAB1* upregulation in a CXCR4^{bright}CD5^{dim} subpopulation might contribute to CLL cells' ability to return to immune niches. To examine this, we created a *GAB1* knockout MEC1 cell line (MEC1^{GAB1-KO}) using CRISPR-Cas9 technology (Figure 14 D). The MEC1 wild type (MEC1^{WT}) and MEC1^{GAB1-KO} cells were labeled with two different colors (CellTrace dyes: CFSE or FarRed), mixed in ratio 1:1, validated by flow cytometry and injected into the tail vein of NSG mice (Figure 14 E). Mice were euthanized after 6 hours and infiltration of various organs was examined by flow-cytometry. This revealed that MEC1^{GAB1-KO} cells were significantly retained in peripheral blood and relatively less in the spleen in comparison to control cells (Figure 14 F). The infiltration of bone marrow or liver did not differ and the number of MEC1 in these organs was low (Figure 14 F). Similarly, the silencing of *GAB1* by siRNA in primary CLL cells impaired migration to the spleen of NSG mice (Figure 14 G). In addition, the localization of CLL cells in the spleen exhibits the tendency for higher accumulation in the trabecular system near the trabecular veins; however, there was no difference in the localization for control cells and cells with low/attenuated expression of *GAB1* (Figure 14 H).

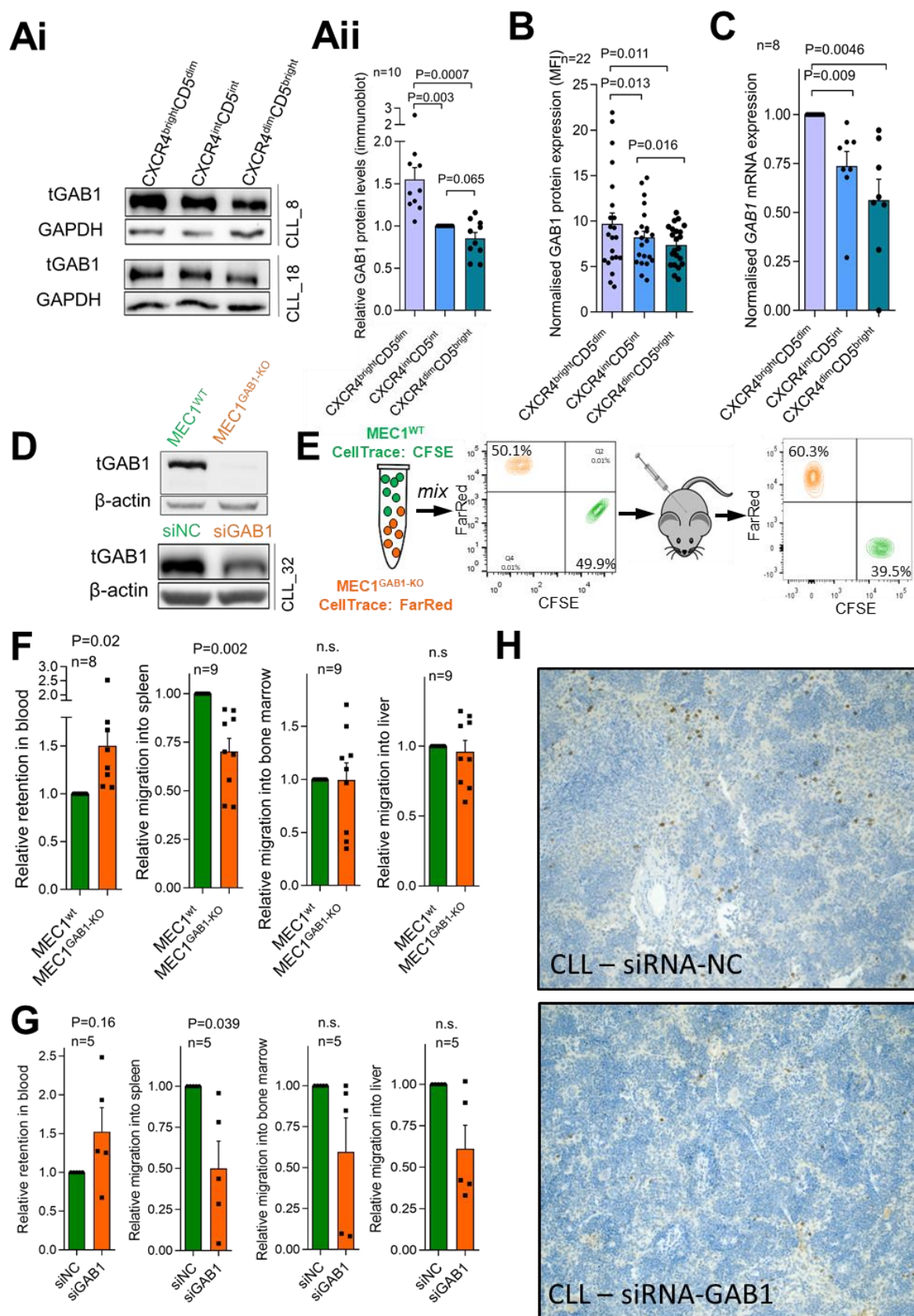


Figure 14. Identification of GAB1 as a regulator of CLL cell migration. (Ai) Representative immunoblot from primary CLL samples sorted according to CXCR4/CD5 expression (purity 99.9%). **(Aii)** Densitometric quantification of relative GAB1 protein level analyzed by immunoblot (n=10). **(B)** Intracellular flow cytometry staining for GAB1 protein in CXCR4/CD5 subpopulations (n=22). Normalized mean fluorescence intensity (MFI) was calculated as the ratio between the sample and control sample stained only with a secondary antibody. **(C)** Validation of *GAB1* mRNA expression (real-time qRT-PCR) in primary CLL samples sorted according to CXCR4/CD5 expression (n=8). **(D)** Representative immunoblot for MEC1^{WT} and MEC1 cells genetically engineered for GAB1 KO, using CRISPR/Cas9 (MEC1^{GAB1-KO}) cells (top); primary CLL cells transfected with siRNA against *GAB1* (siGAB1) or with the control (siNC; bottom), cells were harvested after 48 hours post transfection. **(E)** Schematic design of *in vivo* competitive migration assay of MEC1^{WT} vs MEC1^{GAB1-KO}. Cells were stained with CFSE or FarRed CellTrace dye, and incubated overnight. The next day, an equal number of CFSE- and FarRed-stained cells (each 50x10⁶) were mixed in 1:1 ratio (validated by flow cytometry), and injected into the NSG mice via the tail vein. Organ infiltration was analyzed by gating on human CFSE⁺/FarRed⁺ cells. The plot depicts the CFSE⁺/FarRed⁺ MEC1 cells present in peripheral blood of NSG mice. **(F)** Mice (n=9) from the experiment described in panel [E] were euthanized 6 hours after cell transplantation, and blood, spleen, bone marrow, and liver were analyzed by flow cytometry to detect the presence of viable CFSE⁺ or FarRed⁺ cells. The amount of MEC1 cells in each examined organ site is presented as relative migration of CFSE⁺ vs FarRed⁺ cells. **(G)** An *in vivo* competitive migration assay of primary CLL cells (n=5) transfected with siRNA against *GAB1* (siGAB1) or negative control (siNC). After 48 hours, cells were stained with Sytox Blue and sorted for viable cells, and 10x10⁶ of cells from each condition were processed as described in panel B. *In vivo* migration continued for 4 hours, and blood, spleen, bone marrow, and liver were analyzed by flow cytometry to detect the presence of viable CFSE⁺ or FarRed⁺ cells. A small aliquot of sorted cells was assayed by immunoblot to confirm GAB1 silencing (data not shown). **(H)** Histological evaluation of spleen of NSG mice transplanted with CLL cells transfected with siRNA against *GAB1* (siRNA-GAB1, bottom) or control siRNA (siRNA-NC, top). Cells were incubated for 48 hrs after transfection and then stained with Sytox Blue viability dye and sorted according to viability. Viable CLL cells (10x10⁶ of siRNA-GAB1 or siRNA-NC transfected cells) were transplanted to individual NSG mice via a tail vein injection and sacrificed after 6 hrs. Tissue sections were stained with anti-human-CD45 antibody (brown signal) and the post-counterstaining by using Bluing reagent (Ventana).

7.3. GAB1 is activated in response to various cytokines

Seda V, Vojackova E, Ondrisova L, *et al.* FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia. *Blood*. 2021.

It is known that B cell migration is regulated by a number of chemokines such as SDF1 or CXCL13 that are produced in immune niches.^{16,90,294,295} Indeed, we have confirmed their presence in CLL patients' bone marrow and production by primary human mesenchymal stromal cells, human stromal cell line HS5 (CM^{HS5}), and in the tissues of NSG mice (Figure 15 A-C). However, the involvement of GAB1 in the cytokine-induced signaling process in B cells remained unresolved. Next, we treated MEC1^{WT} cells with SDF1, CXCL13, or CM^{hMSC}, this resulted in rapid GAB1 phosphorylation and activation (measured residues: Y307 and Y627; Figure 16 A), indicating that GAB1 is involved in response to multiple soluble factors. The involvement of GAB1 protein in the migration process towards cytokines SDF1, CXCL13, CM^{hMSC}, or CM^{HS5} was further validated by competitive transwell migration assay of MEC1^{WT} and MEC1^{GAB1-KO} cells. This experiment showed that knock-out of *GAB1* significantly impaired cell migration (Figure 16 B). Importantly, similar data were obtained when *GAB1* was silenced by siRNA in primary CLL cells (Figure 16 C-D). Importantly, the silencing of *GAB1* did not affect the surface levels of CXCR4 and cell viability (Figure 16 E). The treatment with specific CXCR4 inhibitor plerixafor largely blocked GAB1 phosphorylation induced by SDF1 but did not block the phosphorylation induced by conditioned media (Figure 16 F), indicating that GAB1 is involved in the signaling of various cytokines. In addition, MEC1^{GAB1-KO} cells fail to properly polymerize F-actin, after stimulation with conditioned media produced by HS5 cells (Figure 16 G). MEC1^{GAB1-KO} cells' motility speed was significantly lower than MEC1^{WT} ($P < 0.001$; Figure 16 H) and GAB1 knock-out also impaired other cell migration-related characteristics of cells as assessed by holographic microscopy (Table 2). Altogether, this suggests that GAB1 sensitizes CLL cells to homing signals, and influences related cytoskeletal organization.

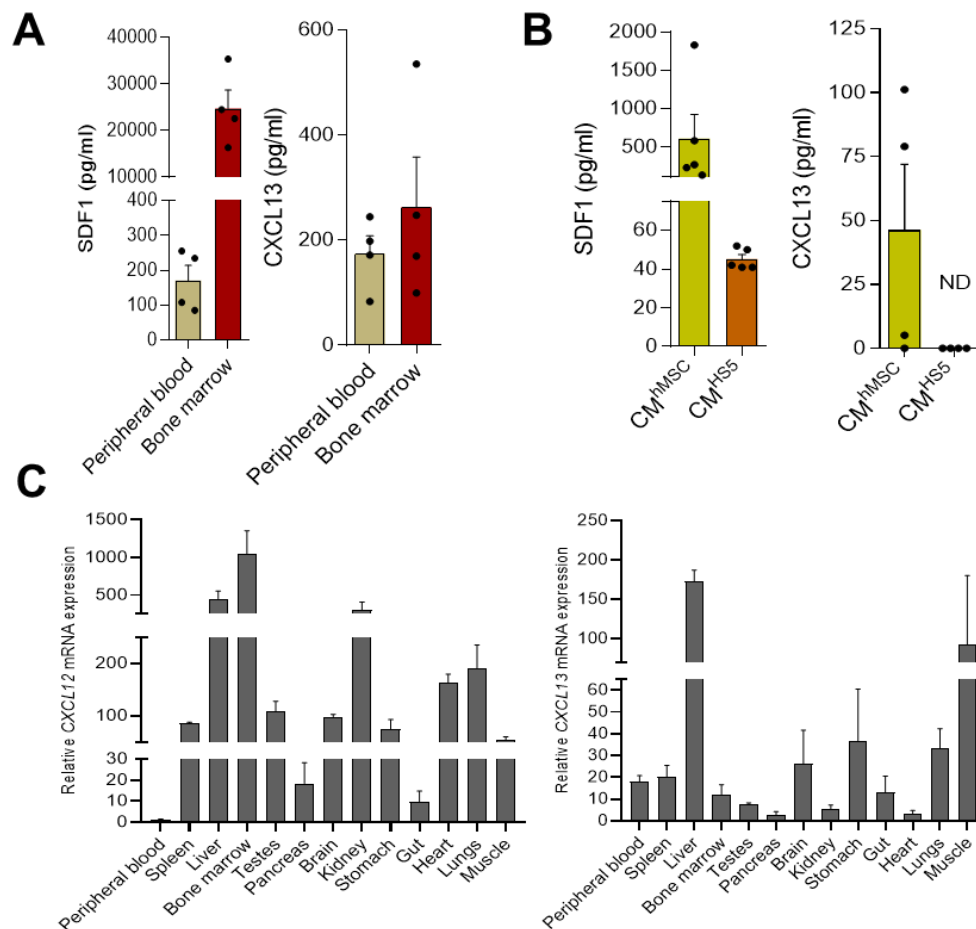


Figure 15. Measurement of SDF1 and CXCL13 concentrations. (A) Serum isolated from peripheral blood or bone marrow of CLL patients (n=4) was tested for SDF1 or CXCL13 levels by enzyme-linked immunosorbent assay (ELISA; R&D Systems). (B) Conditioned media produced by primary human mesenchymal stromal cells (CM^{hMSC}) or by human stromal cell line HS5 (CM^{HS5}) was analyzed for levels of SDF1 or CXCL13. The replicates represent analyses of conditioned media from various passages of the cells. ND stands for “not-detectable”. (C) Relative expression of *SDF1* and *CXCL13* mRNAs in different organs of the NSG mice. For this experiment, 3 NSG mice (without transplantation of malignant cells) were sacrificed, organs homogenized, RNA isolated, and subjected to qRT-PCR.

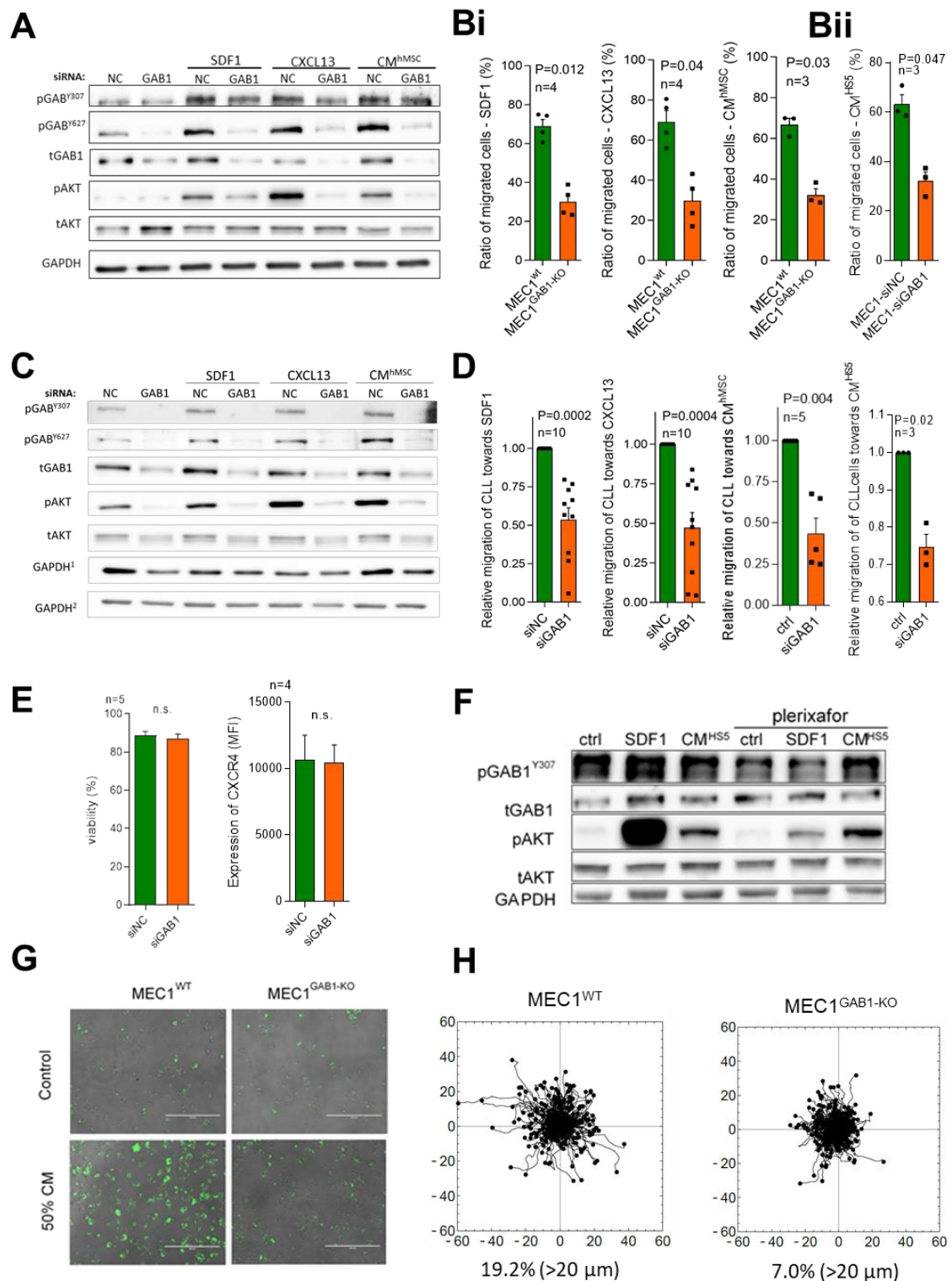


Figure 16. GAB1 is activated in response to various cytokines. (A) Representative immunoblots of MEC1 cells transfected with siRNA against *GAB1* (siGAB1) or the control (siNC). After 48 hours, the cells were treated for 10 minutes with SDF1 (250 ng/mL), CXCL13 (500 ng/mL), or CM (100%) produced by primary human mesenchymal stromal cells (CM^{hMSC}). **(Bi)** An *in vitro* competitive migration assay of MEC1^{WT} vs MEC1^{GAB1-KO} cells (n=4) stained as described in [Figure 14 E]. Migration

toward SDF1 (250 ng/mL), CXCL13 (500 ng/mL), and CM^{hMSC} continued for 6 hours (n=4 for SDF1 and CXCL13; n=3 for CM^{hMSC}). **(Bii)** Competitive migration assay of MEC1 cells transfected with siRNA against *GAB1* (siGAB1) or the control (siNC). After 48 hours, the cells were processed as described in [Figure 14 E]. Migration toward conditioned media produced by HS5 cells continued for 6 hours (n=3). **(C)** Representative immunoblots of primary CLL cells treated for 5 minutes with SDF1 (100 ng/mL), CXCL13 (250 ng/mL), or CM^{hMSC}. The immunoblot contained 2 endogenous controls (GAPDH; glyceraldehyde 3-phosphate dehydrogenase) marked by the index at top, because for technical reasons, pGAB1^{Y627}, tGAB1, tAKT, and tERK (loading control GAPDH¹) were analyzed on the first gel and the remaining proteins (loading control GAPDH²) on the second gel (identical protein loading and conditions). **(D)** *In vitro* competitive migration assay of primary CLL cells transfected by siRNA against *GAB1* (siGAB1) or control siRNA (siNC). Cells were stained as described in [Figure 14 E] and loaded onto a Transwell 48 hours after transfection. Migration continued for 6 hours toward SDF1 (100 ng/mL), CXCL13 (250 ng/mL), CM^{hMSC} or CM^{HS5} (n=10 for SDF1 and CXCL13; n=5 for CM^{hMSC}; n=3 for CM^{HS5}). **(E)** Analysis of cell viability and CXCR4 cell-surface expression in primary CLL cells transfected with siRNA against *GAB1* (siGAB1) or with control scrambled siRNA (siNC). Viability was measured by DiOC6/PI staining. **(F)** Representative immunoblot from primary CLL cells treated with 50% conditioned media produced by human stromal cell line HS5 (CM^{HS5}), SDF1 (100 ng/ml), or their combination with CXCR4-inhibitor plerixafor. Plerixafor (5 µg/ml) was added 2 hours prior to the cell treatment with SDF1 or conditioned media to ensure full CXCR4 inhibition. The control stands for cells treated with vehicle and cultured on plastic. **(G)** Representative image of F-actin polymerization in MEC1^{wt} and MEC1^{GAB1-KO} cells after treatment with 50% conditioned media produced by HS5 cells (15 min). MEC1 cells were subsequently fixed, and polymerized F-actin was visualized by fluorescent microscopy after Phalloidin-488 staining. **(H)** Representative rose plots with 5 min migration trajectories of MEC1^{wt} vs. MEC1^{GAB1-KO} cells in the presence of conditioned media produced by HS5 cells (shifted to a common origin, axis in µm). The percentage below the figure indicates the fraction of cells with trajectory >20 µm at 5 min in MEC1^{wt} vs. MEC1^{GAB1-KO} cells (19.2% vs. 7%, respectively; P<0.0001).

Parameter	MEC1 ^{wt} cells (mean ± s.e.m.)	MEC1 ^{GAB1-KO} cells (mean ± s.e.m.)	ANOVA P-value
Circularity*	0.652 ± 0.004	0.653 ± 0.004	n.s.
Elongation*	0.323 ± 0.008	0.332 ± 0.008	n.s.
Mass [pg]*	200.6 ± 5.3	198.7 ± 4.8	n.s.
Spreading [µm ² /pg]*	1.75 ± 0.02	1.57 ± 0.02	n.s.
Displacement [µm/60 s]*	3.75 ± 0.14	2.71 ± 0.10	0.035
Persistence*	0.69 ± 0.01	0.60 ± 0.01	0.036
Number of movies	6	6	
Number of tracked cells	195	186	
Number of time point measurements	11 939	15 184	
s.e.m. stands for standard error of the mean			
* For the description of the categories see chapter 6. Material and Methods			

Table 2. Statistical analysis of all measurements for cell motility assay

7.4. Identification of FoxO1 as a transcription factor of *GAB1*

Seda V, Vojackova E, Ondrisova L, *et al.* FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia. *Blood*. 2021.

To reveal potential transcriptional regulator of *GAB1* expression which will be relevant in the context of CXCR4/CD5 subpopulations, we compared the list of our differentially expressed genes (Figure 13 B) with the list of transcription factors.²⁹⁶ This identified 20 annotated transcription factors significantly differentially expressed in CXCR4/CD5 subpopulations (Figure 17 A). We further selected FoxO1 as a potential regulator of *GAB1* expression, since it was previously identified as regulator of T cell homing,³⁴ migration of B cells within the germinal centers,^{197,201} and is involved in malignant B cell biology.^{198,202} We have also identified multiple putative evolutionary conserved binding sites upstream from the *GAB1* promotor (Figure 17 B-C). Chromatin immunoprecipitation validated two of these sites as FoxO1 enriched (Figure 17 D).

Next, we silenced the FoxO1 expression by siRNA which resulted in repression of *GAB1* mRNA and protein levels (Figure 18 A). Similar data were obtained after the treatment with FoxO1 inhibitor (Figure 18 B). The existence of a FoxO1-GAB1 axis is supported by a general positive correlation of *GAB1* and FoxO1 mRNA levels (n=42) and protein levels (n=22) in CLL cells (Figure 18 C-D), and the higher levels of both molecules in CXCR4^{bright}CD5^{dim} cells (Figure 18 E-G). Moreover, genetically engineered MEC1 cells without FoxO1 (MEC1^{FoxO1-KO}) exhibited lower *GAB1* levels (Figure 18 H), and similarly to *GAB1* knock-out, exhibited impaired migration to immune niches of NSG mice (Figure 18 I). In addition, the migration of MEC1^{FoxO1-KO} cells or primary CLL cells with silenced *FoxO1* towards SDF1, CXCL13 or CM^{hMSC} was also impaired (Figure 18 J-K), which also mimics the MEC1^{GAB1-KO} cell phenotype.

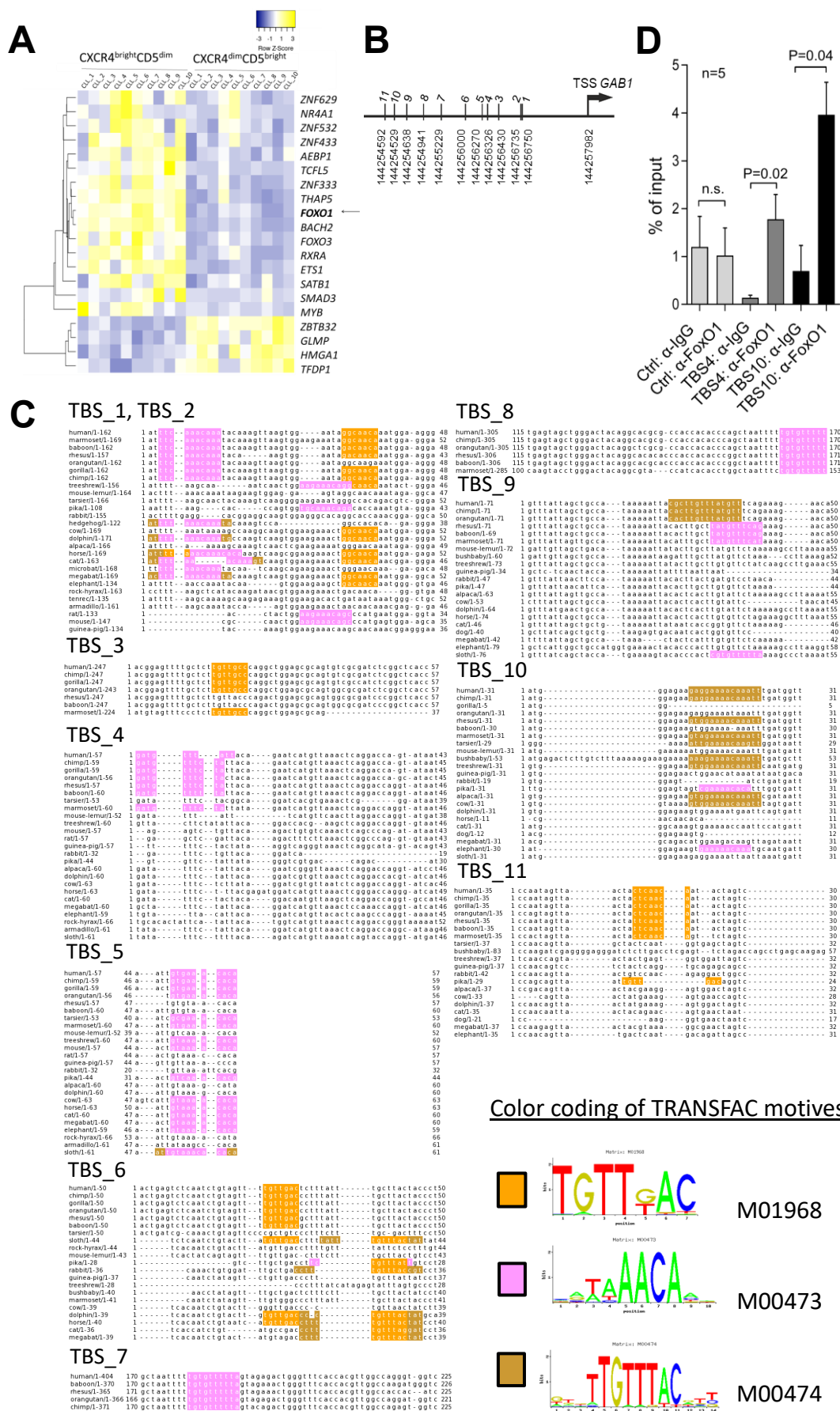


Figure 17. FoxO1 binds to a promotor region in *GAB1* promotor. (A) Heat map of differentially expressed transcription factors (RNAseq; Illumina) in 10 primary CLL samples sorted according to CXCR4/CD5 expression (fold change, ≥ 2 ; $P < 0.001$). The list of all known human transcriptional factors was obtained from Lambert *et al.*²⁹⁶ (B) Map of predicted transcription binding sites of FoxO1 protein in 3.5 kb promoter region of the *GAB1* gene (ContraV3). Predicted transcription binding sites are number 1-11 (at the top), and their genomic position is indicated. (C) The predicted evolutionary conserved transcription factor binding sites (TBS) for FoxO1 in 3.5 kb upstream region from the transcriptional start site of the *GAB1* gene. Different colors refer to different FoxO1 binding motives depicted in bottom right corner (ContraV3 online tool, 2017).²⁹⁷ (D) Chromatin immunoprecipitation analysis of FoxO1 binding upstream of the transcription start site of *GAB1* gene (n=5; numbering of binding sites correspond to [B]). The results TBSs number 4 and 10 are represented as the DNA input percentage, and error bars indicate the standard error of the mean. Control samples (Ctrl: α -IgG; Ctrl: α -FoxO1) were analyzed by primers that amplified a region in the *GAB1* second intron, which does not contain any predicted binding site for FoxO1. Data for other predicted TBSs were not analyzed or were inconclusive (not shown).

We further investigated the mechanisms ensuring the *GAB1* and FoxO1 upregulation in CXCR4^{bright}CD5^{dim} CLL cells and their lower levels in CXCR4^{dim}CD5^{bright} cells. It is known that FoxO1 levels are negatively regulated by active AKT, which provides phosphorylation at S256, leading to loss of FoxO1 interaction with DNA, translocation to the cytoplasm, and subsequent degradation.^{298,299} Indeed, the crosslinking of BCR or co-culture of primary CLL cells with stromal cells resulted in strong AKT activation, FoxO1 degradation, and concurrent decrease of *GAB1* mRNA and protein levels (Figure 19 A-B).

On contrary, the selective inhibition of AKT resulted in FoxO1 dephosphorylation and ~2-fold increase in *GAB1* mRNA and protein levels (Figure 19 C). The expression of other selected BCR/microenvironment-related proteins such as SYK, PLC γ , or CD79 α was not affected by AKT inhibition (Figure 19 D). These data suggest that AKT is a crucial negative *GAB1* level regulator acting via reducing its transcriptional activator levels, FoxO1. On the other hand, relatively low AKT activity in CXCR4^{bright}CD5^{dim} cells (Figure 19 E) allows for an increase in FoxO1 and induction of *GAB1*.

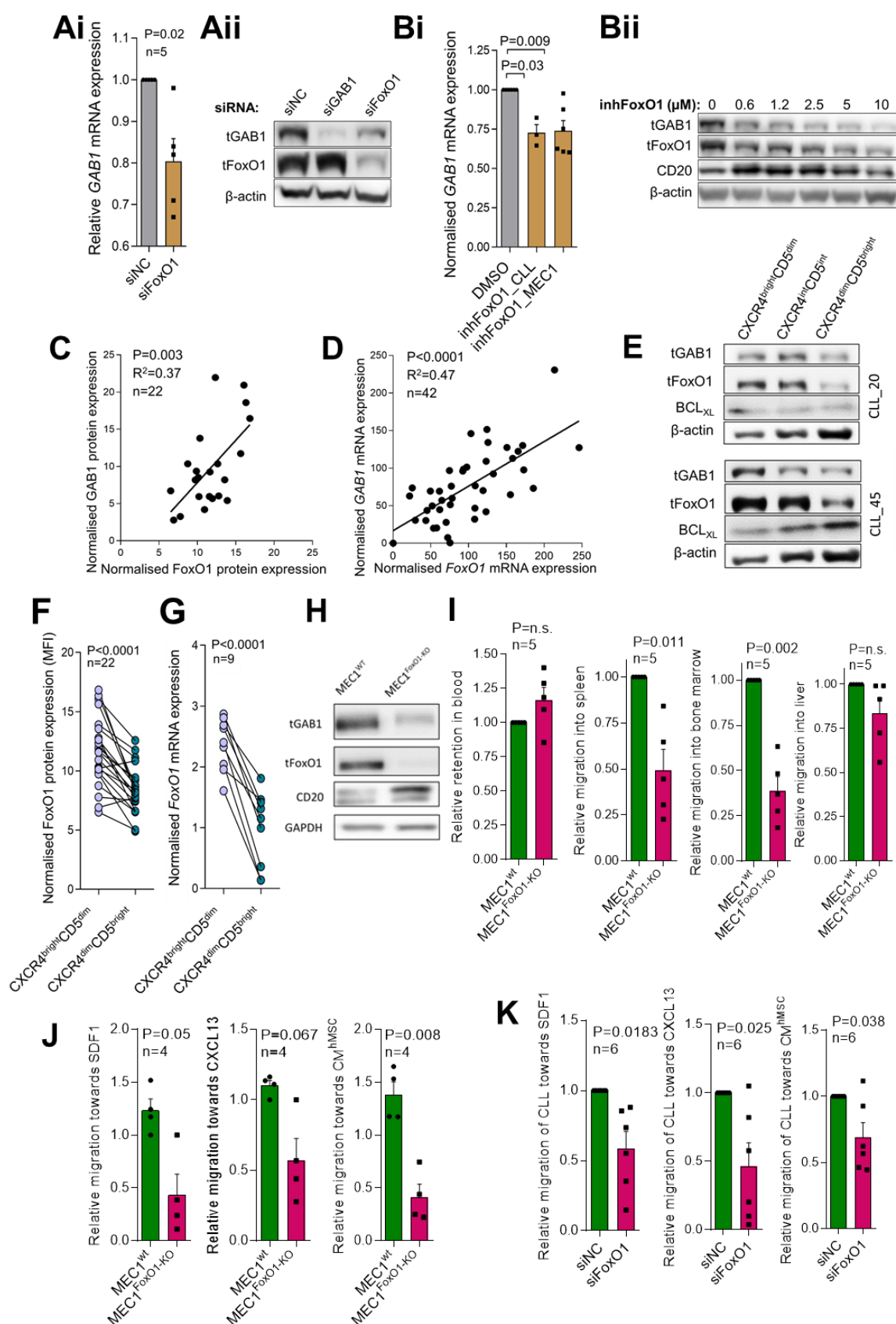


Figure 18. FoxO1 modulation affects GAB1 levels and migration of CLL cells. (Ai) The effect of siRNA against *FoxO1* (siFoxO1) on *GAB1* mRNA expression in MEC1

cells compared with cell transfection with a control scrambled siRNA (siNC; 48 hours after transfection, n=5). **(Aii)** Representative immunoblot of *FoxO1* silencing by siRNA (siFoxO1) and its impact on GAB1 protein level in MEC1 cells (48 hours). The siRNA against *GAB1* (siGAB1) serves as a positive control and control scrambled siRNA (siNC) as a negative control. **(Bi)** Statistical analysis of FoxO1 inhibitor's effect (inhFoxO1; 1 μ M) on *GAB1* mRNA expression in primary CLL (n=3; 48 hours) and MEC1 (n=6; 48 hours) cells. *GAB1* mRNA expression in each control sample (dimethyl sulfoxide [DMSO] treated) was set at 1. **(Bii)** Representative immunoblot of GAB1 levels in primary CLL cells treated *in vitro* with various doses of FoxO1 inhibitor (inhFoxO1, 0.6-10 μ M; 48 hours). CD20 induction was used as a positive control, because FoxO1 acts as a transcriptional repressor of CD20.³⁰⁰ **(C)** *GAB1* and *FoxO1* mRNA expression correlation analysis in primary CLL cells (n=42; real-time qRT-PCR). **(D)** Correlation of GAB1 and FoxO1 intracellular protein levels assessed by flow cytometry (n=22). **(E)** Representative immunoblot from intraclonal CLL cell subpopulations sorted according to CXCR4/CD5 expression (purity >99.9%). Subpopulation sorting was performed according to the gating strategy in [Figure 13 A]. The levels of B-cell lymphoma extra-large (BCL_{XL}) served as a positive control for microenvironmental interactions. **(F, G)** Protein and mRNA levels of FoxO1 in CXCR4/CD5 subpopulations. Protein quantification was assessed by intracellular staining (n=22; flow cytometry). As a control, an identical CXCR4/CD5-stained sample was incubated with a secondary antibody only. *FoxO1* mRNA levels in CXCR4/CD5 sorted subpopulations were measured by qRT-PCR (n=9). **(H)** Representative immunoblot for the effect of FoxO1 attenuation by CRISPR-Cas9 knock-out in MEC1 cells (MEC1^{WT} vs MEC1^{FoxO1-KO} cells). **(I)** *In vivo* competitive migration assay of MEC1^{WT} vs. MEC1^{FoxO1-KO} cells. Cells were processed as described in [Figure 14 E] and injected in tail vein of NSG mice for competitive migration assay. Mice were sacrificed after 6 hrs, and blood, spleen, bone marrow, and liver were analyzed by flow cytometry for the presence of viable CFSE or FarRed positive cells. The amount of MEC1 cells in each examined site is presented as relative migration between CFSE vs. FarRed positive cells. **(J)** *In vitro* competitive migration assay of MEC1^{WT} vs. MEC1^{FoxO1-KO}. Cells were stained as described in [Figure 14 E] and loaded on transwell. Migration (n=4) towards SDF1 (250 ng/ml), or CXCL13 (500 ng/ml), or conditioned media produced by human bone mesenchymal stromal cells (CM^{hMSC}) was allowed for 6 hrs, and the ratio of passed cells was assessed by flow cytometry. The number of migrated cells was normalized to the migration of cells applied to transwell without chemokines in the bottom chamber. **(K)** Competitive migration assay of CLL cells after *FoxO1* silencing by siRNA (siFoxO1) compared to cells transfected with control siRNA (siNC). Cells were incubated for 48 hrs and then stained as described in [Figure 14 E] and loaded on transwell. Migration (n=6) towards SDF1 (100 ng/ml), or CXCL13 (250 ng/ml), or conditioned media produced by human bone mesenchymal stromal cells (CM^{hMSC}) was allowed for 6 hrs, and the ratio of migrated cells was assessed by flow cytometry.

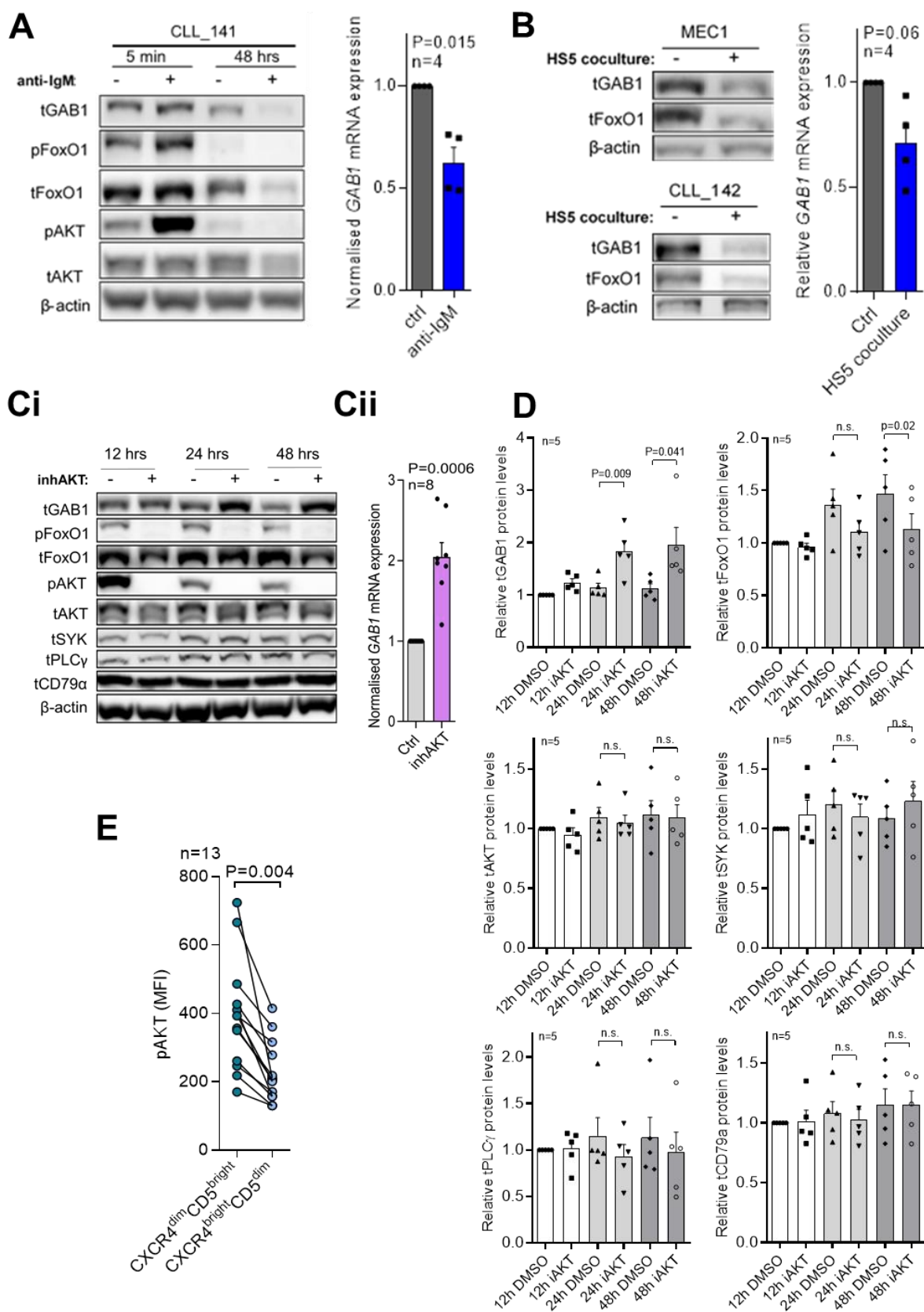


Figure 19. FoxO1 is downregulated in response to B cell activation. (A) Representative immunoblot for FoxO1 and GAB1 levels in primary CLL cells stimulated

with anti-IgM (10 µg/ml) for 5 min or 48 hrs. And *GAB1* mRNA expression from CLL cells (n=4) treated with anti-IgM for 48 hrs. Ctrl stands for cells incubated in culture media only. **(B)** Representative immunoblot of GAB1 and FoxO1 protein levels after coculture of MEC1 cells or primary CLL cells with HS5 stromal cells for 48 hrs. Primary CLL cells were seeded on a confluent layer of HS5 cells in an approx. 5:1 ratio (CLL:HS5). After 48 hrs cell suspension was stained with antibody against CD105 (a specific marker of stromal cells) and sorted by FACS (purity >99,9% of CD19+CD5+ cells). And *GAB1* mRNA expression after coculture of primary CLL cells (n=4) with HS5 stromal cells for 48 hrs. **(Ci)** Representative immunoblot from MEC1 cells treated with a selective inhibitor of AKT (inhAKT; MK-2206 2HCl; 1 µM) or vehicle (DMSO). The primary antibody against pFoxO1 detected phosphorylation on S256, which led to inhibition of FoxO1 activity. **(Cii)** *GAB1* mRNA expression in MEC1 cells treated with a selective AKT inhibitor [treated as in Ci] for 24 hours (n=8). **(D)** Densitometric quantification of tGAB1, pFoxO1, tFoxO1, pAKT, tAKT, tSYK, tPLCγ, tCD79a protein levels in MEC1 cells treated with 1 µM inhibitor of AKT (iAKT) or vehicle (DMSO) for 12-48 hours. MEC1 cells were used in this experiment, since primary CLL cells have relatively very low basal AKT phosphorylation levels to be inhibited (data not shown).

7.5. GAB1 increases the „tonic“ AKT activity

Seda V, Vojackova E, Ondrisova L, *et al.* FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia. *Blood*. 2021.

It has been previously published that GAB1 is activated after BCR crosslinking and serves as an important docking protein allowing for propagation of the ERK or PI3K/AKT signaling.^{172,176,293} In line with this, we observed that GAB1 silencing impaired AKT phosphorylation after BCR cross-linking (Figure 20 A). Interestingly, we noticed that the GAB1 level modulation also affected the basal AKT phosphorylation levels irrespective of BCR cross-linking (Figure 20 A), hinting at a possible role in “tonic” AKT signaling. To investigate this further, we transiently over-expressed GAB1 by a plasmid construct which resulted in induced AKT phosphorylation (Figure 20 B). Analogically, the silencing of *GAB1* by siRNA reduced AKT phosphorylation (Figure 20 C). This was further validated on MEC1 and OSU-CLL cell lines with tetracycline-inducible shRNA against *GAB1* (Figure 20 D). In these experiments, we also looked at the phosphorylation of the p85 subunit of PI3K, which serves as a link between GAB1 and PI3K-dependent AKT phosphorylation.¹⁶⁹ In line with our results, the p85 was activated after GAB1 over-expression and reduced after *GAB1* silencing (Figures 20 B-C). This indicates that GAB1-induced p85 phosphorylation increase is a possible mechanism behind the observed increase in AKT phosphorylation.

Next, we tested the growth capacity of MEC1^{WT} versus MEC1^{GAB1-KO} cells after their engraftment in mice for three weeks. Similarly to the short-term migration assay, the MEC1^{WT} and MEC1^{GAB1-KO} cells (each traced with green fluorescent protein [GFP] or Azurite) were mixed in ratio 1:1 and injected into the tail vein of NSG mice. This experiment revealed a higher proliferation rate of MEC1^{WT} in comparison to MEC1^{GAB1-KO} cells (Figure 20 E), suggesting that GAB1 knock-out negatively affects general cell fitness.

The functionality of the proposed FoxO1-GAB1-pAKT axis was further investigated by analyzing the MEC1^{FoxO1-KO} cells, which exhibited reduced GAB1 levels and impaired AKT phosphorylation compared to MEC1^{WT} (Figure 20 Fi). A similar phenotype was also obtained after *FoxO1* silencing by siRNA in primary CLL cells (Figure 20 Fi) and MEC1 cells or primary CLL cells treatment with FoxO1 inhibitor (Figure 20 Fii). Altogether, this suggests that increased FoxO1/GAB1 levels in CXCR4^{bright}CD5^{dim} CLL cells are contributing to “tonic” pAKT activity.

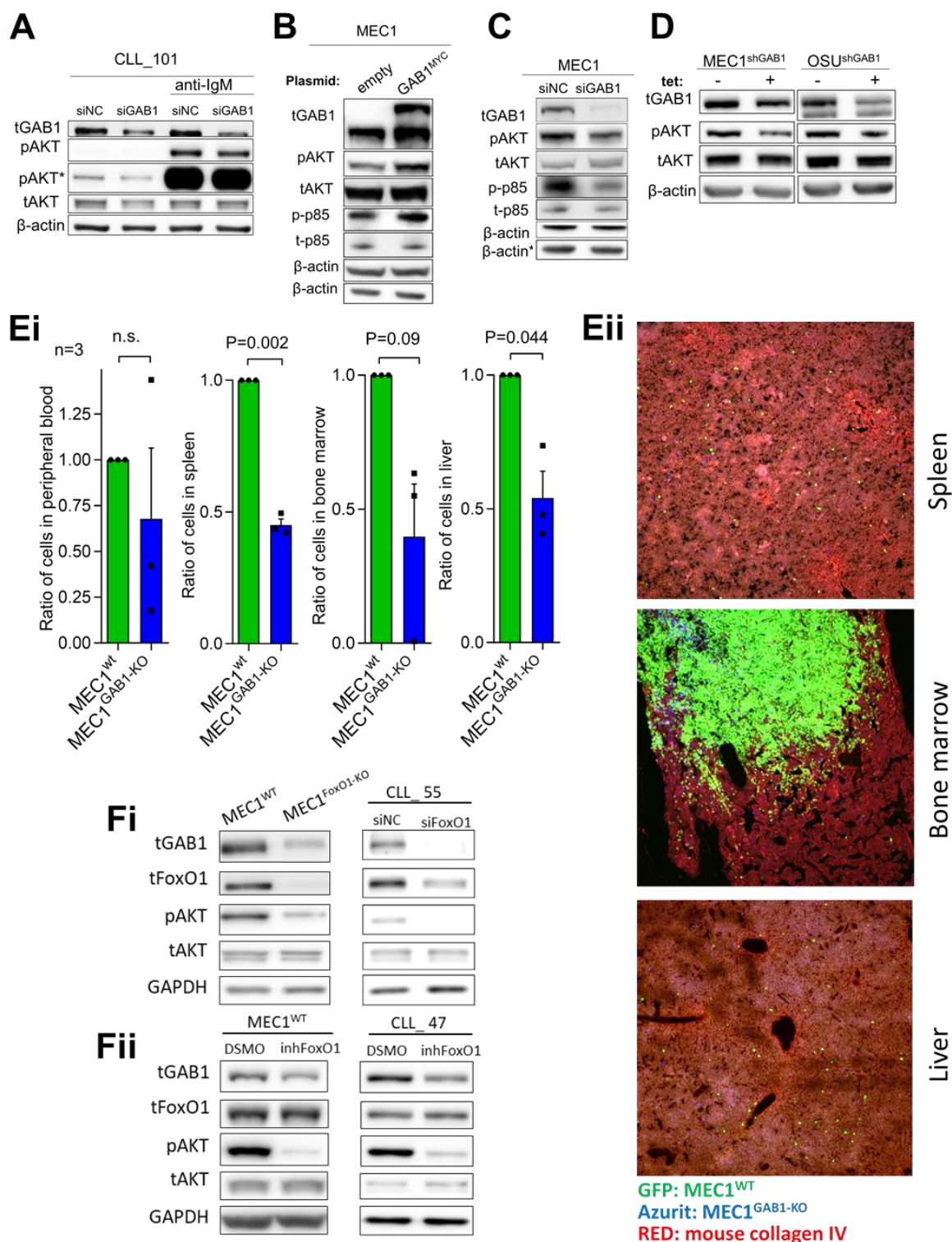


Figure 20. GAB1 increases the „tonic“ AKT activity. (A) Representative immunoblots of primary CLL cells transfected with siRNA against *GAB1* (siGAB1) or the negative control (siNC). Cells were harvested after 48 hours, divided into equal aliquots, and exposed to vehicle (control samples) or anti-IgM for 5 minutes (10 mg/mL). *pAKT (S473) is the same immunoblot as pAKT but was developed for a prolonged time.

(B) Representative immunoblot of MEC1 cells transfected with a plasmid for GAB1 protein overexpression tagged with MYC sequence (GAB1^{MYC}, top band) or with an empty vector (empty), and cultured for 48 hours. * β -actin was the endogenous control for p-p85 and t-p85 assayed on a separate gel with identical loading. **(C)** Representative immunoblot of MEC1 cells transfected with siRNA against *GAB1* (siGAB1) or the negative control (siNC) and harvested 48 hours after transfection. * β -actin, as in panel [B]. **(D)** Representative immunoblot of MEC1 and OSU-CLL cells (CLL-derived cell line) with downmodulated GAB1 levels. MEC1 and OSU-CLL cells were infected with a lentiviral construct (permanent cell line) for tetracycline-inducible shRNA against GAB1 (shGAB1). shRNA expression was induced by adding tetracycline to medium (tet⁺) for 48 hours, and control cells were treated with vehicle (tet⁻). **(Ei)** Effect of GAB1 KO on MEC1 cell growth *in vivo*. MEC1^{WT} and MEC1^{GAB1-KO} cells were transduced with vectors expressing GFP or Azurite, mixed 1:1, and injected via tail vein into NSG mice (n=3). Mice were euthanized 3 weeks after injection, and organs were analyzed by flow cytometry for the presence of MEC1^{WT} and MEC1^{GAB1-KO} cells labeled by GFP or Azurite in blood, spleen, bone marrow, and liver. We also performed a switch of fluorescent labels to exclude any potential effect of a specific fluorescent proteins on the cells (not shown). **(Eii)** Representative images from fluorescence microscopy of the spleen, bone marrow, and liver of NSG transplant recipients, as described in panel [Ei] (original magnification, x20). **(Fi)** Representative immunoblot for the effect of FoxO1 attenuation by KO in MEC1 cells (MEC1^{WT} vs MEC1^{FoxO1-KO} cells) or by siRNA in primary CLL cells. Primary CLL cells were transfected with siRNA against *FoxO1* (siFoxO1) or with control (siNC) and analyzed by immunoblot 48 hours later. **(Fii)** Representative immunoblots for MEC1 cells and primary CLL cells treated with FoxO1 inhibitor (inhFoxO1; 0.5 μ M, 24 hours).

7.6. Ibrutinib treatment induces compensatory GAB1-mediated AKT activation

Seda V, Vojackova E, Ondrisova L, *et al.* FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia. *Blood*. 2021.

Ibrutinib therapy inhibits CLL cells homing,^{126,301} and we hypothesized that this could affect the FoxO1-GAB1-pAKT axis. To investigate this, we treated MEC1 cells with ibrutinib *in vitro* which resulted in increased FoxO1 and GAB1 protein and mRNA accumulation (Figure 21 A), albeit the kinetics of mRNA and protein induction is not identical, and the GAB1 protein levels are sustained for longer. Similarly, treating CLL patients with ibrutinib *in vivo* leads to increased FoxO1 and GAB1 protein and mRNA levels (Figure 21 B). Notably, after an initial drop in AKT phosphorylation, we observed restoration of AKT phosphorylation following several days of continuous MEC1 cell exposure to ibrutinib *in vitro* (Figure 21 Ai) or after several days or weeks of ibrutinib therapy in CLL patients *in vivo* (Figure 21 Bi). Importantly, this ibrutinib-induced AKT phosphorylation did not lead to FoxO1 translocation into the cytoplasm and FoxO1 remained in the nucleus, allowing its function as a transcriptional factor (Figure 21 C). This is in line with already published data suggesting that the translocation of FoxO1 into cytosol upon AKT activation is impaired in B cell lymphomas,¹⁹⁸ and the FoxO1 activity is not mutually exclusive with low-level pAKT. Importantly, the GAB1 knock-down prevented the restoration of AKT phosphorylation in ibrutinib treated cells (Figure 21 D). Altogether this suggests that increasing GAB1 levels in CLL cells during ibrutinib therapy provides “tonic” pAKT activity and might compensate for the lack of AKT activation via BCR/microenvironmental interactions during ibrutinib therapy.

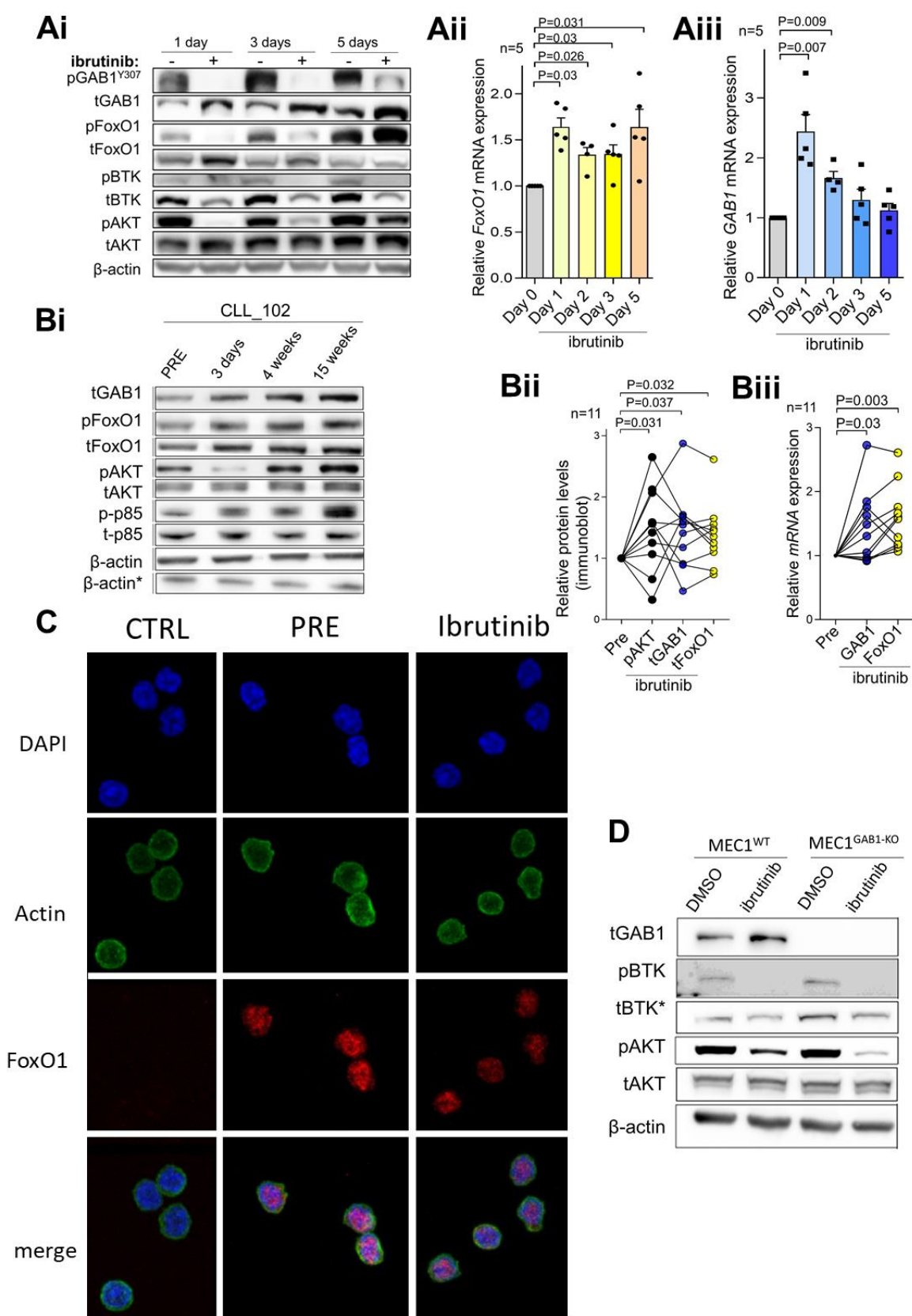


Figure 21. Ibrutinib treatment induces compensatory GAB1-mediated AKT activation. (Ai) Representative immunoblot of MEC1 cells treated with ibrutinib (2 μ M)

or vehicle (DMSO) for the indicated time points. Ibrutinib and DMSO were freshly supplemented every 24 hours, and the cells were sampled at the indicated time points for immunoblot analysis. **(Aii, Aiii)** *FoxO1* and *GAB1* mRNA expression in MEC1 cells treated with ibrutinib or DMSO, as described in panel A (n=5). **(Bi)** Representative immunoblot for primary CLL cells isolated from peripheral blood of patient before (Pre) and during ibrutinib therapy. * β -actin was the endogenous control for p-p85 and t-p85, assayed on a separate gel with identical loading. **(Bii)** Statistical analysis of normalized densitometric data from immunoblots of CLL samples (n=11) obtained from patients before (Pre) and during ibrutinib therapy. The data are relative to expression before ibrutinib administration (set at 1). **(Biii)** *GAB1* and *FoxO1* mRNA expression in primary samples isolated from patients with CLL before (Pre) and during ibrutinib therapy (n=11). The data are relative to expression before ibrutinib (set as 1). **(C)** Representative confocal microscopy picture of primary CLL sample isolated from the patient before (pre) and during ibrutinib therapy (week 2). In control sample (CTRL) no anti-FoxO1 primary antibody was used. Blue staining (DAPI) represents the nucleus, green represents the cytoskeleton (actin), and red staining represents FoxO1. **(D)** Representative immunoblot of MEC1^{WT} and MEC1^{GAB1-KO} cells treated with ibrutinib (1 μ M) or vehicle (DMSO) for 7 days. *Decrease in total BTK protein level is a common effect of ibrutinib, as described previously.³⁰²

7.7. GAB1 inhibitors suppress migration and induce cell apoptosis

Seda V, Vojackova E, Ondrisova L, *et al.* FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia. *Blood*. 2021.

Mraz M, **Seda V**, Ondrisova L. Inhibitors for Treatment of Hematological Malignancies. Applicant 1: Masaryk University, Brno, CZ; Applicant 2: The University Hospital Brno, CZ. Application Number: 16920831; Approved for use through 11/30/2020. OMB 0651-0032, U.S. Patent and Trademark Office.

In the previous part, we showed that GAB1 upregulation during ibrutinib therapy might act as a mechanism to compensate for the lack of pAKT levels. Here we hypothesized that this could be targeted therapeutically by recently synthesized GAB1 inhibitors targeting the PH-domain.¹⁸⁰ We observed that these compounds led to dose-dependent inhibition of GAB1 phosphorylation, AKT, and ERK inhibition (Figure 22 A), and decreased CLL cell viability (Figure 22 B). Because of the need to use high micromolar concentrations, we tested their specificity on MEC1^{WT} and MEC1^{GAB1-KO} cells. This treatment demonstrated larger pERK/pAKT repression and apoptosis in MEC1^{WT} cells than in MEC1^{GAB1-KO} cells (Figure 22 C-D). Next, we observed that these inhibitors also inhibited CLL cells' migration towards SDF1, CXCL13, CM^{hMSC}, CM^{HS5} (Figure 22 E) in a fashion similar to ibrutinib (Figure 22 F). GAB1 inhibition impaired BCR-induced (anti-IgM) AKT phosphorylation (Figure 22 G) and Ca²⁺ influx induced by SDF1 chemokine (Figure 22 H).

In the next part, we tested the possible use of novel GAB1 inhibitors to block processes like migration, AKT activation, and compensatory activation of AKT during ibrutinib therapy. Importantly, the GAB1 inhibitor was able to block the “compensatory” AKT phosphorylation induced by ibrutinib (Figure 23 A) and increased apoptosis of MEC1 cells exposed to ibrutinib for an extended time (Figure 23 B). To test this in primary CLL cells undergoing spontaneous apoptosis within days, CLL cells were cultured in media containing interleukin 4 (IL4), which partially prevented their spontaneous apoptosis. We used this condition to treat them with vehicle or ibrutinib for 6 days to allow FoxO1-GAB1 induction. Subsequently, these cells were treated with GAB1 inhibitors (or vehicle) for additional 48 hours and examined for cell viability. This experiment showed that CLL cell killing was increased by GAB1 inhibitors combined with ibrutinib compared to ibrutinib or GAB1 inhibitor alone (P<0.001; Figure 23 C). In addition, the cultivation without IL4 allow only short-term experiments. Under these conditions the GAB1 inhibitors show an ability to kill primary CLL cells within 48 hours,

however, this is not synergistic with ibrutinib (Figure 23 D). This is an expected result since in such a short time of ibrutinib exposure, the GAB1 inhibitor acts very similarly to a “BCR inhibitor” in terms of inhibiting “tonic” pAKT levels, and ibrutinib does not yet induce compensatory AKT phosphorylation via GAB1. Altogether, GAB1 inhibition can be used to inhibit CLL migration, basal or BCR-induced AKT activity, and adaptive AKT activation during ibrutinib therapy. In addition, despite the need to use high micromolar concentrations, the GAB1 inhibition represents a novel concept that targets an adaptor protein required for multiple cellular processes like migration or ERK and AKT activation. And this may represent a promising therapeutic strategy not only in the treatment of CLL but also in other blood malignancies like mantle cell lymphoma or acute myeloid leukemia (Figure 23 E).

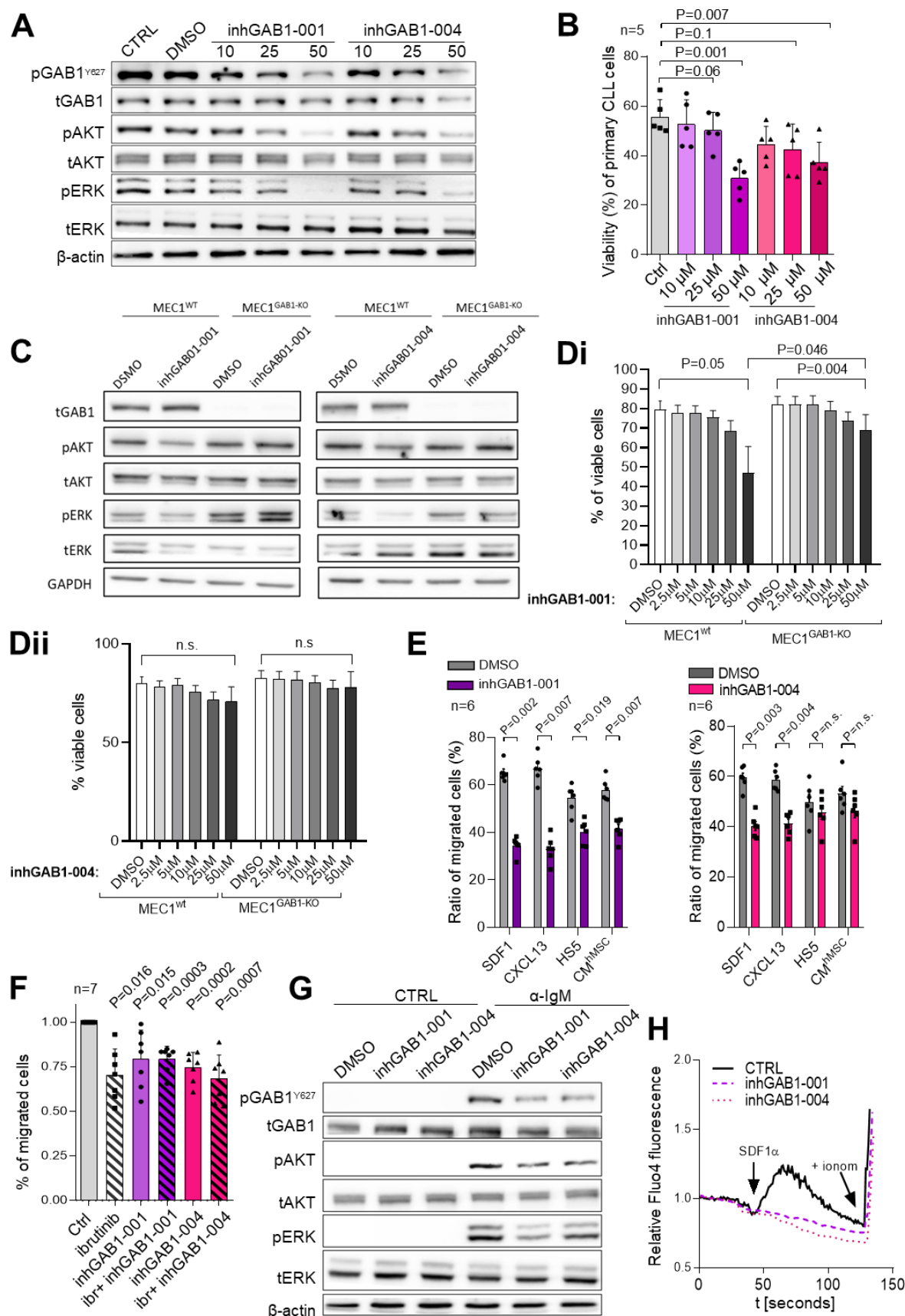


Figure 22. GAB1 inhibitors suppress migration and AKT activity. (A) Representative immunoblots from primary CLL cells treated for 6 hours with various doses (10, 25, and

50 μ M) of 2 GAB1 inhibitors or with the same volume of vehicle (DMSO) or with culture medium only (CTRL). This short-term treatment (6 hours) did not significantly impact cell viability (data not shown). **(B)** The effect of various doses of GAB1 inhibitors on CLL cells viability after 48 hours (n=5). The primary CLL cells were treated as described in panel [A]. **(C)** Immunoblot for MEC1^{WT} and MEC1^{GAB1-KO} cells treated for 6 hours with 50 μ M inhibitor GAB1-001 (left) or GAB1-004 (right). **(D)** The effect of various doses (2.5-50 μ M) of inhibitor GAB1-001 (Di) or GAB1-004 (Dii) on the viability of MEC1^{WT} and MEC1^{GAB1-KO} cells (72 hours, n=7 replicates). **(E)** Effect of a GAB1 inhibitors on primary CLL cells migration (n=6). Cells were treated with inhGAB1-001 (25 μ M) or inhGAB1-004 (25 μ M) for 12 hours. Cells were subsequently stained with CellTrace dye and loaded into a transwell, and migration continued for 4 hours toward SDF1 (100 ng/mL), CXCL13 (250 ng/mL), 50% CM (produced by HS5 stroma cells), or 100% CM produced by primary human bone marrow MSCs (CM^{hMSC}). **(F)** GAB1 inhibitor effect on primary CLL cell migration (n=7). Cells were treated with inhGAB1-001 (25 μ M) or inhGAB1-004 (25 μ M) or ibrutinib (1 μ M) or their combination for 12 hours. Cells were subsequently stained with CellTrace dye and loaded into a transwell, and migration continued for 4 hours toward 50% CM (produced by HS5 stroma cells). **(G)** Representative immunoblot from primary CLL cells treated with GAB1 inhibitors (inhGAB1-001 or inhGAB1-004; 25 μ M) and stimulated with anti-IgM. Primary CLL cells were treated as described in panel A, and cells were divided into equal aliquots and exposed to vehicle (CTRL) or anti-IgM (10 μ g/mL, 5 minutes). **(H)** Effect of GAB1 inhibitors (6 hours pretreatment; 25 μ M) on calcium influx in primary CLL cells after SDF1 stimulation (100 ng/mL; arrow). Ionomycin (indicated by + ionom arrow) was added as a positive control.

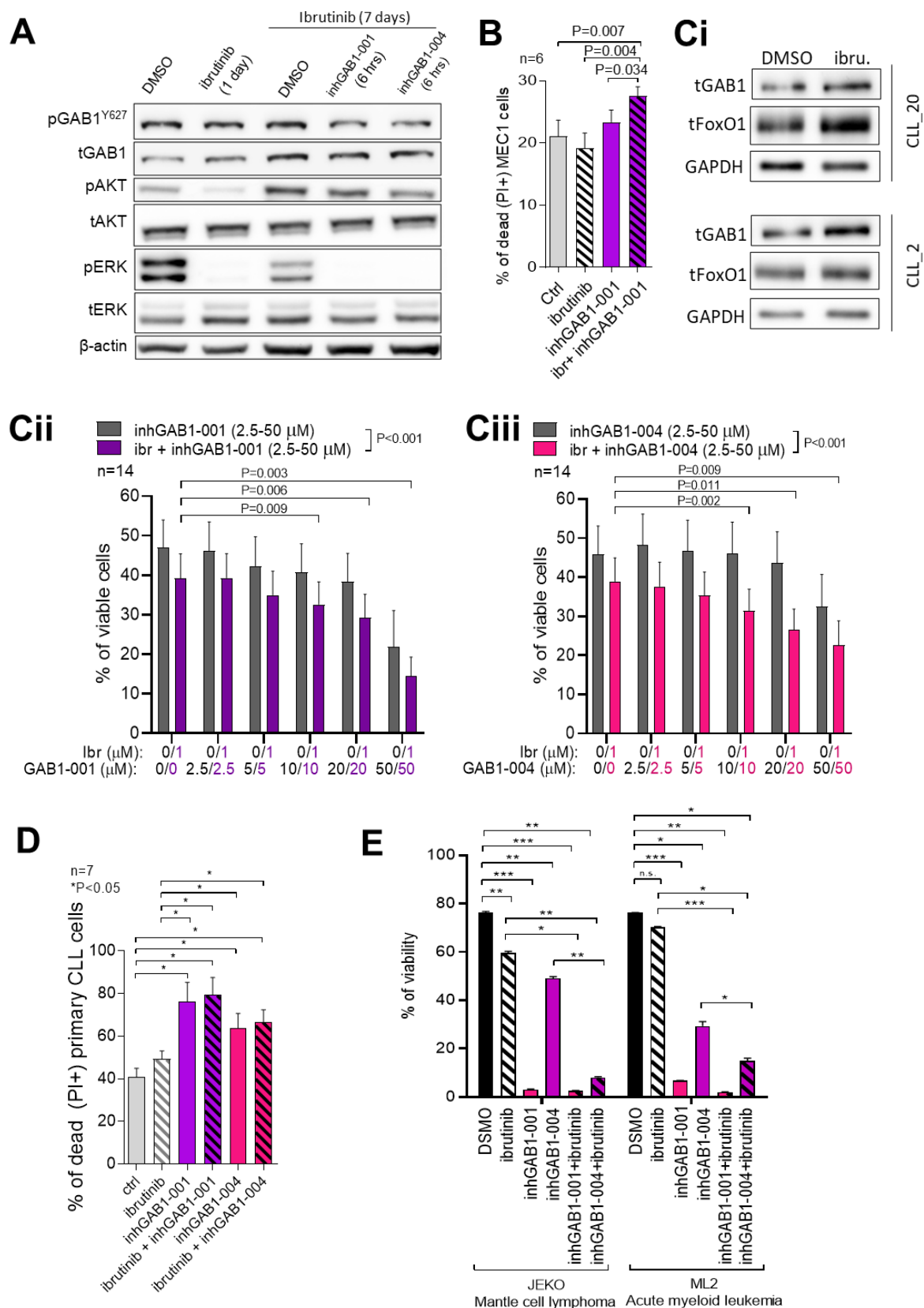


Figure 23. GAB1 inhibitors induce CLL cell apoptosis. (A) Representative immunoblots of MEC1 cells pretreated with ibrutinib (1 μ M) for 24 hours or 7 days and subsequently exposed to vehicle (DMSO) or GAB1 inhibitors for 6 hours (inhGAB1-001

or inhGAB1-004; 25 μ M). **(B)** Statistical analysis of MEC1 cells' viability (n=6). Cells were pretreated for 7 days with ibrutinib (1 μ M; fresh ibrutinib added every 48 hours) or vehicle (DMSO; added every 48 hours), and subsequently exposed to GAB1 inhibitor (inhGAB1-001; 25 μ M) for another 48 hours. **(Ci)** Representative immunoblot of primary CLL cells treated with 1 μ M ibrutinib (ibru.) or equal volume of DMSO for 6 days. Cells were incubated in media containing 5 ng/ml IL4. **(Cii, Ciii)** Effect of GAB1 inhibitors GAB1-001 (Cii) or GAB1-004 (Ciii), alone or in combination with ibrutinib on the viability of primary CLL cells (n=14). CLL cells were cultured in medium with IL4 (5 ng/mL) and simultaneously treated with ibrutinib (ibr; 1 μ M) or vehicle (DMSO) for 6 days. Subsequently, the cells were treated with a combination of GAB1 inhibitors (2.5-50 μ M) and ibrutinib (1 μ M), vehicle (DMSO), ibrutinib alone (1 μ M), or GAB1 inhibitor alone for an additional 48 hours. The color of the numbers below the graphs indicates the combination of drugs for each column. Viability was assessed by DiOC6/PI staining. The statistical significance of differences between the escalating doses of GAB1 inhibitor and GAB1 inhibitor plus ibrutinib was evaluated by 2-way analysis of variance with the Tukey test (both $P < 0.001$); the combination for individual columns was evaluated by paired Student t test (all $P < 0.05$). Ctrl, negative control treated only with equal volume of DMSO. **(D)** Statistical analysis of primary CLL cells' viability (n=7) after 48 hours treatment with ibrutinib (1 μ M) or two different GAB1 inhibitors (inhGAB1-001 or inhGAB1-004; 25 μ M) or their combinations. **(E)** Statistical analysis of JEKO (derived from mantle cell lymphoma) or ML2 (derived from acute myeloid leukemia) cells' viability (n=3) after 48 hours treatment with ibrutinib (1 μ M) or two different GAB1 inhibitors (inhGAB1-001 or inhGAB1-004; 25 μ M) or their combinations.

8. Discussion

In the presented thesis and published manuscript Seda *et al.*,³⁵ we show that GAB1 is upregulated by FoxO1 in the CXCR4^{bright}CD5^{dim} subpopulation, which exhibits higher migration potential towards immune niches. We observed that GAB1 increases the homing capacity of CLL cells via influencing chemokine responsiveness and F-actin polymerization. The increased GAB1 levels were demonstrated to positively regulate basal AKT activity, which was underscored in the context of ibrutinib therapy. This kind of treatment induced the FoxO1-GAB1 axis which resulted in the adaptive restoration of AKT phosphorylation. We have also demonstrated that GAB1 activity can be blocked by novel inhibitors targeting its PH domain.

It has been hypothesized that the CLL cell recirculation can be studied by analyzing CXCR4/CD5 intraclonal subpopulations. The original experiments with blood samples from patients who consumed heavy water (²H₂O) identified that CXCR4^{dim}CD5^{bright} cells represent a fraction of cells that exhibited the highest proportion of incorporated ²H.¹²² And since the proliferation of B cells and CLL cells occurs predominantly inside the lymphoid organs,³⁰³ it is believed that CXCR4^{dim}CD5^{bright} cells represent recent emigrants from immune niches. On the contrary, CXCR4^{bright}CD5^{dim} cells should be more responsive to microenvironmental chemoattractant SDF1 due to higher CXCR4 levels.^{122,124,126} Indeed, the CXCR4^{bright}CD5^{dim} subpopulation had a significantly higher capacity to migrate into the spleen from peripheral blood of NSG mice than CXCR4^{dim}CD5^{bright} cells. And the similar pattern was observed *in vitro* in transwell competitive migration assay towards chemoattractants such as SDF1, CXCL13, and conditioned media produced by human mesenchymal stromal cells.

We have previously analyzed CXCR4^{dim}CD5^{bright} cells to understand the pathways activated in the microenvironment.^{13,123,127} Here we hypothesized that analyzing the “resting” non-proliferative CXCR4^{bright}CD5^{dim} subpopulation could help to understand the factors influencing their return back to immune niches. RNA sequencing of 20 sorted subpopulations (paired samples from 10 CLL patients) identified 147 genes differentially expressed in CXCR4/CD5 subpopulations which were annotated in databases as migration-related. Among them, we noticed the upregulation of *GAB1* in the CXCR4^{bright}CD5^{dim} subpopulation, which we further identified as a CLL cell migration-regulator. The genetic ablation of *GAB1* in CLL cell line

MEC1 largely inhibited their migration towards SDF1, CXCL13, conditioned media produced by primary human mesenchymal stromal cells, and similar results were obtained with siRNA against *GAB1* in primary CLL cells. This was further supported by an *in vivo* competitive migration assay in NSG mice showing that *GAB1* knock-out cells or cells after *GAB1* silencing by siRNA exhibit impaired migration of malignant B cells to the spleen. This is in line with data from muscle progenitor cells,²⁹⁰ where genetic ablation of either *GAB1* or *CXCR4* resulted in highly similar (but not identical) defects in migration to specific tissue locations, suggesting that *GAB1* has a non-redundant role in the *CXCR4* pathway, but also in other cell-migration mechanisms. Indeed, we further observed that *CXCR4* ligand SDF1, induced *GAB1* phosphorylation within minutes, and similar activation was observed after treatment with CXCL13 or conditioned media produced by primary human mesenchymal stromal cells. Importantly, the treatment of MEC1 cells with specific *CXCR4* inhibitor plerixafor largely blocked *GAB1* activation induced by SDF1 but did not block the phosphorylation induced by conditioned media, indicating that *GAB1* is involved signal integration of various chemokine-receptors. We further demonstrated that *GAB1* levels positively affect F-actin polymerization and cell motility in response to chemokines. This could be functionally linked to the *GAB1* acting as a cell-membrane docking site for multiple proteins involved in migration such as PI3K,^{169,185} ERK,³⁰⁴ PLC γ , Crk, or CrkL proteins.³⁰⁵ Indeed, we have shown here that *GAB1* positively affects the activity of PI3K, a known regulator of F-actin polymerization and CLL and T cell migration.^{185,306,307} Taken together, the data show that *GAB1* positively regulates the response of CLL cells to multiple chemokines, and its accumulation in cells increases their homing capacity.

To reveal potential transcriptional regulator that regulates *GAB1* overexpression in CXCR4^{bright}CD5^{dim} cells, we overlapped our list of genes differentially expressed in CXCR4/CD5 subpopulations with the list of known transcriptional factors.²⁹⁶ We further selected FoxO1 (from 20 candidates) as a potential regulator of *GAB1* expression, since it was previously identified as a regulator of T cell homing,³⁴ migration of B cells within the germinal centers,^{197,201} and has multiple putative evolutionary conserved binding sites upstream from the *GAB1* promotor. Our further experiments identified that FoxO1 is accumulated in CXCR4^{bright}CD5^{dim} cells and directly transcriptionally activates *GAB1*. It is known that AKT activity negatively regulates FoxO1 by mediating inhibitory FoxO1^{S256} phosphorylation, resulting in its subsequent translocation from the nucleus to the cytoplasm and further degradation.^{195,196} In the context of migration, the long-term circulating cells usually exhibit

just weak activation of AKT,¹²³ which allows for FoxO1 stabilization and subsequent upregulation of *GAB1* and increased cell homing capacity to immune niches. Whereas in the microenvironment AKT is robustly phosphorylated which leads to FoxO1 degradation and subsequent *GAB1* down-modulation. Indeed, a strong AKT phosphorylation induction in CLL cells after adhesion or BCR activation led to FoxO1 degradation and concurrent *GAB1* down-modulation. In addition, FoxO1's genetic ablation or its silencing by siRNA also resulted in decreased B cell migration capacity in a fashion similar to *GAB1* knock-out. The positive role of FoxO1 in migration was previously demonstrated during migration of dendritic cell,²⁰⁰ T cell,³⁴ and normal B cell migration within the germinal centers.^{197,201} In our data, several migration-related genes (such as *ITGB2*, *PIK3CA*, *NF1*, *ETS1*, *ST14*, *LRRK2*, *APBB2*), which are putative FoxO1 targets, were differentially expressed in CXCR4/CD5 subpopulations ($P < 0.001$). This suggests that FoxO1 might contribute to the regulation of CLL migration by inducing *GAB1* and also other genes.

In B cells *GAB1* function was originally linked to BCR activation, as it contains multiple binding sites for SH2 domain-containing proteins,¹⁷² which is crucial especially in PI3K docking to the B cell membrane connecting BCR-activation with AKT phosphorylation.¹⁶⁹ Surprisingly, here we have identified another *GAB1* function by describing its role in increasing the so-called “tonic” PI3K/AKT signaling. Constitutive PI3K activity was previously observed in most peripheral blood CLL samples,³⁰⁸⁻³¹¹ but here we identified that this basal phosphorylation of PI3K subunit p85 is regulated by *GAB1* levels. This is of note since “tonic” PI3K/AKT signaling was demonstrated to be essential for B cell survival.²⁵³ In the context of CXCR4/CD5 subpopulations, the upregulated *GAB1* in CXCR4^{bright}CD5^{dim} CLL cells in peripheral blood not only contributes to higher migration capacity but also supports “tonic” PI3K/AKT activity. This might help to explain the previously noted association of higher *GAB1* levels with unfavorable prognosis in CLL.²⁹³ Altogether, these data suggest an interdependent balance of AKT activity and the FoxO1-*GAB1* axis, which maintains “tonic” pAKT in CLL cells that do not have access to microenvironmental interactions.

This is important in the context of ibrutinib therapy since this kind of treatment prevents CLL cells from homing.^{126,301} Lack of activation factors in peripheral blood thus strengthens the FoxO1 transcriptional activity, leading to *GAB1* upregulation and compensatory activation of AKT. This mechanism of AKT activation is surprising since FoxO1 activity was shown to be mutually exclusive with PI3K activity.³¹² However, we have shown here that weak or moderate AKT activity is not mutually exclusive with FoxO1 nuclear localization in CLL. A similar trend

was observed in some B cell lymphomas that tend to gain an oncogenic constitutive FoxO1 nuclear activity even in the presence of robust AKT activity.¹⁹⁸ Interestingly, FoxO1 was described as a positive regulator of proteins arresting the cell cycle. Therefore it is plausible that in the CXCR4^{bright}CD5^{dim} subpopulation FoxO1 has a dual role – on one hand, it upregulates proteins involved in the cell cycle arrest,³¹² to prevent the proliferation of misplaced cells, and on the other hand, it upregulates GAB1 to increase CLL cell homing and sustain their survival by maintaining “tonic” AKT activity.

Recently, new GAB1 inhibitors targeting its PH domain were synthesized and we demonstrated that they can suppress CLL cell migration towards microenvironmental cytokines like SDF1, CXCL13, or conditioned media produced by primary human mesenchymal stromal cells. Importantly, GAB1 inhibitors also impaired BCR induced AKT activity, and compensatory AKT phosphorylation during ibrutinib treatment, which was accompanied by decreased viability of treated cells. We also observed that targeting of GAB1 PH domain negatively affected ERK phosphorylation, which is in line with previously published data demonstrating that ERK activation in an SHP2-dependent manner is highly dependent on the NH₂-terminal PH domain of GAB1.^{179,304,313} In addition, GAB1 might represent a promising therapeutic target whose inhibition exhibit a different toxicity profile to commonly used drugs targeting PI3K or CXCR4 proteins. This will, however, require further development of more potent GAB1 inhibitors since their effect on CLL cell migration, pAKT suppression, and cell viability was observed only at higher concentrations.

In summary, in this thesis we described a novel FoxO1-GAB1-dependent mechanism of CLL cell homing and maintaining of “tonic” AKT activity. We showed that CLL cells isolated from patients undergoing ibrutinib treatment exhibit compensatory activation of AKT and that this could be targeted by novel GAB1 inhibitors (Figure 24). In the end, we showed that targeting of GAB1 may represent a promising strategy also in other hematological malignancies such as mantle cell lymphoma or acute myeloid leukemia.

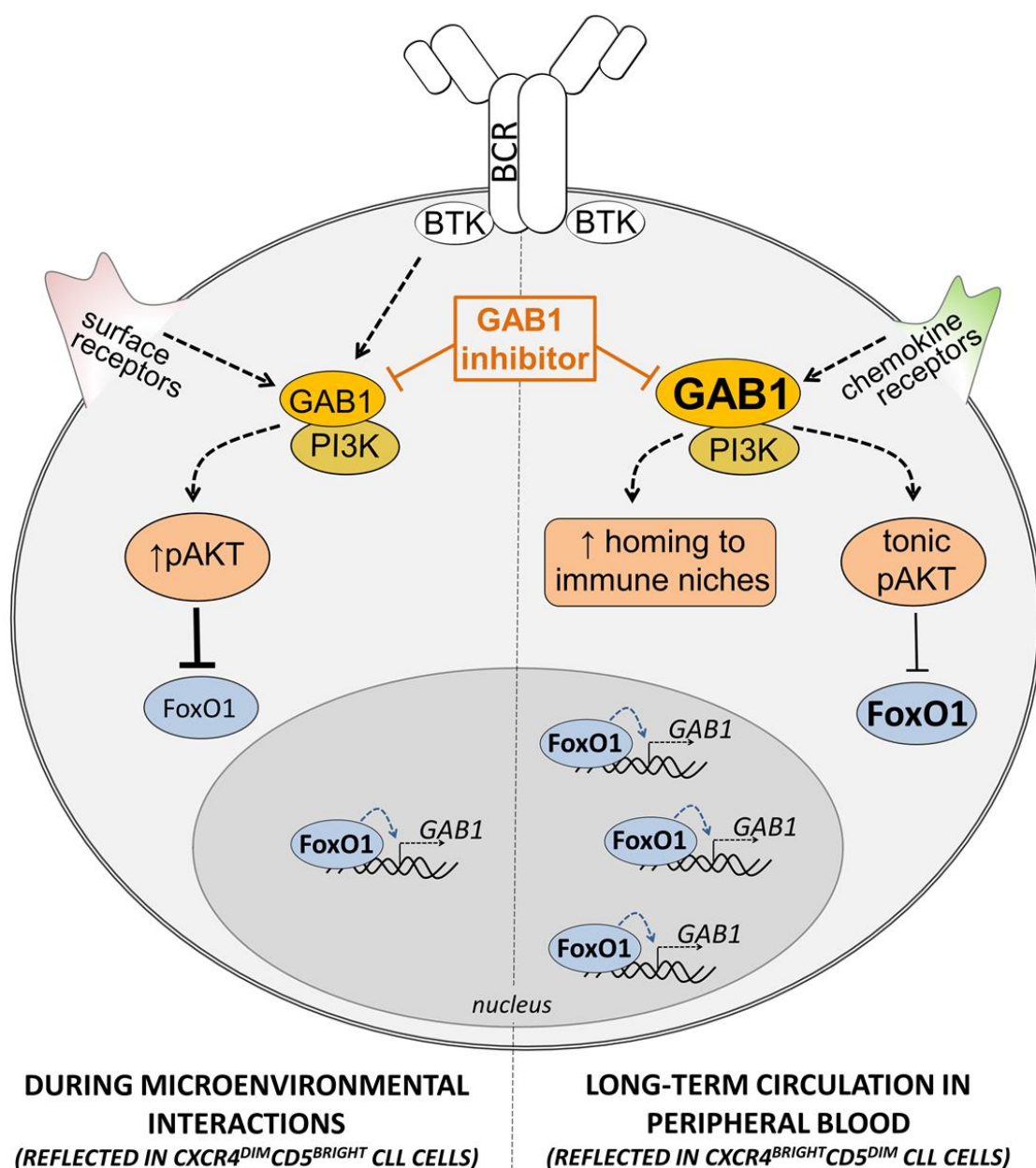


Figure 24. GAB1 regulation by FoxO1 and its role in CLL migration and tonic AKT activity: a novel therapeutic target. GAB1 is upregulated by FoxO1 during the transition of intraclonal CLL cell subpopulation from CXCR4^{dim}CD5^{bright} into CXCR4^{bright}CD5^{dim} cells. This increases the homing capacity of CXCR4^{bright}CD5^{dim} cells and also sustains pro-survival tonic AKT activity. During microenvironmental interactions (reflected in CXCR4^{dim}CD5^{bright} cells) GAB1 is involved in signaling from BCR and other cell surface receptors, and subsequent AKT activation represses GAB1 transcription via FoxO1. GAB1 inhibitors impair cell migration, inhibit tonic or BCR-induced AKT phosphorylation, and block compensatory AKT activity during ibrutinib therapy.

9. Conclusion

The results presented in this thesis are explaining the largely unknown mechanism of how CLL cells regulate their decision to return to the immune niches and how they balance their survival in peripheral blood. We have shown that the CXCR4^{bright}CD5^{dim} cell subpopulation exhibits higher migration capacity towards microenvironmental cytokines like SDF1 or CXCL13 and into immune niches of NSG mice. The sequencing of CXCR4/CD5 intraclonal subpopulations revealed differential expression of dozens of migration-related genes, including *GAB1*, which was upregulated in the CXCR4^{bright}CD5^{dim} subpopulation. This is of note since GAB1 protein was demonstrated to act as a migration regulator in non-immune cells.^{290,291} However, its role in lymphocyte migration was not investigated. Our experiments in CLL cells demonstrated that GAB1 protein is activated in response to various chemoattractants, and its expression positively affects cell migration towards cytokines such as SDF1 or CXCL13 or towards immune niches of NSG mice. We found that gradual GAB1 accumulation in CLL cells outside of immune niches is mediated by FoxO1, given that it transcriptionally activates the *GAB1* gene. We have also demonstrated that GAB1 upregulation takes a role in increasing basal AKT phosphorylation, which is required to sustain CLL cell survival.³¹¹ This is important in the context of ibrutinib therapy since this kind of treatment prevents CLL cells from homing. The analysis of primary CLL samples collected before and during ibrutinib therapy demonstrated the induction of the FoxO1-GAB1 pathway and subsequent compensatory activation of the AKT signaling. We have further shown that novel inhibitors targeting the GAB1 PH domain suppress cell migration, tonic AKT activity, and compensatory activation of AKT during ibrutinib treatment. The treatment of primary CLL cells with GAB1 inhibitors reduced their viability and this was synergic in combination with ibrutinib. Despite the necessity to improve attributes of GAB1 inhibitor, our proposed model represents a novel and promising strategy not only for the treatment of CLL but possibly also for the treatment of other hematological malignancies such as mantle cell lymphoma or acute myeloid leukemia.

10. Literature

1. Kipps TJ, Stevenson FK, Wu CJ, et al. Chronic lymphocytic leukaemia. *Nature reviews Disease primers*. 2017;3:17008.
2. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*. 2008;111(12):5446-5456.
3. Rai KR, Sawitsky A. A review of the prognostic role of cytogenetic, phenotypic, morphologic, and immune function characteristics in chronic lymphocytic leukemia. *Blood Cells*. 1987;12(2):327-338.
4. Binet JL, Auquier A, Dighiero G, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer*. 1981;48(1):198-206.
5. Rossi D, Gaidano G. ATM and chronic lymphocytic leukemia: mutations, and not only deletions, matter. *Haematologica*. 2012;97(1):5-8.
6. Puente XS, Pinyol M, Quesada V, et al. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature*. 2011;475(7354):101-105.
7. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America*. 2002;99(24):15524-15529.
8. Abruzzo LV, Herling CD, Calin GA, et al. Trisomy 12 chronic lymphocytic leukemia expresses a unique set of activated and targetable pathways. *Haematologica*. 2018;103(12):2069-2078.
9. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood*. 1999;94(6):1848-1854.
10. Damle RN, Wasil T, Fais F, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood*. 1999;94(6):1840-1847.
11. Crespo M, Bosch F, Villamor N, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med*. 2003;348(18):1764-1775.
12. Messmer BT, Messmer D, Allen SL, et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *J Clin Invest*. 2005;115(3):755-764.
13. Pavlasova G, Borsky M, Seda V, et al. Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis. *Blood*. 2016;128(12):1609-1613.
14. Panayiotidis P, Jones D, Ganeshaguru K, Foroni L, Hoffbrand AV. Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro. *Br J Haematol*. 1996;92(1):97-103.
15. Herishanu Y, Perez-Galan P, Liu D, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood*. 2011;117(2):563-574.
16. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood*. 2000;96(8):2655-2663.
17. Mraz M, Zent CS, Church AK, et al. Bone marrow stromal cells protect lymphoma B-cells from rituximab-induced apoptosis and targeting integrin alpha-4-beta-1 (VLA-4) with natalizumab can overcome this resistance. *British journal of haematology*. 2011;155(1):53-64.
18. Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *The New England journal of medicine*. 2013;369(1):32-42.
19. Furman RR, Sharman JP, Coutre SE, et al. Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. *The New England journal of medicine*. 2014;370(11):997-1007.

20. Ponader S, Chen SS, Buggy JJ, et al. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood*. 2012;119(5):1182-1189.
21. Ondrisova L, Mraz M. Genetic and Non-Genetic Mechanisms of Resistance to BCR Signaling Inhibitors in B Cell Malignancies. *Front Oncol*. 2020;10:591577.
22. Girard JP, Springer TA. High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. *Immunol Today*. 1995;16(9):449-457.
23. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7(9):678-689.
24. Arbones ML, Ord DC, Ley K, et al. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity*. 1994;1(4):247-260.
25. Lafouresse F, Bellard E, Laurent C, et al. L-selectin controls trafficking of chronic lymphocytic leukemia cells in lymph node high endothelial venules in vivo. *Blood*. 2015;126(11):1336-1345.
26. Ivetic A, Hoskins Green HL, Hart SJ. L-selectin: A Major Regulator of Leukocyte Adhesion, Migration and Signaling. *Front Immunol*. 2019;10:1068.
27. Fors BP, Goodarzi K, von Andrian UH. L-selectin shedding is independent of its subsurface structures and topographic distribution. *Journal of immunology*. 2001;167(7):3642-3651.
28. Rosen SD. Ligands for L-selectin: homing, inflammation, and beyond. *Annu Rev Immunol*. 2004;22:129-156.
29. Kahn J, Ingraham RH, Shirley F, Migaki GI, Kishimoto TK. Membrane proximal cleavage of L-selectin: identification of the cleavage site and a 6-kD transmembrane peptide fragment of L-selectin. *The Journal of cell biology*. 1994;125(2):461-470.
30. Kahn J, Walcheck B, Migaki GI, Jutila MA, Kishimoto TK. Calmodulin regulates L-selectin adhesion molecule expression and function through a protease-dependent mechanism. *Cell*. 1998;92(6):809-818.
31. Liu S, Kiick K. Architecture effects on L-selectin shedding induced by polypeptide-based multivalent ligands. *Polym Chem*. 2011;2(7):1513-1522.
32. Chen JR, Gu BJ, Dao LP, Bradley CJ, Mulligan SP, Wiley JS. Transendothelial migration of lymphocytes in chronic lymphocytic leukaemia is impaired and involved down-regulation of both L-selectin and CD23. *British journal of haematology*. 1999;105(1):181-189.
33. Fabre S, Carrette F, Chen J, et al. FOXO1 regulates L-Selectin and a network of human T cell homing molecules downstream of phosphatidylinositol 3-kinase. *J Immunol*. 2008;181(5):2980-2989.
34. Kerdiles YM, Beisner DR, Tinoco R, et al. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nat Immunol*. 2009;10(2):176-184.
35. Seda V, Vojackova E, Ondrisova L, et al. FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia. *Blood*. 2021.
36. Zhang Y, Wang H. Integrin signalling and function in immune cells. *Immunology*. 2012;135(4):268-275.
37. Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. *Annual review of immunology*. 2007;25:619-647.
38. Harzschel A, Zucchetto A, Gattei V, Hartmann TN. VLA-4 Expression and Activation in B Cell Malignancies: Functional and Clinical Aspects. *Int J Mol Sci*. 2020;21(6).
39. Alon R, Dustin ML. Force as a facilitator of integrin conformational changes during leukocyte arrest on blood vessels and antigen-presenting cells. *Immunity*. 2007;26(1):17-27.
40. Hughes PE, Diaz-Gonzalez F, Leong L, et al. Breaking the integrin hinge. A defined structural constraint regulates integrin signaling. *The Journal of biological chemistry*. 1996;271(12):6571-6574.
41. Calderwood DA. Integrin activation. *J Cell Sci*. 2004;117(Pt 5):657-666.
42. O'Toole TE, Mandelman D, Forsyth J, Shattil SJ, Plow EF, Ginsberg MH. Modulation of the affinity of integrin alpha IIb beta 3 (GPIIb-IIIa) by the cytoplasmic domain of alpha IIb. *Science*. 1991;254(5033):845-847.

43. Spaargaren M, Beuling EA, Rurup ML, et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *The Journal of experimental medicine*. 2003;198(10):1539-1550.
44. Takagi J, Petre BM, Walz T, Springer TA. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell*. 2002;110(5):599-511.
45. Calderwood DA, Fujioka Y, de Pereda JM, et al. Integrin beta cytoplasmic domain interactions with phosphotyrosine-binding domains: a structural prototype for diversity in integrin signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100(5):2272-2277.
46. Lad Y, Kiema T, Jiang P, et al. Structure of three tandem filamin domains reveals auto-inhibition of ligand binding. *EMBO J*. 2007;26(17):3993-4004.
47. Tadokoro S, Shattil SJ, Eto K, et al. Talin binding to integrin beta tails: a final common step in integrin activation. *Science*. 2003;302(5642):103-106.
48. Critchley DR. Genetic, biochemical and structural approaches to talin function. *Biochem Soc Trans*. 2005;33(Pt 6):1308-1312.
49. Oxley CL, Anthis NJ, Lowe ED, Vakonakis I, Campbell ID, Wegener KL. An integrin phosphorylation switch: the effect of beta3 integrin tail phosphorylation on Dok1 and talin binding. *The Journal of biological chemistry*. 2008;283(9):5420-5426.
50. Millon-Fremillon A, Bouvard D, Grichine A, Manet-Dupe S, Block MR, Albiges-Rizo C. Cell adaptive response to extracellular matrix density is controlled by ICAP-1-dependent beta1-integrin affinity. *The Journal of cell biology*. 2008;180(2):427-441.
51. Hughes PE, Renshaw MW, Pfaff M, et al. Suppression of integrin activation: a novel function of a Ras/Raf-initiated MAP kinase pathway. *Cell*. 1997;88(4):521-530.
52. Carman CV, Springer TA. Integrin avidity regulation: are changes in affinity and conformation underemphasized? *Curr Opin Cell Biol*. 2003;15(5):547-556.
53. Springer TA. Adhesion receptors of the immune system. *Nature*. 1990;346(6283):425-434.
54. Arroyo AG, Yang JT, Rayburn H, Hynes RO. Differential requirements for alpha4 integrins during fetal and adult hematopoiesis. *Cell*. 1996;85(7):997-1008.
55. Imai Y, Shimaoka M, Kurokawa M. Essential roles of VLA-4 in the hematopoietic system. *Int J Hematol*. 2010;91(4):569-575.
56. Chen CC, Manning AM. Transcriptional regulation of endothelial cell adhesion molecules: a dominant role for NF-kappa B. *Agents Actions Suppl*. 1995;47:135-141.
57. Gattei V, Bulian P, Del Principe MI, et al. Relevance of CD49d protein expression as overall survival and progressive disease prognosticator in chronic lymphocytic leukemia. *Blood*. 2008;111(2):865-873.
58. Berlin-Rufenach C, Otto F, Mathies M, et al. Lymphocyte migration in lymphocyte function-associated antigen (LFA)-1-deficient mice. *The Journal of experimental medicine*. 1999;189(9):1467-1478.
59. Carrasco YR, Fleire SJ, Cameron T, Dustin ML, Batista FD. LFA-1/ICAM-1 interaction lowers the threshold of B cell activation by facilitating B cell adhesion and synapse formation. *Immunity*. 2004;20(5):589-599.
60. Hartmann TN, Grabovsky V, Wang W, et al. Circulating B-cell chronic lymphocytic leukemia cells display impaired migration to lymph nodes and bone marrow. *Cancer Res*. 2009;69(7):3121-3130.
61. Partridge MA, Marcantonio EE. Initiation of attachment and generation of mature focal adhesions by integrin-containing filopodia in cell spreading. *Mol Biol Cell*. 2006;17(10):4237-4248.
62. Morgan MR, Humphries MJ, Bass MD. Synergistic control of cell adhesion by integrins and syndecans. *Nat Rev Mol Cell Biol*. 2007;8(12):957-969.
63. Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol*. 2010;11(4):288-300.
64. Dwyer SF, Gelman IH. Cross-Phosphorylation and Interaction between Src/FAK and MAPKAP5/PRAK in Early Focal Adhesions Controls Cell Motility. *J Cancer Biol Res*. 2014;2(1).

65. Watanabe T, Tsuda M, Makino Y, et al. Crk adaptor protein-induced phosphorylation of Gab1 on tyrosine 307 via Src is important for organization of focal adhesions and enhanced cell migration. *Cell Res.* 2009;19(5):638-650.
66. Thomas JW, Ellis B, Boerner RJ, Knight WB, White GC, 2nd, Schaller MD. SH2- and SH3-mediated interactions between focal adhesion kinase and Src. *The Journal of biological chemistry.* 1998;273(1):577-583.
67. Calalb MB, Polte TR, Hanks SK. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Molecular and cellular biology.* 1995;15(2):954-963.
68. Owen JD, Ruest PJ, Fry DW, Hanks SK. Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. *Molecular and cellular biology.* 1999;19(7):4806-4818.
69. Calalb MB, Zhang X, Polte TR, Hanks SK. Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. *Biochemical and biophysical research communications.* 1996;228(3):662-668.
70. Schlaepfer DD, Hanks SK, Hunter T, van der Geer P. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature.* 1994;372(6508):786-791.
71. Hanks SK, Ryzhova L, Shin NY, Brabek J. Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. *Front Biosci.* 2003;8:d982-996.
72. Liu ZX, Yu CF, Nickel C, Thomas S, Cantley LG. Hepatocyte growth factor induces ERK-dependent paxillin phosphorylation and regulates paxillin-focal adhesion kinase association. *The Journal of biological chemistry.* 2002;277(12):10452-10458.
73. Turner CE. Paxillin and focal adhesion signalling. *Nat Cell Biol.* 2000;2(12):E231-236.
74. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annual review of immunology.* 2014;32:659-702.
75. Bachelier F, Graham GJ, Locati M, et al. An atypical addition to the chemokine receptor nomenclature: IUPHAR Review 15. *Br J Pharmacol.* 2015;172(16):3945-3949.
76. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. *Nature immunology.* 2001;2(2):123-128.
77. Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science.* 1996;272(5263):872-877.
78. Chatterjee S, Behnam Azad B, Nimmagadda S. The intricate role of CXCR4 in cancer. *Adv Cancer Res.* 2014;124:31-82.
79. Kucia M, Jankowski K, Reza R, et al. CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. *J Mol Histol.* 2004;35(3):233-245.
80. Nagasawa T, Hirota S, Tachibana K, et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature.* 1996;382(6592):635-638.
81. Ma Q, Jones D, Springer TA. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity.* 1999;10(4):463-471.
82. Okada T, Ngo VN, Ekland EH, et al. Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. *The Journal of experimental medicine.* 2002;196(1):65-75.
83. Netelenbos T, Zuijderduijn S, Van Den Born J, et al. Proteoglycans guide SDF-1-induced migration of hematopoietic progenitor cells. *J Leukoc Biol.* 2002;72(2):353-362.
84. Sadir R, Baleux F, Grosdidier A, Imberty A, Lortat-Jacob H. Characterization of the stromal cell-derived factor-1 α -heparin complex. *The Journal of biological chemistry.* 2001;276(11):8288-8296.
85. Netelenbos T, van den Born J, Kessler FL, et al. Proteoglycans on bone marrow endothelial cells bind and present SDF-1 towards hematopoietic progenitor cells. *Leukemia.* 2003;17(1):175-184.

86. Wysoczynski M, Reza R, Ratajczak J, et al. Incorporation of CXCR4 into membrane lipid rafts primes homing-related responses of hematopoietic stem/progenitor cells to an SDF-1 gradient. *Blood*. 2005;105(1):40-48.
87. Le Y, Honczarenko M, Glodek AM, Ho DK, Silberstein LE. CXC chemokine ligand 12-induced focal adhesion kinase activation and segregation into membrane domains is modulated by regulator of G protein signaling 1 in pro-B cells. *Journal of immunology*. 2005;174(5):2582-2590.
88. Burger JA, Burger M, Kipps TJ. Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. *Blood*. 1999;94(11):3658-3667.
89. Ghobrial IM, Bone ND, Stenson MJ, et al. Expression of the chemokine receptors CXCR4 and CCR7 and disease progression in B-cell chronic lymphocytic leukemia/ small lymphocytic lymphoma. *Mayo Clin Proc*. 2004;79(3):318-325.
90. Burkle A, Niedermeier M, Schmitt-Graff A, Wierda WG, Keating MJ, Burger JA. Overexpression of the CXCR5 chemokine receptor, and its ligand, CXCL13 in B-cell chronic lymphocytic leukemia. *Blood*. 2007;110(9):3316-3325.
91. Singh S, Singh R, Sharma PK, et al. Serum CXCL13 positively correlates with prostatic disease, prostate-specific antigen and mediates prostate cancer cell invasion, integrin clustering and cell adhesion. *Cancer Lett*. 2009;283(1):29-35.
92. Olsen RS, Nijm J, Andersson RE, Dimberg J, Wagsater D. Circulating inflammatory factors associated with worse long-term prognosis in colorectal cancer. *World J Gastroenterol*. 2017;23(34):6212-6219.
93. Liu JF, Lee CW, Lin CY, et al. CXCL13/CXCR5 Interaction Facilitates VCAM-1-Dependent Migration in Human Osteosarcoma. *Int J Mol Sci*. 2020;21(17).
94. Biswas S, Roy Chowdhury S, Mandal G, Purohit S, Gupta A, Bhattacharyya A. RelA driven co-expression of CXCL13 and CXCR5 is governed by a multifaceted transcriptional program regulating breast cancer progression. *Biochim Biophys Acta Mol Basis Dis*. 2019;1865(2):502-511.
95. Heinig K, Gatjen M, Grau M, et al. Access to follicular dendritic cells is a pivotal step in murine chronic lymphocytic leukemia B-cell activation and proliferation. *Cancer Discov*. 2014;4(12):1448-1465.
96. Allen CD, Cyster JG. Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Seminars in immunology*. 2008;20(1):14-25.
97. Muller G, Lipp M. Signal transduction by the chemokine receptor CXCR5: structural requirements for G protein activation analyzed by chimeric CXCR1/CXCR5 molecules. *Biol Chem*. 2001;382(9):1387-1397.
98. Alley J, Bloom L, Kasaian M, et al. A human CXCL13-induced actin polymerization assay measured by fluorescence plate reader. *Assay Drug Dev Technol*. 2010;8(1):73-84.
99. Saez de Guinoa J, Barrio L, Mellado M, Carrasco YR. CXCL13/CXCR5 signaling enhances BCR-triggered B-cell activation by shaping cell dynamics. *Blood*. 2011;118(6):1560-1569.
100. Forster R, Schubel A, Breitfeld D, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell*. 1999;99(1):23-33.
101. McHeik S, Van Eeckhout N, De Poorter C, Gales C, Parmentier M, Springael JY. Coexpression of CCR7 and CXCR4 During B Cell Development Controls CXCR4 Responsiveness and Bone Marrow Homing. *Front Immunol*. 2019;10:2970.
102. Reif K, Ekland EH, Ohl L, et al. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature*. 2002;416(6876):94-99.
103. Stebegg M, Kumar SD, Silva-Cayetano A, Fonseca VR, Linterman MA, Graca L. Regulation of the Germinal Center Response. *Front Immunol*. 2018;9:2469.
104. Yoshida R, Imai T, Hieshima K, et al. Molecular cloning of a novel human CC chemokine EBI1-ligand chemokine that is a specific functional ligand for EBI1, CCR7. *The Journal of biological chemistry*. 1997;272(21):13803-13809.

105. Yan Y, Chen R, Wang X, et al. CCL19 and CCR7 Expression, Signaling Pathways, and Adjuvant Functions in Viral Infection and Prevention. *Front Cell Dev Biol.* 2019;7:212.
106. Manzo A, Paoletti S, Carulli M, et al. Systematic microanatomical analysis of CXCL13 and CCL21 in situ production and progressive lymphoid organization in rheumatoid synovitis. *European journal of immunology.* 2005;35(5):1347-1359.
107. Luther SA, Tang HL, Hyman PL, Farr AG, Cyster JG. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proceedings of the National Academy of Sciences of the United States of America.* 2000;97(23):12694-12699.
108. Carlsen HS, Haraldsen G, Brandtzaeg P, Baekkevold ES. Disparate lymphoid chemokine expression in mice and men: no evidence of CCL21 synthesis by human high endothelial venules. *Blood.* 2005;106(2):444-446.
109. Yoshida R, Nagira M, Kitaura M, Imagawa N, Imai T, Yoshie O. Secondary lymphoid-tissue chemokine is a functional ligand for the CC chemokine receptor CCR7. *The Journal of biological chemistry.* 1998;273(12):7118-7122.
110. Forster R, Davalos-Miszlitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol.* 2008;8(5):362-371.
111. Schwab SR, Cyster JG. Finding a way out: lymphocyte egress from lymphoid organs. *Nature immunology.* 2007;8(12):1295-1301.
112. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annual review of immunology.* 2005;23:127-159.
113. Liu Y, Wada R, Yamashita T, et al. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *The Journal of clinical investigation.* 2000;106(8):951-961.
114. Allende ML, Dreier JL, Mandala S, Proia RL. Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. *The Journal of biological chemistry.* 2004;279(15):15396-15401.
115. Matloubian M, Lo CG, Cinamon G, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature.* 2004;427(6972):355-360.
116. Lee MJ, Van Brocklyn JR, Thangada S, et al. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science.* 1998;279(5356):1552-1555.
117. Thuy AV, Reimann CM, Hemdan NY, Graler MH. Sphingosine 1-phosphate in blood: function, metabolism, and fate. *Cell Physiol Biochem.* 2014;34(1):158-171.
118. Murata N, Sato K, Kon J, Tomura H, Okajima F. Quantitative measurement of sphingosine 1-phosphate by radioreceptor-binding assay. *Anal Biochem.* 2000;282(1):115-120.
119. Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y, Cyster JG. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science.* 2005;309(5741):1735-1739.
120. Capitani N, Patrussi L, Trentin L, et al. S1P1 expression is controlled by the pro-oxidant activity of p66Shc and is impaired in B-CLL patients with unfavorable prognosis. *Blood.* 2012;120(22):4391-4399.
121. Brown JR, Byrd JC, Coutre SE, et al. Idelalisib, an inhibitor of phosphatidylinositol 3-kinase p110delta, for relapsed/refractory chronic lymphocytic leukemia. *Blood.* 2014;123(22):3390-3397.
122. Calissano C, Damle RN, Marsilio S, et al. Intraclonal complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. *Molecular medicine.* 2011;17(11-12):1374-1382.
123. Pavlasova G, Borsky M, Svobodova V, et al. Rituximab primarily targets an intra-clonal BCR signaling proficient CLL subpopulation characterized by high CD20 levels. *Leukemia.* 2018;32(9):2028-2031.
124. Herndon TM, Chen SS, Saba NS, et al. Direct in vivo evidence for increased proliferation of CLL cells in lymph nodes compared to bone marrow and peripheral blood. *Leukemia.* 2017;31(6):1340-1347.

125. Bartholdy BA, Wang X, Yan XJ, et al. CLL intraclonal fractions exhibit established and recently acquired patterns of DNA methylation. *Blood Adv.* 2020;4(5):893-905.
126. Chen SS, Chang BY, Chang S, et al. BTK inhibition results in impaired CXCR4 chemokine receptor surface expression, signaling and function in chronic lymphocytic leukemia. *Leukemia.* 2016;30(4):833-843.
127. Sharma S, Pavlasova GM, Seda V, et al. miR-29 Modulates CD40 Signaling in Chronic Lymphocytic Leukemia by Targeting TRAF4: an Axis Affected by BCR inhibitors. *Blood.* 2020.
128. Janeway CA, Murphy K, Travers P, Walport MJ. *Janeway's immunobiology.* Vol 8: Garland Science Taylor and Francis Group; 2008.
129. Gupta SK, Viswanatha DS, Patel KP. Evaluation of Somatic Hypermutation Status in Chronic Lymphocytic Leukemia (CLL) in the Era of Next Generation Sequencing. *Front Cell Dev Biol.* 2020;8:357.
130. Efremov DG, Ivanovski M, Batista FD, Pozzato G, Burrone OR. IgM-producing chronic lymphocytic leukemia cells undergo immunoglobulin isotype-switching without acquiring somatic mutations. *J Clin Invest.* 1996;98(2):290-298.
131. Dal Porto JM, Gauld SB, Merrell KT, Mills D, Pugh-Bernard AE, Cambier J. B cell antigen receptor signaling 101. *Mol Immunol.* 2004;41(6-7):599-613.
132. Reth M, Nielsen P. Signaling circuits in early B-cell development. *Advances in immunology.* 2014;122:129-175.
133. Flaswinkel H, Reth M. Dual role of the tyrosine activation motif of the Ig-alpha protein during signal transduction via the B cell antigen receptor. *The EMBO journal.* 1994;13(1):83-89.
134. Monroe JG. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat Rev Immunol.* 2006;6(4):283-294.
135. Singer SJ, Nicolson GL. The fluid mosaic model of the structure of cell membranes. *Science.* 1972;175(4023):720-731.
136. Harwood NE, Batista FD. Early events in B cell activation. *Annu Rev Immunol.* 2010;28:185-210.
137. Yang J, Reth M. Oligomeric organization of the B-cell antigen receptor on resting cells. *Nature.* 2010;467(7314):465-469.
138. Ziegler CGK, Kim J, Piersanti K, et al. Constitutive Activation of the B Cell Receptor Underlies Dysfunctional Signaling in Chronic Lymphocytic Leukemia. *Cell Rep.* 2019;28(4):923-937 e923.
139. Gupta N, DeFranco AL. Lipid rafts and B cell signaling. *Semin Cell Dev Biol.* 2007;18(5):616-626.
140. Cheng AM, Rowley B, Pao W, Hayday A, Bolen JB, Pawson T. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature.* 1995;378(6554):303-306.
141. Khan WN, Sideras P, Rosen FS, Alt FW. The role of Bruton's tyrosine kinase in B-cell development and function in mice and man. *Ann N Y Acad Sci.* 1995;764:27-38.
142. Pao LI, Famiglietti SJ, Cambier JC. Asymmetrical phosphorylation and function of immunoreceptor tyrosine-based activation motif tyrosines in B cell antigen receptor signal transduction. *J Immunol.* 1998;160(7):3305-3314.
143. Kurosaki T, Johnson SA, Pao L, Sada K, Yamamura H, Cambier JC. Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. *The Journal of experimental medicine.* 1995;182(6):1815-1823.
144. Tolar P, Pierce SK. A conformation-induced oligomerization model for B cell receptor microclustering and signaling. *Curr Top Microbiol Immunol.* 2010;340:155-169.
145. Fujimoto M, Fujimoto Y, Poe JC, et al. CD19 regulates Src family protein tyrosine kinase activation in B lymphocytes through processive amplification. *Immunity.* 2000;13(1):47-57.
146. Maity PC, Yang J, Klaesener K, Reth M. The nanoscale organization of the B lymphocyte membrane. *Biochim Biophys Acta.* 2015;1853(4):830-840.
147. Klasener K, Maity PC, Hobeika E, Yang J, Reth M. B cell activation involves nanoscale receptor reorganizations and inside-out signaling by Syk. *Elife.* 2014;3:e02069.

148. Depoil D, Fleire S, Treanor BL, et al. CD19 is essential for B cell activation by promoting B cell receptor-antigen microcluster formation in response to membrane-bound ligand. *Nature immunology*. 2008;9(1):63-72.
149. Chung EY, Psathas JN, Yu D, Li Y, Weiss MJ, Thomas-Tikhonenko A. CD19 is a major B cell receptor-independent activator of MYC-driven B-lymphomagenesis. *The Journal of clinical investigation*. 2012;122(6):2257-2266.
150. Chong YP, Mulhern TD, Cheng HC. C-terminal Src kinase (CSK) and CSK-homologous kinase (CHK)--endogenous negative regulators of Src-family protein kinases. *Growth Factors*. 2005;23(3):233-244.
151. Hata A, Sabe H, Kurosaki T, Takata M, Hanafusa H. Functional analysis of Csk in signal transduction through the B-cell antigen receptor. *Mol Cell Biol*. 1994;14(11):7306-7313.
152. Shrivastava P, Katagiri T, Ogimoto M, Mizuno K, Yakura H. Dynamic regulation of Src-family kinases by CD45 in B cells. *Blood*. 2004;103(4):1425-1432.
153. Pao LI, Cambier JC. Syk, but not Lyn, recruitment to B cell antigen receptor and activation following stimulation of CD45- B cells. *J Immunol*. 1997;158(6):2663-2669.
154. Majeti R, Xu Z, Parslow TG, et al. An inactivating point mutation in the inhibitory wedge of CD45 causes lymphoproliferation and autoimmunity. *Cell*. 2000;103(7):1059-1070.
155. Yang J, Nie J, Ma X, Wei Y, Peng Y, Wei X. Targeting PI3K in cancer: mechanisms and advances in clinical trials. *Molecular cancer*. 2019;18(1):26.
156. Cuevas BD, Lu Y, Mao M, et al. Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase. *The Journal of biological chemistry*. 2001;276(29):27455-27461.
157. Mocsai A, Ruland J, Tybulewicz VL. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat Rev Immunol*. 2010;10(6):387-402.
158. Cambier JC, Pleiman CM, Clark MR. Signal transduction by the B cell antigen receptor and its coreceptors. *Annual review of immunology*. 1994;12:457-486.
159. Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. *Biochimica et biophysica acta*. 1998;1436(1-2):127-150.
160. Czech MP. PIP2 and PIP3: complex roles at the cell surface. *Cell*. 2000;100(6):603-606.
161. Kurosaki T. Regulation of B-cell signal transduction by adaptor proteins. *Nat Rev Immunol*. 2002;2(5):354-363.
162. Okada T, Maeda A, Iwamatsu A, Gotoh K, Kurosaki T. BCAP: the tyrosine kinase substrate that connects B cell receptor to phosphoinositide 3-kinase activation. *Immunity*. 2000;13(6):817-827.
163. Hodson DJ, Turner M. The role of PI3K signalling in the B cell response to antigen. *Adv Exp Med Biol*. 2009;633:43-53.
164. Liu Y, Rohrschneider LR. The gift of Gab. *FEBS Lett*. 2002;515(1-3):1-7.
165. Holgado-Madruga M, Emler DR, Moscatello DK, Godwin AK, Wong AJ. A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature*. 1996;379(6565):560-564.
166. van der Voort R, Keehnen RM, Beuling EA, Spaargaren M, Pals ST. Regulation of cytokine signaling by B cell antigen receptor and CD40-controlled expression of heparan sulfate proteoglycans. *The Journal of experimental medicine*. 2000;192(8):1115-1124.
167. Kuwano Y, Fujimoto M, Watanabe R, et al. The involvement of Gab1 and PI 3-kinase in beta1 integrin signaling in keratinocytes. *Biochemical and biophysical research communications*. 2007;361(1):224-229.
168. Zheng Y, An H, Yao M, et al. Scaffolding adaptor protein Gab1 is required for TLR3/4- and RIG-I-mediated production of proinflammatory cytokines and type I IFN in macrophages. *Journal of immunology*. 2010;184(11):6447-6456.
169. Ingham RJ, Santos L, Dang-Lawson M, et al. The Gab1 docking protein links the b cell antigen receptor to the phosphatidylinositol 3-kinase/Akt signaling pathway and to the SHP2 tyrosine phosphatase. *The Journal of biological chemistry*. 2001;276(15):12257-12265.
170. Rodrigues GA, Falasca M, Zhang Z, Ong SH, Schlessinger J. A novel positive feedback loop mediated by the docking protein Gab1 and phosphatidylinositol 3-kinase in epidermal growth factor receptor signaling. *Molecular and cellular biology*. 2000;20(4):1448-1459.

171. Isakoff SJ, Cardozo T, Andreev J, et al. Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel in vivo assay in yeast. *The EMBO journal*. 1998;17(18):5374-5387.
172. Ingham RJ, Holgado-Madruga M, Siu C, Wong AJ, Gold MR. The Gab1 protein is a docking site for multiple proteins involved in signaling by the B cell antigen receptor. *The Journal of biological chemistry*. 1998;273(46):30630-30637.
173. Schaeper U, Gehring NH, Fuchs KP, Sachs M, Kempkes B, Birchmeier W. Coupling of Gab1 to c-Met, Grb2, and Shp2 mediates biological responses. *J Cell Biol*. 2000;149(7):1419-1432.
174. Yu CF, Roshan B, Liu ZX, Cantley LG. ERK regulates the hepatocyte growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. *The Journal of biological chemistry*. 2001;276(35):32552-32558.
175. Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R, Wong AJ. Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94(23):12419-12424.
176. Yart A, Laffargue M, Mayeux P, et al. A critical role for phosphoinositide 3-kinase upstream of Gab1 and SHP2 in the activation of ras and mitogen-activated protein kinases by epidermal growth factor. *The Journal of biological chemistry*. 2001;276(12):8856-8864.
177. Ong SH, Hadari YR, Gotoh N, Guy GR, Schlessinger J, Lax I. Stimulation of phosphatidylinositol 3-kinase by fibroblast growth factor receptors is mediated by coordinated recruitment of multiple docking proteins. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98(11):6074-6079.
178. Cunnick JM, Mei L, Doupnik CA, Wu J. Phosphotyrosines 627 and 659 of Gab1 constitute a bisphosphoryl tyrosine-based activation motif (BTAM) conferring binding and activation of SHP2. *The Journal of biological chemistry*. 2001;276(26):24380-24387.
179. Cai T, Nishida K, Hirano T, Khavari PA. Gab1 and SHP-2 promote Ras/MAPK regulation of epidermal growth and differentiation. *The Journal of cell biology*. 2002;159(1):103-112.
180. Chen L, Du-Cuny L, Moses S, et al. Novel inhibitors induce large conformational changes of GAB1 pleckstrin homology domain and kill breast cancer cells. *PLoS computational biology*. 2015;11(1):e1004021.
181. Zhang SQ, Tsiaras WG, Araki T, et al. Receptor-specific regulation of phosphatidylinositol 3'-kinase activation by the protein tyrosine phosphatase Shp2. *Molecular and cellular biology*. 2002;22(12):4062-4072.
182. Gual P, Giordano S, Williams TA, Rocchi S, Van Obberghen E, Comoglio PM. Sustained recruitment of phospholipase C-gamma to Gab1 is required for HGF-induced branching tubulogenesis. *Oncogene*. 2000;19(12):1509-1518.
183. Gu H, Neel BG. The "Gab" in signal transduction. *Trends in cell biology*. 2003;13(3):122-130.
184. Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. *Cellular signalling*. 2002;14(5):381-395.
185. Ali AY, Wu X, Eissa N, et al. Distinct roles for phosphoinositide 3-kinases gamma and delta in malignant B cell migration. *Leukemia*. 2018;32(9):1958-1969.
186. Alessi DR, James SR, Downes CP, et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balph. *Curr Biol*. 1997;7(4):261-269.
187. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 2005;307(5712):1098-1101.
188. Klippel A, Kavanaugh WM, Pot D, Williams LT. A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Molecular and cellular biology*. 1997;17(1):338-344.
189. Park SG, Long M, Kang JA, et al. The kinase PDK1 is essential for B-cell receptor mediated survival signaling. *PLoS One*. 2013;8(2):e55378.
190. Liu P, Gan W, Chin YR, et al. PtdIns(3,4,5)P3-Dependent Activation of the mTORC2 Kinase Complex. *Cancer Discov*. 2015;5(11):1194-1209.

191. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell*. 2007;129(7):1261-1274.
192. Kuo YC, Huang KY, Yang CH, Yang YS, Lee WY, Chiang CW. Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. *The Journal of biological chemistry*. 2008;283(4):1882-1892.
193. Gao T, Furnari F, Newton AC. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol Cell*. 2005;18(1):13-24.
194. Wang Y, Zhou Y, Graves DT. FOXO transcription factors: their clinical significance and regulation. *Biomed Res Int*. 2014;2014:925350.
195. Zhang X, Gan L, Pan H, et al. Phosphorylation of serine 256 suppresses transactivation by FKHR (FOXO1) by multiple mechanisms. Direct and indirect effects on nuclear/cytoplasmic shuttling and DNA binding. *J Biol Chem*. 2002;277(47):45276-45284.
196. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*. 1999;96(6):857-868.
197. Sander S, Chu VT, Yasuda T, et al. PI3 Kinase and FOXO1 Transcription Factor Activity Differentially Control B Cells in the Germinal Center Light and Dark Zones. *Immunity*. 2015;43(6):1075-1086.
198. Kabrani E, Chu VT, Tasouri E, et al. Nuclear FOXO1 promotes lymphomagenesis in germinal center B cells. *Blood*. 2018.
199. Trinh DL, Scott DW, Morin RD, et al. Analysis of FOXO1 mutations in diffuse large B-cell lymphoma. *Blood*. 2013;121(18):3666-3674.
200. Xiao W, Dong G, Pacios S, et al. FOXO1 deletion reduces dendritic cell function and enhances susceptibility to periodontitis. *Am J Pathol*. 2015;185(4):1085-1093.
201. Dengler HS, Baracho GV, Omori SA, et al. Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation. *Nature immunology*. 2008;9(12):1388-1398.
202. Beekman R, Chapaprieta V, Russinol N, et al. The reference epigenome and regulatory chromatin landscape of chronic lymphocytic leukemia. *Nature medicine*. 2018;24(6):868-880.
203. Hermida MA, Dinesh Kumar J, Leslie NR. GSK3 and its interactions with the PI3K/AKT/mTOR signalling network. *Adv Biol Regul*. 2017;65:5-15.
204. Wang R, Xia L, Gabrilove J, Waxman S, Jing Y. Downregulation of Mcl-1 through GSK-3beta activation contributes to arsenic trioxide-induced apoptosis in acute myeloid leukemia cells. *Leukemia*. 2013;27(2):315-324.
205. Maccario H, Perera NM, Davidson L, Downes CP, Leslie NR. PTEN is destabilized by phosphorylation on Thr366. *Biochem J*. 2007;405(3):439-444.
206. Koo J, Wu X, Mao Z, Khuri FR, Sun SY. Rictor Undergoes Glycogen Synthase Kinase 3 (GSK3)-dependent, FBXW7-mediated Ubiquitination and Proteasomal Degradation. *The Journal of biological chemistry*. 2015;290(22):14120-14129.
207. Gregory MA, Qi Y, Hann SR. Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. *The Journal of biological chemistry*. 2003;278(51):51606-51612.
208. Wei W, Jin J, Schlisio S, Harper JW, Kaelin WG, Jr. The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. *Cancer Cell*. 2005;8(1):25-33.
209. Hoffmeister L, Diekmann M, Brand K, Huber R. GSK3: A Kinase Balancing Promotion and Resolution of Inflammation. *Cells*. 2020;9(4).
210. Flugel D, Gorlach A, Michiels C, Kietzmann T. Glycogen synthase kinase 3 phosphorylates hypoxia-inducible factor 1alpha and mediates its destabilization in a VHL-independent manner. *Molecular and cellular biology*. 2007;27(9):3253-3265.
211. Christian F, Smith EL, Carmody RJ. The Regulation of NF-kappaB Subunits by Phosphorylation. *Cells*. 2016;5(1).
212. Ougolkov AV, Bone ND, Fernandez-Zapico ME, Kay NE, Billadeau DD. Inhibition of glycogen synthase kinase-3 activity leads to epigenetic silencing of nuclear factor kappaB target

- genes and induction of apoptosis in chronic lymphocytic leukemia B cells. *Blood*. 2007;110(2):735-742.
213. Hendriks RW, Yuvaraj S, Kil LP. Targeting Bruton's tyrosine kinase in B cell malignancies. *Nature reviews Cancer*. 2014;14(4):219-232.
 214. Rawlings DJ, Scharenberg AM, Park H, et al. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science*. 1996;271(5250):822-825.
 215. Baba Y, Hashimoto S, Matsushita M, et al. BLNK mediates Syk-dependent Btk activation. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98(5):2582-2586.
 216. Kurosaki T, Kurosaki M. Transphosphorylation of Bruton's tyrosine kinase on tyrosine 551 is critical for B cell antigen receptor function. *The Journal of biological chemistry*. 1997;272(25):15595-15598.
 217. Park H, Wahl MI, Afar DE, et al. Regulation of Btk function by a major autophosphorylation site within the SH3 domain. *Immunity*. 1996;4(5):515-525.
 218. Middendorp S, Dingjan GM, Maas A, Dahlenborg K, Hendriks RW. Function of Bruton's tyrosine kinase during B cell development is partially independent of its catalytic activity. *Journal of immunology*. 2003;171(11):5988-5996.
 219. Chiu CW, Dalton M, Ishiai M, Kurosaki T, Chan AC. BLNK: molecular scaffolding through 'cis'-mediated organization of signaling proteins. *EMBO J*. 2002;21(23):6461-6472.
 220. Weber M, Treanor B, Depoil D, et al. Phospholipase C-gamma2 and Vav cooperate within signaling microclusters to propagate B cell spreading in response to membrane-bound antigen. *The Journal of experimental medicine*. 2008;205(4):853-868.
 221. Craxton A, Jiang A, Kurosaki T, Clark EA. Syk and Bruton's tyrosine kinase are required for B cell antigen receptor-mediated activation of the kinase Akt. *The Journal of biological chemistry*. 1999;274(43):30644-30650.
 222. Broad LM, Braun FJ, Lievremont JP, Bird GS, Kurosaki T, Putney JW, Jr. Role of the phospholipase C-inositol 1,4,5-trisphosphate pathway in calcium release-activated calcium current and capacitative calcium entry. *The Journal of biological chemistry*. 2001;276(19):15945-15952.
 223. Smyth JT, Hwang SY, Tomita T, DeHaven WI, Mercer JC, Putney JW. Activation and regulation of store-operated calcium entry. *J Cell Mol Med*. 2010;14(10):2337-2349.
 224. Park YJ, Yoo SA, Kim M, Kim WU. The Role of Calcium-Calcieneurin-NFAT Signaling Pathway in Health and Autoimmune Diseases. *Front Immunol*. 2020;11:195.
 225. Rosse C, Linch M, Kermorgant S, Cameron AJ, Boeckeler K, Parker PJ. PKC and the control of localized signal dynamics. *Nat Rev Mol Cell Biol*. 2010;11(2):103-112.
 226. Sugawara H, Kurosaki M, Takata M, Kurosaki T. Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor. *EMBO J*. 1997;16(11):3078-3088.
 227. Hashimoto A, Okada H, Jiang A, et al. Involvement of guanosine triphosphatases and phospholipase C-gamma2 in extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase activation by the B cell antigen receptor. *The Journal of experimental medicine*. 1998;188(7):1287-1295.
 228. Kang SW, Wahl MI, Chu J, et al. PKCbeta modulates antigen receptor signaling via regulation of Btk membrane localization. *EMBO J*. 2001;20(20):5692-5702.
 229. Tarakhovsky A. Xid and Xid-like immunodeficiencies from a signaling point of view. *Curr Opin Immunol*. 1997;9(3):319-323.
 230. Koyama M, Oka T, Ouchida M, et al. Activated proliferation of B-cell lymphomas/leukemias with the SHP1 gene silencing by aberrant CpG methylation. *Lab Invest*. 2003;83(12):1849-1858.
 231. Oka T, Ouchida M, Koyama M, et al. Gene silencing of the tyrosine phosphatase SHP1 gene by aberrant methylation in leukemias/lymphomas. *Cancer Res*. 2002;62(22):6390-6394.
 232. Cui B, Chen L, Zhang S, et al. MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia. *Blood*. 2014;124(4):546-554.

233. DeFranco AL, Chan VW, Lowell CA. Positive and negative roles of the tyrosine kinase Lyn in B cell function. *Semin Immunol.* 1998;10(4):299-307.
234. Hibbs ML, Tarlinton DM, Armes J, et al. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell.* 1995;83(2):301-311.
235. Nishizumi H, Taniuchi I, Yamanashi Y, et al. Impaired proliferation of peripheral B cells and indication of autoimmune disease in lyn-deficient mice. *Immunity.* 1995;3(5):549-560.
236. Chan VW, Meng F, Soriano P, DeFranco AL, Lowell CA. Characterization of the B lymphocyte populations in Lyn-deficient mice and the role of Lyn in signal initiation and down-regulation. *Immunity.* 1997;7(1):69-81.
237. Tibaldi E, Brunati AM, Zonta F, et al. Lyn-mediated SHP-1 recruitment to CD5 contributes to resistance to apoptosis of B-cell chronic lymphocytic leukemia cells. *Leukemia.* 2011;25(11):1768-1781.
238. Nishizumi H, Horikawa K, Mlinaric-Rascan I, Yamamoto T. A double-edged kinase Lyn: a positive and negative regulator for antigen receptor-mediated signals. *J Exp Med.* 1998;187(8):1343-1348.
239. Vivier E, Daeron M. Immunoreceptor tyrosine-based inhibition motifs. *Immunol Today.* 1997;18(6):286-291.
240. Brauweiler A, Tamir I, Dal Porto J, et al. Differential regulation of B cell development, activation, and death by the src homology 2 domain-containing 5' inositol phosphatase (SHIP). *J Exp Med.* 2000;191(9):1545-1554.
241. Gary-Gouy H, Harriague J, Dalloul A, Donnadieu E, Bismuth G. CD5-negative regulation of B cell receptor signaling pathways originates from tyrosine residue Y429 outside an immunoreceptor tyrosine-based inhibitory motif. *J Immunol.* 2002;168(1):232-239.
242. Blasioli J, Paust S, Thomas ML. Definition of the sites of interaction between the protein tyrosine phosphatase SHP-1 and CD22. *J Biol Chem.* 1999;274(4):2303-2307.
243. Mizuno K, Tagawa Y, Mitomo K, et al. Src homology region 2 (SH2) domain-containing phosphatase-1 dephosphorylates B cell linker protein/SH2 domain leukocyte protein of 65 kDa and selectively regulates c-Jun NH2-terminal kinase activation in B cells. *J Immunol.* 2000;165(3):1344-1351.
244. Tamir I, Dal Porto JM, Cambier JC. Cytoplasmic protein tyrosine phosphatases SHP-1 and SHP-2: regulators of B cell signal transduction. *Curr Opin Immunol.* 2000;12(3):307-315.
245. Sato S, Jansen PJ, Tedder TF. CD19 and CD22 expression reciprocally regulates tyrosine phosphorylation of Vav protein during B lymphocyte signaling. *Proceedings of the National Academy of Sciences of the United States of America.* 1997;94(24):13158-13162.
246. Cerna K, Oppelt J, Chochola V, et al. MicroRNA miR-34a downregulates FOXP1 during DNA damage response to limit BCR signalling in chronic lymphocytic leukaemia B cells. *Leukemia.* 2019;33(2):403-414.
247. Ouchida R, Lu Q, Liu J, et al. FcμR interacts and cooperates with the B cell receptor To promote B cell survival. *J Immunol.* 2015;194(7):3096-3101.
248. Nguyen TT, Klasener K, Zurn C, et al. The IgM receptor FcμR limits tonic BCR signaling by regulating expression of the IgM BCR. *Nat Immunol.* 2017;18(3):321-333.
249. Seda V, Mraz M. B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells. *European journal of haematology.* 2015;94(3):193-205.
250. Lam KP, Kuhn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell.* 1997;90(6):1073-1083.
251. Roose JP, Diehn M, Tomlinson MG, et al. T cell receptor-independent basal signaling via Erk and Abl kinases suppresses RAG gene expression. *PLoS Biol.* 2003;1(2):E53.
252. Burger JA, Chiorazzi N. B cell receptor signaling in chronic lymphocytic leukemia. *Trends in immunology.* 2013;34(12):592-601.
253. Srinivasan L, Sasaki Y, Calado DP, et al. PI3 kinase signals BCR-dependent mature B cell survival. *Cell.* 2009;139(3):573-586.
254. Agathangelidis A, Darzentas N, Hadzidimitriou A, et al. Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. *Blood.* 2012;119(19):4467-4475.

255. Hoogeboom R, van Kessel KP, Hochstenbach F, et al. A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. *The Journal of experimental medicine*. 2013;210(1):59-70.
256. Landau DA, Carter SL, Stojanov P, et al. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell*. 2013;152(4):714-726.
257. Nadeu F, Delgado J, Royo C, et al. Clinical impact of clonal and subclonal TP53, SF3B1, BIRC3, NOTCH1, and ATM mutations in chronic lymphocytic leukemia. *Blood*. 2016;127(17):2122-2130.
258. Gomes de Castro MA, Wildhagen H, Sograte-Idrissi S, et al. Differential organization of tonic and chronic B cell antigen receptors in the plasma membrane. *Nature communications*. 2019;10(1):820.
259. Duhren-von Minden M, Ubelhart R, Schneider D, et al. Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature*. 2012;489(7415):309-312.
260. Rai KR, Peterson BL, Appelbaum FR, et al. Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia. *The New England journal of medicine*. 2000;343(24):1750-1757.
261. Ghia P, Hallek M. Management of chronic lymphocytic leukemia. *Haematologica*. 2014;99(6):965-972.
262. Roberts AW, Davids MS, Pagel JM, et al. Targeting BCL2 with Venetoclax in Relapsed Chronic Lymphocytic Leukemia. *The New England journal of medicine*. 2016;374(4):311-322.
263. de Rooij MF, Kuil A, Geest CR, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood*. 2012;119(11):2590-2594.
264. Brown JR. Ibrutinib (PCI-32765), the first BTK (Bruton's tyrosine kinase) inhibitor in clinical trials. *Curr Hematol Malig Rep*. 2013;8(1):1-6.
265. Woyach JA, Smucker K, Smith LL, et al. Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. *Blood*. 2014;123(12):1810-1817.
266. Kim JH, Kim WS, Ryu K, Kim SJ, Park C. CD79B limits response of diffuse large B cell lymphoma to ibrutinib. *Leuk Lymphoma*. 2016;57(6):1413-1422.
267. Zhao X, Lwin T, Silva A, et al. Unification of de novo and acquired ibrutinib resistance in mantle cell lymphoma. *Nature communications*. 2017;8:14920.
268. Hoellenriegel J, Meadows SA, Sivina M, et al. The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood*. 2011;118(13):3603-3612.
269. Edelmann J, Holzmann K, Miller F, et al. High-resolution genomic profiling of chronic lymphocytic leukemia reveals new recurrent genomic alterations. *Blood*. 2012;120(24):4783-4794.
270. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(39):13944-13949.
271. Yalniz FF, Wierda WG. Targeting BCL2 in Chronic Lymphocytic Leukemia and Other Hematologic Malignancies. *Drugs*. 2019;79(12):1287-1304.
272. Mraz M, Malinova K, Kotaskova J, et al. miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. *Leukemia*. 2009;23(6):1159-1163.
273. Mraz M, Malinova K, Mayer J, Pospisilova S. MicroRNA isolation and stability in stored RNA samples. *Biochemical and biophysical research communications*. 2009;390(1):1-4.
274. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. 2011. 2011;17(1):3 %J EMBnet.journal.
275. Hackenberg M, Sturm M, Langenberger D, Falcon-Perez JM, Aransay AM. miRanalyzer: a microRNA detection and analysis tool for next-generation sequencing experiments. *Nucleic Acids Res*. 2009;37(Web Server issue):W68-76.
276. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014;30(15):2114-2120.

277. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 2014;42(Database issue):D68-73.
278. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014;15(12):550.
279. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* 2010;26(1):139-140.
280. Huber W, Carey VJ, Gentleman R, et al. Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods.* 2015;12(2):115-121.
281. Babicki S, Arndt D, Marcu A, et al. Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res.* 2016;44(W1):W147-153.
282. Shultz LD, Lyons BL, Burzenski LM, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol.* 2005;174(10):6477-6489.
283. Wiederschain D, Wee S, Chen L, et al. Single-vector inducible lentiviral RNAi system for oncology target validation. *Cell Cycle.* 2009;8(3):498-504.
284. Carey MF, Peterson CL, Smale ST. Chromatin immunoprecipitation (ChIP). *Cold Spring Harb Protoc.* 2009;2009(9):pdb prot5279.
285. Strbkova L, Zicha D, Vesely P, Chmelik R. Automated classification of cell morphology by coherence-controlled holographic microscopy. *J Biomed Opt.* 2017;22(8):1-9.
286. Zicha D, Genot E, Dunn GA, Kramer IM. TGFbeta1 induces a cell-cycle-dependent increase in motility of epithelial cells. *J Cell Sci.* 1999;112 (Pt 4):447-454.
287. Dunn GA, Zicha D. Dynamics of fibroblast spreading. *J Cell Sci.* 1995;108 (Pt 3):1239-1249.
288. Bagnara D, Kaufman MS, Calissano C, et al. A novel adoptive transfer model of chronic lymphocytic leukemia suggests a key role for T lymphocytes in the disease. *Blood.* 2011;117(20):5463-5472.
289. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet.* 2000;25(1):25-29.
290. Vasyutina E, Stebler J, Brand-Saberi B, Schulz S, Raz E, Birchmeier C. CXCR4 and Gab1 cooperate to control the development of migrating muscle progenitor cells. *Genes & development.* 2005;19(18):2187-2198.
291. Sang H, Li T, Li H, Liu J. Down-regulation of Gab1 inhibits cell proliferation and migration in hilar cholangiocarcinoma. *PloS one.* 2013;8(11):e81347.
292. Wang X, Peng J, Yang Z, et al. Elevated expression of Gab1 promotes breast cancer metastasis by dissociating the PAR complex. *J Exp Clin Cancer Res.* 2019;38(1):27.
293. Mraz M, Chen L, Rassenti LZ, et al. miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1. *Blood.* 2014;124(1):84-95.
294. Niemann CU, Herman SE, Maric I, et al. Disruption of in vivo Chronic Lymphocytic Leukemia Tumor-Microenvironment Interactions by Ibrutinib--Findings from an Investigator-Initiated Phase II Study. *Clin Cancer Res.* 2016;22(7):1572-1582.
295. Sivina M, Xiao L, Kim E, et al. CXCL13 plasma levels function as a biomarker for disease activity in patients with chronic lymphocytic leukemia. *Leukemia.* 2020.
296. Lambert SA, Jolma A, Campitelli LF, et al. The Human Transcription Factors. *Cell.* 2018;172(4):650-665.
297. Kreft L, Soete A, Hulpiau P, Botzki A, Saeys Y, De Bleser P. ConTra v3: a tool to identify transcription factor binding sites across species, update 2017. *Nucleic acids research.* 2017;45(W1):W490-W494.
298. Zhang X, Tang N, Hadden TJ, Rishi AK. Akt, FoxO and regulation of apoptosis. *Biochimica et biophysica acta.* 2011;1813(11):1978-1986.
299. Deng Y, Wang F, Hughes T, Yu J. FOXOs in cancer immunity: Knowns and unknowns. *Seminars in cancer biology.* 2018;50:53-64.
300. Pyrzynska B, Dwojak M, Zerrouqi A, et al. FOXO1 promotes resistance of non-Hodgkin lymphomas to anti-CD20-based therapy. *Oncoimmunology.* 2018;7(5):e1423183.

301. Byrd JC, Brown JR, O'Brien S, et al. Ibrutinib versus ofatumumab in previously treated chronic lymphoid leukemia. *The New England journal of medicine*. 2014;371(3):213-223.
302. Cervantes-Gomez F, Kumar Patel V, Bose P, Keating MJ, Gandhi V. Decrease in total protein level of Bruton's tyrosine kinase during ibrutinib therapy in chronic lymphocytic leukemia lymphocytes. *Leukemia*. 2016;30(8):1803-1804.
303. Os A, Burgler S, Ribes AP, et al. Chronic lymphocytic leukemia cells are activated and proliferate in response to specific T helper cells. *Cell Rep*. 2013;4(3):566-577.
304. Meng S, Chen Z, Munoz-Antonia T, Wu J. Participation of both Gab1 and Gab2 in the activation of the ERK/MAPK pathway by epidermal growth factor. *Biochem J*. 2005;391(Pt 1):143-151.
305. Roy NH, MacKay JL, Robertson TF, Hammer DA, Burkhardt JK. Crk adaptor proteins mediate actin-dependent T cell migration and mechanosensing induced by the integrin LFA-1. *Sci Signal*. 2018;11(560).
306. Thomas MS, Mitchell JS, DeNucci CC, Martin AL, Shimizu Y. The p110gamma isoform of phosphatidylinositol 3-kinase regulates migration of effector CD4 T lymphocytes into peripheral inflammatory sites. *J Leukoc Biol*. 2008;84(3):814-823.
307. Martin AL, Schwartz MD, Jameson SC, Shimizu Y. Selective regulation of CD8 effector T cell migration by the p110 gamma isoform of phosphatidylinositol 3-kinase. *Journal of immunology*. 2008;180(4):2081-2088.
308. Ringshausen I, Schneller F, Bogner C, et al. Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. *Blood*. 2002;100(10):3741-3748.
309. Herman SE, Sun X, McAuley EM, et al. Modeling tumor-host interactions of chronic lymphocytic leukemia in xenografted mice to study tumor biology and evaluate targeted therapy. *Leukemia*. 2013;27(12):2311-2321.
310. Zhuang J, Hawkins SF, Glenn MA, et al. Akt is activated in chronic lymphocytic leukemia cells and delivers a pro-survival signal: the therapeutic potential of Akt inhibition. *Haematologica*. 2010;95(1):110-118.
311. Gobessi S, Laurenti L, Longo PG, et al. Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukemia B cells. *Leukemia*. 2009;23(4):686-697.
312. Szydlowski M, Kiliszek P, Sewastianik T, et al. FOXO1 activation is an effector of SYK and AKT inhibition in tonic BCR signal-dependent diffuse large B-cell lymphomas. *Blood*. 2016;127(6):739-748.
313. Aasrum M, Odegard J, Sandnes D, Christoffersen T. The involvement of the docking protein Gab1 in mitogenic signalling induced by EGF and HGF in rat hepatocytes. *Biochim Biophys Acta*. 2013;1833(12):3286-3294.

URL:

1. PIP2 cleavage to IP3 and DAG (Internet). [Cited 7. June 2021]. Retrieved from: https://cs.wikipedia.org/wiki/Soubor:PIP2_cleavage_to_IP3_and_DAG.jpg

11. List of abbreviations

AKT	Protein kinase B (also known as PKB)
BCA1	B cell-attracting chemokine 1 (also known as CXCL13)
BCAP	B-cell adaptor for phosphoinositide-3-kinase
BCL2	B-cell lymphoma 2
BCL _{XL}	B-cell lymphoma-extra large
BCR	B cell receptor
BLNK	B-cell linker
BTK	Bruton's tyrosine kinase
CD19	B-Lymphocyte surface antigen, B4
CD45	pan-B cell marker, B220
CD62L	Leukocyte-selectin, also known as L-selectin
CEBP	CCAAT-enhancer-binding proteins
CFSE	Carboxyfluorescein succinimidyl ester
ChIP	Chromatin immunoprecipitation
CLL	Chronic lymphocytic leukemia
CM ^{hMSC}	Conditioned media produced by primary human mesenchymal stromal cells
CM ^{HS5}	Conditioned media produced by HS5 cells
CSK	C-terminal Src tyrosine kinase
c-Src	cellular-Src
DAG	diacylglycerol
DiOC6	3,3'-dihexyloxacarbocyanine iodide
DMSO	Dimethyl sulfoxide
DOK1	Docking protein 1
EGF	Epidermal growth factor
ELISA	Enzyme-Linked ImmunoSorbent Assay

ERK	Extracellular signal-regulated kinase
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FcγR	Fc receptor for IgG
FcμR	Fc receptor for IgM
FCR	Therapy consists of fludarabine, cyclophosphamide, and rituximab
FFPE	Formalin-fixed paraffin-embedded
FLNA	Filamin A, alpha
FoxO1	Forkhead box protein O1 (also known as FKHR)
FSC-A	Forward scatter area
GAB1	GRB2-associated-binding protein 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GlyCAM-1	Glycosylated cell adhesion molecule 1
GPR183	G-protein coupled receptor 183
GRB2	Growth factor receptor-bound protein 2
GSK3	Glycogen Synthase Kinase 3
HEV	High endothelial venules
HIF1	Hypoxia-inducible factor 1
HIV	Human Immunodeficiency Virus
HS5	Human stromal cell line 5
ICAM	Intercellular Adhesion Molecule
ICAP1	Integrin cytoplasmic domain-associated protein 1
Ig	Immunoglobulin
IGHV	Immunoglobulin heavy chain variable region
IL4	Interleukin 4
IL6R	Interleukin 6 receptor
inhGAB1-001	Inhibitor of GAB1 protein no. 001
inhGAB1-004	Inhibitor of GAB1 protein no. 004

IP3	inositol triphosphate
ITAM	Immunoreceptor tyrosine-based motive
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
JAK	Janus kinase
LFA1	Leukocyte-function-associated antigen 1
MAPK	Mitogen-activated protein kinases
MadCAM-1	Mucosal addressin cell adhesion molecule 1
MCL1	Induced myeloid leukemia cell differentiation protein
MEC1 ^{FoxO1-KO}	MEC1 cell line where the <i>FoxO1</i> gene was genetically knocked-out
MEC1 ^{GAB1-KO}	MEC1 cell line where the <i>GAB1</i> gene was genetically knocked-out
MEC1 ^{WT}	MEC1 wild type cell line
MFI	Mean fluorescent intensity
mTORC2	Mammalian target of rapamycin complex 2
MYLK	Myosin Light Chain Kinase
NCK2	NCK adaptor protein 2
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor-kappa B
NSG	NOD scid gamma mouse
PDK1	Phosphoinositide-dependent protein kinase 1
PH	Pleckstrin homology domain
PHLPP	PH domain leucine-rich repeat protein phosphatase
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PP2A	Protein phosphatase 2A
PRAK	p38-regulated and activated kinase

PTEN	Phosphatase and tensin homolog
RICTOR	Rapamycin-insensitive companion of mammalian target of rapamycin
S1P	Sphingosine-1-phosphate
S1PR1	Sphingosine-1 phosphate receptor
SDF1	Stromal cell-derived factor 1 (also known as CXCL12)
SEMA4F	Semaforin-4F
SERPINF1	Serpin Family F Member 1
SHIP1	Src homology 2 domain containing inositol polyphosphate 5-phosphatase 1
SHP1	Src homology region 2 domain-containing phosphatase-1
SHP2	Src homology region 2 domain-containing phosphatase-2
SIN1	Stress-activated map kinase-interacting protein 1
STIM	Stromal interaction molecules
SYK	Spleen tyrosine kinase
TBS	Transcription binding site
TLR	Toll-like receptor
VCAM1	Vascular cell adhesion molecule 1
Vin	Vinculin
VLA4	Very late antigen 4
ZAP70	Zeta-chain-associated protein kinase 70

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Seda, V., E. Vojackova, L. Ondrisova, L. Kostalova, S. Sharma, T. Loja, G. Pavlasova, D. Zicha, M. K. Peskova, J. Krivanek, K. Liskova, L. Kren, V. Benes, K. Litzmanova, M. Borsky, J. Oppelt, J. Verner, S. Pospisilova, Y. Brychtova, A. Panovska, Z. Tan, S. Zhang, M. Doubek, K. Cerna, J. Mayer and M. Mraz (2021). "FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia." *Blood*.

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Summary of Patent application	

15. List of author's publications

Doctoral student is the first author of 2 publications and co-author of another 6 publications already published in foreign impacted journals (total impact factor: 93.61, cited by 223 on Web of Science [without self-citations: 215]; h index 7). First author review was published in European Journal of Haematology (IF 2015: 2.54; medicine: Q1, hematology: Q2). First author original article was published in Blood (IF 2020: 17.79; immunology: Q1, hematology: Q1; cell biology: Q1). Other co-authored publications were published in Blood, Leukemia or Haematologica journals. Student is also author of one review written in the Czech language (shared first authorship). The data were presented on 1 foreign and 2 domestic conferences (as first author) and on another 37 conferences (as co-author). Data were presented by student in 5 educational lectures. Student is also co-author of the international Patent Application.

15.1. Articles in impacted journals

- 1) **Seda, V.**, E. Vojackova, L. Ondrisova, L. Kostalova, S. Sharma, T. Loja, G. Pavlasova, D. Zicha, M. K. Peskova, J. Krivanek, K. Liskova, L. Kren, V. Benes, K. Litzmanova, M. Borsky, J. Oppelt, J. Verner, S. Pospisilova, Y. Brychtova, A. Panovska, Z. Tan, S. Zhang, M. Doubek, K. Cerna, J. Mayer and M. Mraz (2021). "FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia." Blood. **IF₂₀₂₀=17.794**
- 2) Sharma, S., G. M. Pavlasova, **V. Seda**, K. A. Cerna, E. Vojackova, D. Filip, L. Ondrisova, V. Sandova, L. Kostalova, P. F. Zeni, M. Borsky, J. Oppelt, K. Liskova, L. Kren, A. Janikova, S. Pospisilova, S. M. Fernandes, M. Shehata, L. Z. Rassenti, U. Jaeger, M. Doubek, M. S. Davids, J. R. Brown, J. Mayer, T. J. Kipps and M. Mraz (2021). "miR-29 modulates CD40 signaling in chronic lymphocytic leukemia by targeting TRAF4: an axis affected by BCR inhibitors." Blood 137(18): 2481-2494. **IF₂₀₂₀=17.794**
- 3) Cerna, K., J. Oppelt, V. Chochola, K. Musilova, **V. Seda**, G. Pavlasova, L. Radova, M. Arigoni, R. A. Calogero, V. Benes, M. Trbusek, Y. Brychtova, M. Doubek, J. Mayer, S. Pospisilova and M. Mraz (2019). "MicroRNA miR-34a downregulates FOXP1 during DNA damage response to limit BCR signalling in chronic lymphocytic leukaemia B cells." Leukemia 33(2): 403-414. **IF₂₀₁₉=8.665**

- 4) Boudny, M., J. Zemanova, P. Khirsariya, M. Borsky, J. Verner, J. Cerna, A. Oltova, **V. Seda**, M. Mraz, J. Jaros, Z. Jaskova, M. Spunarova, Y. Brychtova, K. Soucek, S. Drapela, M. Kasparkova, J. Mayer, K. Paruch and M. Trbusek (2019). "Novel CHK1 inhibitor MU380 exhibits significant single-agent activity in TP53-mutated chronic lymphocytic leukemia cells." *Haematologica* 104(12): 2443-2455. **IF₂₀₁₉=7.116**
- 5) Pavlasova, G., M. Borsky, V. Svobodova, J. Oppelt, K. Cerna, J. Novotna, **V. Seda**, M. Fojtova, J. Fajkus, Y. Brychtova, M. Doubek, S. Pospisilova, J. Mayer and M. Mraz (2018). "Rituximab primarily targets an intra-clonal BCR signaling proficient CLL subpopulation characterized by high CD20 levels." *Leukemia* 32(9): 2028-2031. **IF₂₀₁₈=9.944**
- 6) Musilova, K., J. Devan, K. Cerna, **V. Seda**, G. Pavlasova, S. Sharma, J. Oppelt, R. Pytlik, V. Prochazka, Z. Prouzova, M. Trbusek, L. Zlamalikova, K. Liskova, L. Kruzova, M. Jarosova, A. Mareckova, C. Kornauth, I. Simonitsch-Klupp, A. I. Schiefer, O. Merkel, H. Mocikova, P. Burda, K. Machova Polakova, L. Kren, J. Mayer, C. S. Zent, M. Trneny, A. G. Evans, A. Janikova and M. Mraz (2018). "miR-150 downregulation contributes to the high-grade transformation of follicular lymphoma by upregulating FOXP1 levels." *Blood* 132(22): 2389-2400. **IF₂₀₁₈=16.562**
- 7) Pavlasova, G., M. Borsky, **V. Seda**, K. Cerna, J. Osickova, M. Doubek, J. Mayer, R. Calogero, M. Trbusek, S. Pospisilova, M. S. Davids, T. J. Kipps, J. R. Brown and M. Mraz (2016). "Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis." *Blood* 128(12): 1609-1613. **IF₂₀₁₆=13.164**
- 8) **Seda, V.** and M. Mraz (2015). "B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells." *Eur J Haematol* 94(3): 193-205. **IF₂₀₁₅=2.610**

15.2. Articles in domestic journals

- 1) Košťálová L, **Šeda V**, Mráz M. (2019). "Komplexní mechanismy účinku inhibitorů „BCR signalizace“ a vzniku rezistence na tuto cílenou léčbu u chronické lymfocytární leukemie." *Transfuze a hematologie dnes*, No. 4, p. 301-308. (Shared first authorship.)

15.3. Conference contributions

- 1) **Seda V**, Vojackova E, Ondrisova L, Kostalova L, Sharma S, Loja T, Zicha D, Verner J, Pospisilova S, Brychtova Y, Panovska A, Tan Z, Zhang S, Doubek M, Mayer J, Mraz M. FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic

- Lymphocytic Leukemia: Novel Therapeutic Target. The European Hematology Association (EHA), online congress 2021.
- 2) Vojáčková E, Verner J, Lišková K, Dorazilová J, Francová HS, Loja T, **Šeda V**, Šandová V, Pavlasová GM, Sharma S, Zeni PF, Filip D, Ondrišová L, Košťálová L, Vojtová L, Křen L a Mráz M. Development of novel patient-derived xenograft model of chronic lymphocytic leukemia. In CEITEC PhD Conference, Brno, Feb. 2020. 2020.
 - 3) Sharma S, Pavlasova GM, **Seda V**, Cerna KA, Vojackova E, Ondrisova L, Sandova V, Zeni PF, Kostalova L, Pospisilova S, Fernandez SM, Shehata M, Jager U, Doubek M, Davids MS, Brown JR, Mayer J, Kipps TJ a Mraz M. mir-29 influences CD40 signaling in chronic lymphocytic leukemia (CLL). In 25th Congress of European Hematology Association, Amsterdam, Nizozemsko, in HemaSphere. 2020.
 - 4) Sharma S, Pavlasová GM, **Šeda V**, Vojáčková E, Ondrišová L, Šandová V, Zeni PF, Filip D, Košťálová L a Mráz M. mir-29 regulates the CD40 signaling in the microenvironment of chronic lymphocytic leukemia. In CEITEC PhD Conference, Brno, 2020. 2020.
 - 5) Kostalova L, **Seda V**, Ondrisova L, Vojackova E, Filip D, Sandova V, Sharma S and Mraz M. Regulation of BCR signaling during therapy in chronic lymphocytic leukemia. In XIX. Interdisciplinary Meeting of Young Biologists, Biochemists and Chemists. Milovy. 2020.
 - 6) Musilova K, Cerna KA, Sharma S, **Seda V**, Pavlasova GM, Oppelt J, Filip D, Sandova V, Ondrisova L, Pospisilova S, Vojackova E, Zeni PF, Mayer J, Mraz M. B Cell Receptor Signalling Regulation by Non-coding RNAs. In EU-LIFE Scientific Meeting 2019, Cambridge, UK. 2019.
 - 7) Ondrisova L, **Seda V**, Cerna KA, Pavlasova G, Vojackova E, Oppelt J, Filip D, Sandova V, Mayer J, Mraz M: Mechanism of leukemia cell adaptation to targeted therapy in chronic lymphocytic leukemia. XIX. Interdisciplinary meeting of young life scientists. Devět Skal Milovy, Czech Republic (May 20-23, 2019) in Czech Chemical Society Symposium Series (2019); 17: 27-28.
 - 8) Cerna KA, Oppelt J, Chochola V, Musilova K, **Seda V**, Pavlasova G, Radova L, Arigoni M, Calogero RA, Benes V, Trbusek M, Brychtova Y, Doubek M, Mayer J, Pospisilova S, Mraz M. MicroRNA miR-34a downregulates FOXP1 during DNA damage response to limit BCR signalling in chronic lymphocytic leukaemia B cells. *Leukemia*. London: Nature Publishing Group, 2019, roč. 33, č. 2, s. 403-414. ISSN 0887-6924. doi:10.1038/s41375-018-0230-x.

- 9) Boudny M, Zemanova J, Khirsariya PK, Borsky M, Verner J, Cerna J, **Seda V**, Mraz M, Oltova A, Jaros J, Jaskova Z, Spunarova M, Brychtova Y, Mayer J, Paruch K, Trbusek M. Potenciál inhibice proteinu checkpoint kinase 1 u chronické lymfocytární leukémie s mutacemi v genu TP53. In XXXIII. Olomoucké hematologické dny, Olomouc. 2019.
- 10) Černá KA, Musilová K, Pavlasová GM, **Šeda V**, Oppelt J, Filip D, Šandová V, Vojáčková E, Sharma S, Zeni PF, Mayer J, Mraz M. The surprising role of microRNAs in the DNA damage and B cell receptor signalling in malignant B cells. In XXXIII. Olomoucké hematologické dny, Olomouc. 2019.
- 11) Pavlasova G, Sandova V, Borsky M, Oppelt J, **Seda V**, Brychtova Y, Pospisilova S, Doubek M, Mayer J, Mraz M: CD20 in the context of microenvironmental interactions of malignant B cells: Implications for targeted therapy. In: XXV. Pařížkovy dny, Ostrava 2019, March 6-8.
- 12) Mráz M, Pavlasová G, Svobodová V, Borský M, Oppelt J, Černá K, Musilová K, **Šeda V**, Brychtová Y, Doubek M, Pospíšilová Š, Mayer J. CD20 reguluje signalizaci přes B buněčný receptor v mikroprostředí chronické lymfocytární leukémie. In: I. Český hematologický a transfuziologický sjezd, Praha, 16.-19. září 2018 in Transfuze hematol. dnes 2018; 24, Supplement 2, str. 5-6.
- 13) Pavlasova G, Svobodova V, Borsky M, Oppelt J, Novotna J, Cerna K, Musilova K, **Seda V**, Brychtova Y, Doubek M, Pospisilova S, Mayer J, Mraz M. CD20 is a direct regulator of B-cell receptor signaling in the microenvironment of chronic lymphocytic leukemia. In: 23rd Congress of the European Hematology Association; 2018 June 14–17; Stockholm, Sweden: HemaSphere 2018; 2(S1): p490; Abstract PS1091.
- 14) Musilova K, Devan J, Cerna K, **Seda V**, Pavlasova G, Pytlik R, Prochazka V, Prouzova Z, Trbusek M, Zlamalikova L, Liskova K, Kruzova L, Jarosova M, Kornauth Ch, Simonitsch-Klupp I, Schiefer A-I, Merkel O, Burda P, Machova Polakova K, Mocikova H, Kren L, Mayer J, Zent CS, Trneny M, Evans A, Janikova A, Mraz M. MYC-mediated downregulation of miR-150 contributes to the transformation of follicular lymphoma by upregulating FOXP1 levels. In: 23rd Congress of the European Hematology Association; 2018 June 14–17; Stockholm, Sweden: HemaSphere 2018; 2(S1): p269-270; Abstract PF634.
- 15) Mraz M, Musilova K, Devan J, Cerna K, **Seda V**, Pavlasova G, Pytlik R, Prochazka V, Prouzova Z, Zlamalikova L, Liskova K, Kruzova L, Burda P, Machova Polakova K, Mocikova H, Kren L, Mayer J, Trneny M, Janikova A. MYC-mediated downregulation of miR-150 contributes to the transformation of follicular lymphoma to DLBCL by upregulating FOXP1 levels. In: XXXII. Olomoucké hematologické dny, Olomouc, květen 2018, str. 7-8.

- 16) Boudny M, Zemanova J, Khirsariya PK, Borsky M, Verner J, Cerna J, Mraz M, Oltova A, Jaskova Z, Spunarova M, **Seda V**, Brychtova Y, Mayer J, Paruch K, Trbusek M. Selective CHK1 inhibitor MU380 exhibits single-agent activity against chronic lymphocytic leukemia cells with TP53 mutations. In 23rd Congress of the European Hematology Association, Stockholm, Sweden in HemaSphere. 2018.
- 17) Pavlasova G, Borsky M, Svobodova V, Oppelt J, Cerna K, Novotna J, Musilova K, **Seda V**, Vojackova E, Brychtova E, Doubek M, Pospisilova S, Mayer J, Mraz M. CD20 supports BCR signaling in an intra-clonal aggressive chronic lymphocytic leukemia subpopulation of cells and rituximab primarily targets these BCR-proficient B cells in vivo. In AACR Annual Meeting, Chicago, Illinois, USA (Apr 14-18, 2018) in Proceedings of the Annual Meeting of the American Association for Cancer Research 2018; 59: 260; Abstract 1012.
- 18) Boudný M, Zemanová J, Khirsariya PK, Borský M, Verner J, Černá KA, Mraz M, Oltová A, Jašková Z, Špunarová M, **Šeda V**, Brychtová Y, Mayer J, Paruch K a Trbušek M. Inovativní inhibitor proteinu CHK1 indukuje apoptózu u buněk chronické lymfocytární leukémie s mutacemi v genu TP53. In I. Český hematologický a transfuziologický sjezd, Praha. 2018.
- 19) Musilova K, Pavlasova G, **Seda V**, Cerna K, Vojackova E, Didi J, Pytlík R, Prochazka V, Prouzova Z, Zlamalikova L, Mocikova H, Jarosova M, Evans A, Zent CS, Kren L, Trneny M, Mayer J, Janikova A, Mraz M. Down-regulation of miR-150 and upregulation of its target FOXP1 is associated with transformation of follicular lymphoma. In 14th International Conference on Malignant Lymphoma (14-ICML); Lugano, Switzerland (June 14-17, 2017); Hematological Oncology 2017: 35(S1): 150-151; Abstract nr 142.
- 20) Pavlasova G, Borsky M, Svobodova V, Musilova K, Cerna K, **Seda V**, Osickova J, Brychtova Y, Doubek M, Pospisilova S, Mayer J, Mraz M. BCR signalling proficient chronic lymphocytic leukaemia cells are prone to rituximab mediated elimination in vivo. In 22nd EHA Annual Congress; Madrid, Spain (June 22-25, 2017); Haematologica 2017: 102(s2): 412-412; Abstract E1004.
- 21) Cerna K, Oppelt J, **Seda V**, Musilova K, Pavlasova G, Svobodova V, Mayer J, Pospisilova S, Mraz M. MicroRNA Induced by DNA Damage Response Regulate the Expression of FOXP1 and Therapy Response in CLL. 2nd International Conference on the Long and the Short of Non-Coding RNAs, Heraklion, Greece (June 9-14, 2017); abstract no. 81, p.107.
- 22) Cerna K, Oppelt J, **Seda V**, Musilova K, Pavlasova G, Mayer J, Pospisilova S, Mraz M. MicroRNA Induced by DNA Damage Response Regulate the Expression of FOXP1

- and Therapy Response in CLL. XVII International Workshop on CLL, New York, USA (May 12-15, 2017); Leuk Lymphoma 2017; 58(S1): 73-74; Abstract ID 81.
- 23) Musilova K, Pavlasova G, **Seda V**, Vojackova E, Cerna K, Svobodova V, Pytlik R, Prochazka V, Prouzova Z, Pospisilova S, Zlamalikova L, Mocikova H, Kruzova L, Jarosova M, Evans A, Zent CS, Kren L, Trneny M, Mayer J, Janikova A, Mraz M. Differential expression of microRNAs in transformation of follicular lymphoma to diffuse large B cell lymphoma. In AACR Annual Meeting, Washington, D.C., USA (Apr 1-5, 2017) in Proceedings of the 107th Annual Meeting of the American Association for Cancer Research 2017; 58: 380; Abstract nr 1479.
- 24) Vojáčková E, **Šeda V**, Loja T, Černá K, Musilová K, Pavlasová G, Svobodová V, Sharma S, Mráz M. The role of microenvironment in the regulation of B-cell receptor signalling via modulation of GAB1 protein levels. XVII. Mezioborové setkání mladých biologů, biochemiků a chemiků; Devět Skal Milovy, Czech Republic (May 30- June 1, 2017) in Czech Chemical Society Symposium Series (2017); 15: 42-43.
- 25) **Šeda V**, Vojáčková E, Loja T, Pavlasová G, Černá K, Musilová K, Svobodová V, Sharma S, Mráz M. Regulation of B-cell receptor signalling in chronic lymphocytic leukaemia (CLL) microenvironment via modulation of GAB1 protein levels. CEITEC PHD RETREAT II, Telč, duben 2017, s. 52.
- 26) Musilova K, Pavlasova G, **Seda V**, Cerna K, Vojackova E, Didi J, Pytlik R, Prochazka V, Prouzova Z, Zlamalikova L, Mocikova H, Jarosova M, Evans A, Zent C, Kren L, Trneny M, Mayer J, Janikova A, Mraz M. Down-regulation of miR-150 and up-regulation of its target FOXP1 is associated with transformation of follicular lymphoma. In 14th International Conference on Malignant Lymphoma (14-ICML), Lugano, Switzerland, červen 2017 in Hematological Oncology 2017. 2017.
- 27) Pavlasova G, Musilova K, **Seda V**, Cerna K, Vojackova E, Svobodova V, Mraz M. The identification of combinatorial therapeutic approaches with BCR inhibitors in B cell malignancies [abstract]. In: Proceedings of the AACR Precision Medicine Series: Opportunities and Challenges of Exploiting Synthetic Lethality in Cancer; Jan 4-7, 2017; San Diego, CA. Philadelphia (PA): AACR; Mol Cancer Ther 2017;16(10 Suppl): Abstract nr A34.
- 28) Musilova K, Pavlasova G, **Seda V**, Vojackova E, Cerna K, Didi J, Pytlik R, Prochazka V, Prouzova Z, Zlamalikova L, Pospisilova S, Evans A, Zent C, Kren L, Trneny M, Mayer J, Janikova A, Mraz M. The Role of Microrna-150 in the Prognosis and Transformation of Follicular Lymphoma. In 58th ASH Annual Meeting and Exposition, San Diego, USA (December 3-6, 2016) in Blood 2016; 128 (22): 4125.
- 29) Pavlasova G, Borsky M, Musilova K, **Seda V**, Cerna K, Osickova J, Vojackova E, Brychtova Y, Doubek M, Pospisilova S, Mayer J, Mraz M. Rituximab Preferentially

- Eliminates BCR Signaling Proficient Chronic Lymphocytic Leukemia B Cells In Vivo. In 58th ASH Annual Meeting and Exposition, San Diego, USA (December 3-6, 2016) in Blood 2016; 128 (22): 2030.
- 30) Pavlasova G, Borsky M, **Seda V**, Cerna K, Osickova J, Doubek M, Brychtova Y, Mayer J, Pospisilova S, Davids M, Brown JR a Mraz M. The expression of CD20 on malignant B cells is regulated by chemokine signaling through the CXCR4/SDF1 axis: implications for targeting the microenvironmental interactions. In Cancer Research (2016); 76 (14S): 3291 (AACR Annual Meeting; New Orleans, USA). 2016.
- 31) Pavlasova G, Borsky M, **Seda V**, Cerna K, Doubek M, Mayer J, Pospisilova S, Davids M, Brown JR a Mraz M. Ibrutinib inhibits CD20 up-regulation on CLL B cells mediated by the CXCR4/SDF-1 AXIS. In XXX. Olomoucké hematologické dny. 2016.
- 32) Mraz M, Cerna K, Pavlasova G, Musilova K, **Seda V**, Mayer J, Pospisilova S: MicroRNAs in the microenvironmental interactions of B cell leukemias. XXV. Biochemický sjezd, Praha, Czech Republic (September 13-16, 2016); abstract page 82.
- 33) Musilova K, Pavlasova G, Cerna K, **Seda V**, Jez M, Brychtova Y, Doubek M, Mayer J, Arigoni M, Riccardo F, Calogero R, Pospisilova S, Kipps T, Janikova A, Mraz M: MicroRNA-150 influences microenvironmental interactions and prognosis of follicular lymphoma. ICML 2015: 13th International Conference on Malignant Lymphoma; Lugano, Switzerland (June 16-20); Hematological Oncology 2015: 33(S1): 100-180.
- 34) Cerna K, Oppelt J, Radova L, Musilova K, **Seda V**, Pavlasova G, Jez M, Tom N, Malcikova J, Tichy B, Brychtova Y, Doubek M, Trbusek M, Mayer J, Koca J, Arigoni M, Ricardo F, Calogero R, Pospisilova S, Mraz M: MicroRNAs in DNA damage response and BCR signaling in malignant B cell. Keystone Symposia on Molecular and Cellular Biology 2015: MicroRNAs and Noncoding RNAs in Cancer; Keystone, Colorado (June 7-12, 2015); abstract no. 2027
- 35) Musilova K, Pavlasova G, Didi J, **Seda V**, Cerna K, Jez M, Tom N, Brychtova Y, Doubek M, Mayer J, Arigoni M, Ricardo F, Calogero R, Pospisilova S, Kipps T, Janikova A, Mraz M: Microrna150 Influences Microenvironmental Interactions and Prognosis of B Cell Malignancies. ESH 2015: International Conference on The Tumour Microenvironment in the Haematological Malignancies and its Therapeutic Targeting; Lisbon, Portugal (May 7-9, 2015); abstract no. 3307 27)
- 36) Musilova K, Pavlasova G, Didi J, **Seda V**, Cerna K, Jez M, Tom N, Brychtova Y, Doubek M, Mayer J, Arigoni M, Ricardo F, Calogero R, Pospisilova S, Kipps T, Janikova A, Mraz M: Microrna150 Influences Microenvironmental Interactions and Prognosis of B Cell Malignancies. XXIX. Olomoucké hematologické dny s mezinárodní účastí; Olomouc, Czech Republic (May 31-June 1, 2015)

- 37) Cerna K, Oppelt J, Radova L, Musilova K, **Seda V**, Pavlasova G, Jez M, Tom N, Pardy F, Malcikova J, Plevova K, Tichy B, Brychtova Y, Doubek M, Trbusek M, Mayer J, Koca J, Calogero R, Pospisilova S, Mraz M: MicroRNA involvement in DNA damage response and BCR signaling in malignant B cells. In 106th AACR Annual Meeting; Philadelphia, Pennsylvania, USA (April 18-22, 2015); Cancer Res 2015; 75 (15 Suppl): Abstract nr 3084.
- 38) Pavlasova G, **Seda V**, Borsky M, Cerna K, Osickova J, Mayer J, Pospisilova S, Davids M, Brown JR, Mraz M. Microenvironmental interactions up-regulate CD20 expression in CLL B cells through the CXCR4/SDF-1 axis: implications for CD20-targeting antibodies and the use of BCR-inhibitors in combination. In ASH in Blood, 2015.
- 39) Jez M, Pavlasova G, Cerna K, Musilova K, **Seda V**, Mayer J, Pospisilova S, Mraz M: Focal adhesion kinase regulation and expression in malignant B cells. CEITEC PhD Retreat; Valtice, Czech Republic (April 23-24, 2015); abstract no. L22, p. 55.
- 40) **Seda V**, Jez M, Pavlasova G, Cerna K, Musilova K, Pospisilova S, Doubek M, Brychtova Y, Mayer J, Mraz M: The effect of ZAP-70 on adaptor molecule involved in microenvironmental interactions of malignant B cells. CEITEC0 PhD Retreat; Valtice, Czech Republic (April 23-24, 2015); abstract no. L41, p. 74.

15.4. Educational lectures

- 1) **Seda V**, *FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia: Novel Therapeutic Target*. The European Hematology Association (EHA), section: New biological and translational insights in CLL; online congress; June 2021.
- 2) **Seda V**, *Molecular Pathways Guiding Re-circulation of Leukemic B Cells' to Immune Niches: Therapeutic implications*. Molecular Medicine Seminar CEITEC MU, Brno, Czech Republic; 29th October 2019
- 3) **Seda V**, *Regulation of B-cell receptor signalling in chronic lymphocytic leukaemia (CLL) microenvironment via modulation of GAB1 protein levels*. CEITEC PHD RETREAT II, Telč; April 2017
- 4) **Seda V**, *Crosstalk of microenvironmental interactions and BCR signalling in malignant B cells*. Center of Molecular Biology and Gene Therapy Seminar, Department of Internal Medicine – Hematooncology, University Hospital Brno, Czech Republic; 17th October 2016

- 5) **Seda V**, *Crosstalk of microenvironmental interactions and BCR signalling in malignant B cells*. Molecular Medicine Seminar CEITEC MU, Brno, Czech Republic; 11th October 2016

15.5. Patent application

- 1) Mraz M, **Seda V**, Ondrisova L. *Inhibitors for Treatment of Hematological Malignancies*. Applicant 1: Masaryk University, Brno, CZ; Applicant 2: The University Hospital Brno, CZ. Application Number: 16920831; Approved for use through 11/30/2020. OMB 0651-0032, U.S. Patent and Trademark Office.

16. Summary of the thesis

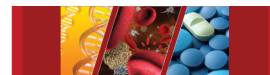
The results presented in this thesis are explaining the largely unknown mechanism of how CLL cells regulate their decision to return to the immune niches and how they balance their survival in peripheral blood. We have experimentally validated that the CXCR4^{bright}CD5^{dim} subpopulation exhibits significantly higher migration capacity towards microenvironmental cytokines like SDF1 or CXCL13 and mouse spleen in comparison to CXCR4^{dim}CD5^{bright}. The sequencing of these two populations further revealed 147 differentially expressed genes annotated in databases as migration-related and among them, we noticed upregulation of *GAB1* mRNA in the CXCR4^{bright}CD5^{dim} subpopulation. Subsequent *in vitro* and *in vivo* experiments proved that GAB1 is activated in response to microenvironmental cytokines and controls the F-actin polymerization, cell motility, and migration. We further demonstrated that the whole mechanism responsible for GAB1 stabilization in long-term circulating cells is dependent on the FoxO1 transcription factor. Next, we observed that GAB1 levels positively correlate with basal AKT phosphorylation, and we demonstrated that the modulation of GAB1 levels *in vivo* may show significant consequences on malignant CLL cell line growth. Our observations were further discussed in the context of targeted therapy by ibrutinib, given that the CLL cells are utilizing the mechanism FoxO1-GAB1 upregulation to compensatory activate the AKT signaling in a relatively short period of time. In the final chapters of this thesis, we demonstrated that this adaptation mechanism can be targeted by novel GAB1 inhibitors. Such treatment inhibited not only AKT activation, but also migration of CLL cells and contributed to increased apoptosis of not only CLL but also other blood cancers such as mantle cell lymphoma or acute myeloid leukemia.

Author contributed to these publications: Seda *et al.*, 2021 (Blood, IF₂₀₂₁=17.794); Sharma, *et al.*, 2021 (Blood, IF₂₀₂₀=17.794); Cerna *et al.*, (Leukemia, IF₂₀₁₉=8.665); Boudny *et al.*, (Haematologica, IF₂₀₁₉=7.116); Pavlasova *et al.*, (Leukemia, IF₂₀₁₈=9.944); Musilova *et al.*, (Blood, IF₂₀₁₈=16.562); Pavlasova *et al.*, (Blood, IF₂₀₁₆=13.164); Seda *et Mraz* (Eur J Hematol, IF₂₀₁₆=2.610).

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17. Supplementary materials

**Supplementary material 1**

REVIEW ARTICLE

B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cellsVaclav Seda¹, Marek Mraz^{1,2,3}¹Central European Institute of Technology, Masaryk University, Brno; ²Department of Internal Medicine, Hematology and Oncology, University Hospital Brno; ³Faculty of Medicine, Masaryk University, Brno, Czech Republic**Abstract**

The physiology of B cells is intimately connected with the function of their B-cell receptor (BCR). B-cell lymphomas frequently (dys)regulate BCR signalling and thus take advantage of this pre-existing pathway for B-cell proliferation and survival. This has recently been underscored by clinical trials demonstrating that small molecules (fosfatinib, ibrutinib, idelalisib) inhibiting BCR-associated kinases (SYK, BTK, PI3K) have an encouraging clinical effect. Here we describe the current knowledge of the specific aspects of BCR signalling in diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, chronic lymphocytic leukaemia (CLL) and normal B cells. Multiple factors can contribute to BCR pathway (dys)regulation in these malignancies and the activation of 'chronic' or 'tonic' BCR signalling. In lymphoma B cells, the balance of initiation, amplitude and duration of BCR activation can be influenced by a specific immunoglobulin structure, the expression and mutations of adaptor molecules (like GAB1, BLNK, GRB2, CARD11), the activity of kinases (like LYN, SYK, PI3K) or phosphatases (like SHIP-1, SHP-1 and PTEN) and levels of microRNAs. We also discuss the crosstalk of BCR with other signalling pathways (NF- κ B, adhesion through integrins, migration and chemokine signalling) to emphasise that the 'BCR inhibitors' target multiple pathways interconnected with BCR, which might explain some of their clinical activity.

Key words B-cell receptor; LYN; spleen tyrosine kinase; bruton tyrosine kinase; ZAP-70; PI(3)K; NF κ B; ibrutinib; miRNA; B cell

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It is known that the pathogenesis of B-cell lymphomas is connected with the differentiation stage of B cell which gives rise to the malignant clone. The proliferation and differentiation of B cells is closely connected with the function and maturation of their B-cell receptor (BCR). It is the variability of the immunoglobulin component of the BCR and the ability of a selected B cell to clonally proliferate in response to infection, which is the stem of adaptive immunity. This defence mechanism includes steps like DNA strand breaks, DNA mutations, loss of genetic material (during rearrangement of immunoglobulin subgenes) and clonal cell proliferation (to produce large amounts of antibodies). This provides essential protection for the organism but is also coupled with risk of this unique molecular pathway's deregulation. The germinal centres in lymph nodes play a central role in these processes and is also a source of many lymphoma types, especially follicular lymphoma (FL), dif-

fuse large B-cell lymphoma (DLBCL) and Burkitt's lymphoma (BL). Recently, the application of RNA interference screens and genome-wide sequencing have provided a fundamental insight into the pathogenesis of these B-cell malignancies. It has been revealed that malignant B cells not only frequently translocate a part of the immunoglobulin gene to upregulate proto-oncogenes (like BCL2, cMYC, BCL6), but also harbour mutations that turn-on the BCR signalling and thus take advantage of this pre-existing 'programme' for B-cell proliferation and survival. These observations have recently been underscored by clinical trials demonstrating that small molecules inhibiting various BCR-associated kinases have encouraging clinical effect. BCR pathway is not only central to the onset of B-cell malignancies, but they retain dependence on its functionality, which is providing a therapeutic target. However, this is only the beginning of an evolving field of research, as better understanding is needed

to reveal (i) how and why malignant B cells are dependent on BCR signalling, (ii) how BCR-signalling crosstalks with other molecular pathways like NF- κ B and B-cell adhesion/migration, and (iii) how to identify patients who would benefit from BCR inhibitors and design combinatorial therapeutic approaches. In this review we will discuss these questions and describe the current knowledge of BCR regulation in normal and (dys)regulation in malignant B cells.

BCR signalling in normal B cells

B-cell receptor is a transmembrane protein complex that controls B-cell fate from the beginning of its expression in the form of pro-BCR and pre-BCR and thus guides cell maturation, survival, anergy and the production of antibodies in plasma B cells [reviewed in (1, 2)]. Each normal mature B cell has a pair of heavy- and light-chain immunoglobulins which are the result of random rearrangement of immunoglobulin subgenes, creating a unique antigen-binding site. This BCR surface component is connected to a network of kinases and phosphatases that tune and amplify its activation. Surface immunoglobulin (Ig) itself does not have kinase activity. This is ensured by non-covalent coupling with the heterodimer of Ig α and Ig β (CD79A, CD79B) proteins. Each of the CD79A and CD79B subunits contain a single immunoreceptor tyrosine-based activation motif (ITAM), which is composed of two conserved tyrosine residues. Antigen-induced BCR aggregation and its conformation changes lead to LYN-mediated phosphorylation of ITAMs, which is the moment of BCR cascade activation. The phosphorylation of ITAMs creates docking sites for SH-2-domain-containing proteins, such as spleen tyrosine kinase (SYK) and further recruitment of more LYN kinase (3). Other tyrosine kinases like FYN and BLK also contribute to this process.

The SYK and Bruton tyrosine kinase (BTK) act as crucial components to couple BCR to more distal signalling, largely via the activation of and interaction with B-cell linker protein (BLNK). Proper BCR signalling requires the presence of BTK, whose inactivation by mutations in mice or humans lead to the absence of mature B cells and agammaglobulinemia due to defects in B-cell development (4). The B cells with non-functional BTK fail to recognise that Ig was successfully rearranged at the pro-B-cell stage due to a lack of BCR signalling and therefore do not proliferate and die of apoptosis. The activation of BTK depends on the ability of SYK to phosphorylate phosphoinositide 3-kinase (PI3K), which is needed to bind PI3K in the proximity of its substrate in the plasma membrane (5). PI3K-binding is mediated by its association with multiple proteins that serve as docking sites, namely CD19, B-cell adaptor for phosphoinositide-3-kinase (BCAP), GRB2-associated-binding protein 1 and 2 (GAB1/2) (6, 7). These adaptor molecules serve in recruiting and assembling BCR signalosome and the amplification of

its signal. The functional significance of this apparent redundancy in PI3K recruitment is largely unknown, but probably defines the amplitude and duration of PI3K activity (8). PI3K recruitment can bypass SYK by LYN binding directly to PI3K, which however, creates only a weak increase in PI3K activity (9). Activated PI3K phosphorylates phosphatidylinositol biphosphate (PIP2) resulting in second messenger PIP3, which serves as a docking site to recruit other effector proteins like BTK and AKT, phospholipase C γ (PLC γ) and protein kinase C β (PKC β) (10). Furthermore, BCR activation affects other signalling pathways, such as Janus kinases, ERK/MAPK, NF- κ B, and mammalian target of rapamycin (mTOR) (Fig. 1) (9, 11).

It is interesting to note that LYN plays a dual role in BCR signalling by triggering an important negative regulatory feedback reaction mediated by phosphatases that limit the BCR activation. This is best illustrated by LYN-deficient mice which, in contrary to an intuitive expectation, have enhanced BCR signalling (12). The function of LYN in ITAM phosphorylation is somehow redundant, but its negative regulatory action is indispensable. The readiness of LYN to perform its function is regulated by a well-known surface molecule present on B cells, namely CD45. This phosphatase removes an inhibitory phosphorylation on LYN (Y508) which is provided by CSK, a C-terminal src tyrosine kinase (13). This is necessary to maintain a balanced amount of 'ready to act' LYN to initiate BCR signalling (14). As mentioned previously, LYN kinase counteracts its own activity to limit BCR pathway by phosphorylating immunoreceptor tyrosine-based motifs (ITIMs) in CD22 and FC γ RIIB. CD22 and FC γ RIIB serve as a docking sites for PTEN and SHIP-1 and 2. These remove the 3' phosphate of phosphatidylinositol (3,4,5)-triphosphate (PIP3) and thus attenuate and balance signalling downstream of PI3K (Fig. 1) (15–17) [reviewed in (18)]. This inhibitory pathway dependent on LYN is also activated by prolonged exposure to autoantigens and is the basis of eliminating auto-reactive B cells.

Taken together, BCR signal transduction is a labyrinth of regulatory cascades working together with other stimuli to control the highly specialised functions of B cells. The above-described process of BCR pathway activation is a very general model which does not take into account that this process can be modulated in the context of lymphatic tissue or peripheral blood and differs based on the maturation stage of the B cell. It is likely that class switching of immunoglobulin and/or its somatic hypermutations might change the nuances in BCR signalling. A key feature of immunological memory is the production of high affinity, isotype-switched antibodies in response to antigenic challenge. In antigen-experienced B cells with class switched Ig from IgM/IgD to IgG, the BCR signalling is not only propagated through CD79A/B but also through the extended cytoplasmic tail of IgG (19). IgG BCRs are enhanced in driving early antigen-dependent events as compared to IgM BCRs

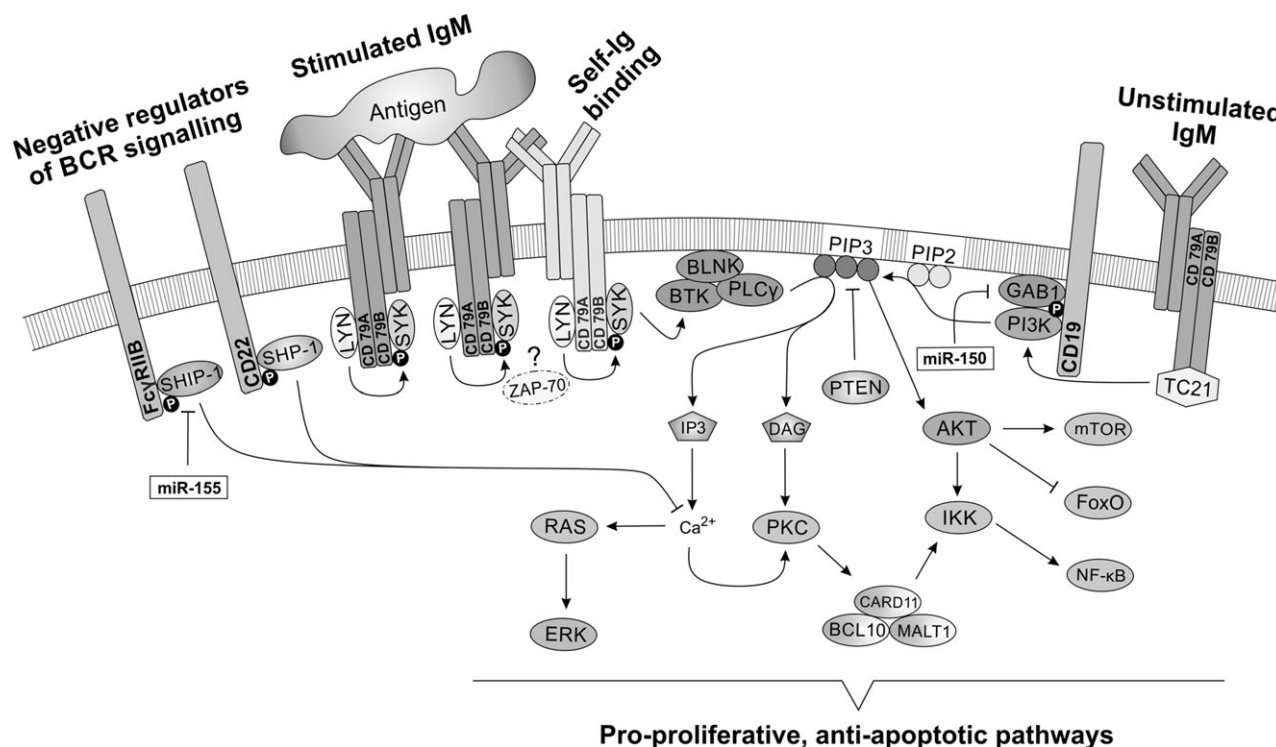


Figure 1 B-cell receptor transduction cascade. BCR signalling is triggered after its engagement by antigens or by self Ig-binding. On the right side of the figure is displayed a possible mechanism of 'tonic' BCR signalling mediated by recruitment of PI3K via TC21. In B cells, the balance of initiation, amplitude and duration of BCR activation can be influenced by a specific immunoglobulin structure, adaptor molecules (like GAB1, BLNK, GRB2, CARD11), the activity of kinases (like LYN, SYK, PI3K) or phosphatases (like SHIP-1, SHP-1 and PTEN) and levels of microRNAs (miR-155, miR-150). BCR activates downstream pathways such as PI3K/AKT, NF-κB or ERK/MAPK, which were described as regulators of B-cell survival and proliferation.

(20). Additionally, Batista and colleagues recently reported differences in the steady-state behaviours of IgM, IgG and IgD in the absence of antigen (21). These differences were influenced by the cytoskeleton-defined network affecting BCR movement by creating boundaries that restrict its diffusion. It has been also proposed that mature B cells have a qualitatively distinct BCR signalling.

It is the mature B cell which has already passed through the checkpoints of pro- and pre-BCR receptor, elimination of auto-reactivity, and thus needs to be maintained as a pool for future selection of antibody-producing clones. It seems logical that in the absence of antigen the BCR signal is not fully turned off but retains a basal activity that could be termed 'tonic'. This has been elegantly demonstrated in mice with conditional deletion of IgM in mature B cells, which resulted in their rapid apoptosis (22). The most straightforward interpretation of this observation is that the BCR provides a survival signal to these cells. Such a signal could be B cell-autonomous and depend upon the assembly of the BCR complex as such or its interaction with other molecules on the B-cell surface. Alternatively, B-cell survival could be mediated by low-level signals generated by a low affinity interaction of BCR with self or environmental antigens. The idea of autonomous tonic signalling is supported by the

observation that all B cells in BCR-less mice were eliminated, irrespective of their individual immunoglobulin heavy/light chain (IGHV/IGL) rearrangements and BCR structure. The B-cell elimination in these mice was based on apoptosis and was relatively rapid (within approx. 3 wks). This would suggest the existence of 'tonic' BCR signalling; however, this is still debated. It has been shown that introducing PI3K may rescue BCR-less mature B cell from apoptosis by directly associating with non-phosphorylated CD79A (23, 24). The rescue of BCR-less B cells cannot be achieved upon activation of the canonical NF-κB pathway (23). How PI3K signalling without a ligand binding to BCR is initiated in resting mature B cells is not understood. In some recently proposed models (25), the antigen receptors are connected to the PI3K pathway by GTPase TC21, which directly recruits the catalytic subunit of PI3K to the non-phosphorylated ITAMs of these receptors (Fig. 1). In this scenario, the PI3K-mediated survival signal is independent of BCR engagement by an external ligand. It is interesting in this context that in T cells lacking T-cell receptor (TCR) expression, the inactivation of a negative regulator of tyrosine kinases, c-src tyrosine kinase (CSK) results in a normal compartment of peripheral blood T cells and analogically inhibition of PTEN can rescue BCR-less B cells (23, 26).

The rapid response to BCR stimulation would in general suggest that there is a pre-existing complex on the membrane, and this could be the basis for 'tonic' signalling. Additionally, it is not clear if the LYN kinase, as the first BCR-responsive kinase, is pre-recruited to the BCR/ITAMs or its recruitment is based on BCR aggregation. The data indicate that once expressed on the surface, the BCR organises protein tyrosine phosphatases, kinases and their substrates into a transducer complex. The tyrosine phosphatases in this complex do not permit strong or constant activation of the signalosome but might allow for some signalling (27). This indirect evidence suggests that alterations in critical BCR features which promote its oligomerisation may lead to spontaneous activation in B cells. Microscopic analysis of BCR organisation on cell membranes led to the observation that cells derived from more aggressive subtypes of DLBCL and cultured *in vitro* more frequently have spontaneously organised clusters of BCRs (28, 29).

These observations are not conclusive but indicate that in malignant B cells, BCR signalling can be initiated by BCR clustering after their engagement by self IGHV binding (Fig. 1) (30). However, it is well accepted that classical antigen-induced BCR signalling plays an important role in lymphomas coupled with infections like mucosa-associated lymphoid tissue lymphoma (MALT) and *Helicobacter pylori*, or splenic marginal zone lymphoma (SMZL) and hepatitis C virus. Additionally, recognising autoantigens and/or aberrant activation of BCR pathway by genetic aberrations are present in other lymphomas.

BCR signalling in lymphomas

The germinal centre reaction in response to antigen is orchestrated by a number of transcription factors [reviewed in (31)] and also turns on activation-induced cytidine deaminase (AID), responsible for somatic hypermutations of DNA. This modifies the specificity of Ig and also allows class switch recombination to IgG/A/E. The germinal centre B cells therefore undergo significant DNA modifications/damage, they have to be naturally resistant to it to a certain extent, and this might be the reason why they are a founding cell for multiple types of lymphomas (FL, DLBCL, BL). AID can create mutations outside Ig and introduce double-strand breaks which lead to chromosomal translocations. Such cells acquire translocations of the heavy chain immunoglobulin gene (IgH) with proto-oncogenes like BCL2 and cMYC, which allows them to avoid cell death. It is important to note that lymphoma B cells retain a second productively rearranged Ig allele allowing them to potentially receive antigen-stimulation or antigen-independent signalling from BCR.

Lymphomas like BL, FL and subtypes of DLBCL typically utilise the IgM constant region in formation of the BCR, although most of these cells are derived from germinal

centre with typical isotype switched Ig to IgG (32). There is a preference to maintain IgM expression on malignant B cells, which is evidenced by the isotype switch of their translocated IgH (like IgH-BCL6 or IgH-cMYC), but no isotype switch of the productively rearranged IgH forming the BCR (33). An explanation for this might be their qualitatively distinct signalling outputs. The IgM membrane possesses only two amino acids on its cytoplasmic end, thus most signals from IgM-BCR emanate from CD79A and CD79B, which trigger pro-survival pathways such as NF- κ B. Whereas IgM BCRs initiate signals exclusively through CD79A/B, IgG-containing BCRs also use other conserved tyrosine residues in the cytoplasmic segments of their immunoglobulin heavy chains. When phosphorylated, these tyrosines recruit the adaptor protein growth factor receptor-bound protein 2 (GRB2), resulting in sustained protein kinase activation and prolonged generation of second messengers, which together culminate in enhanced B cell proliferation (19, 34, 35). IgG-BCR signalling is further mediated via ERK/MAPK and other MAPK pathways, which play an important role in plasmacytic differentiation and cell proliferation (Fig. 1) (36, 37). This indirectly indicates that the preferential presence of IgM on many lymphomas relates to the as yet unknown nature of the antigen and/or signalling which can be provided by IgM rather than IgG.

The presence of IgM vs. IgG also associates with two subtypes of DLBCL, which were described based on gene expression profiling, and further prognostically stratify these patients (38). Germinal centre B-cell-like (GCB) DLBCL subtype is characterised by the expression profile of germinal centre B cells, including the presence of somatic hypermutation and class switching to IgG. Activated B-cell-like (ABC) subtype resembles *in vitro* activated B cells or plasmatic cells, which are dependent on BCR signalling. In these cells, IgM expression is probably maintained as a consequence of genetic deletions within IgH switch regions, resulting in the blocking of class switch recombination to other isotypes (33).

The pioneering work of Dr. Staudt's group took advantage of RNA interference technology and investigated which molecular pathways are most intimately connected to survival and proliferation of ABC- vs. GC B-cell lines. This showed that ABC DLBCL cells rely on the anti-apoptotic NF- κ B pathway for survival, and especially on the presence of its organising complex CBM built from an adaptor molecule CARD11, BCL10 and MALT1. Additionally, the analysis of primary material from patients revealed the presence of mutant forms of CARD11 in ~10% of cases. This was a typical feature of ABC DLBCL and is in line with gene expression analysis which previously pointed to the NF- κ B pathway activation in these cells (38).

CARD11 mutation induces spontaneous aggregation of CBM complex and activates NF- κ B constitutively (39). The addition to the CARD11 pathway is a phenotype that

is positively selected during the evolution of these lymphomas (39). CARD11 mutations may allow the lymphoma cells to engage the NF- κ B pathway in the absence of antigen receptor signals, however, might also sensitise the cells to natural modes of its activation (39). In most ABC DLBCL cells, the CARD11 is not mutated, but the tumour cells' survival still depends on CARD11 and NF- κ B. The activation of NF- κ B can be delivered by multiple signals, but in B cells, it is known to be largely and directly dependent on BCR and BTK (40). The knockdown of any BCR components (IgH, CD79A and CD79B) or proximal effectors (SYK, PLC γ 2, PI3K, PKC β , BTK, BLNK) causes apoptosis in ABC DLBCL (29).

Compelling evidence for the importance of BCR in lymphomas came from targeted sequencing of BCR-involved genes. The sequence and structure of the Ig itself is 'suspicious' in lymphomas, especially in chronic lymphocytic leukaemia (CLL). Most cases of CLL express IgM and IgD, and it was an unexpected observation that the presence or absence of somatic hypermutations in IGHV variable region genes is a robust determinant of patient's prognosis (41). The subsequent identification of quasi-identical BCR Igs among unrelated patients with CLL suggests that either common antigen or common feature of such BCR is involved in CLL pathogenesis. These 'stereotypy' BCRs are present in ~30% of CLL cases and can be further categorised into 'subsets' according to shared Ig structural characteristics (42, 43). The biased BCR repertoire was also observed in mantle cell lymphoma (MCL) (44). However, the functional significance of these subsets and stereotype BCRs is still elusive. In the majority of CLLs with IGHVs lacking somatic hypermutations, these are polyreactive antibodies, recognising self- and exo-antigens, such as DNA, lipopolysaccharides, oxidised low-density lipoprotein and the cytoskeletal antigens myosin and vimentin (45–49). A potential antigen bound by a subset of CLL BCRs was recently discovered, namely a yeast/fungi cell wall component (50), and antigens that are found on apoptotic cells and inflammatory tissues were described for other BCRs expressed in CLL (51). A controversial study also provided evidence for autonomous antigen-independent BCR signalling in CLL (30). It was proposed that this is based on the recognition of an epitope in the BCR itself and was specifically observed for CLL derived Ig. It seems unlikely this would be a sole direct mechanism for CLL onset, but it is noteworthy that mutations in BCR-associated proteins are uncommon in CLL (52, 53). It is tempting to speculate this is because BCR signalling is already activated sufficiently in these malignant cells by either antigen or autonomous signalling. An alternative explanation could be that CLL cells would not tolerate BCR activation by mutations present in some lymphomas, due to their developmental stage. Importantly, BCR-signalling responses in CLL cells are heterogeneous, being more active in cases without somatic hypermutations

in IGHV, and these associate with the clinical outcome (54, 55). Interestingly, the BCR-signalling propensity in CLL can be directly affected by microRNAs and thus influence disease aggressivity (56–58).

Similar to CLL, indolent FL cells rarely harbour mutations in BCR-associated proteins. Their resistance to apoptosis is ensured by SYK expression. In contrast to normal B cells or CLL cells, FL cells have a higher SYK expression, and it is also mostly expressed as its phosphorylated form (59). This event is critical for the activation of the PI3K/AKT pathway and other pro-survival signalling such as mTOR (59, 60). But the mechanism underlining the increase in SYK activity still remains elusive, and it is unclear if it is through upstream deregulation of BCR signalling. Upregulation of SYK has also been observed in MCL, where amplifications of its locus are sometimes present and constitutive activation of NF- κ B and AKT is common (61, 62).

On the contrary, in DLBCL BCR signalling is directly affected by frequent mutations in CD79A/B, an indispensable component of BCR signalling. Mutations in ITAM motifs occur in ~20% of patients with the aggressive ABC subtype primarily affecting tyrosine residue in CD79B either by deletion or by substitution with another amino acid (29). Mutations in CD79A occur rarely as well as complete deletion of CD79B or CD79A (29). Importantly, cells always retain one fully intact CD79A or CD79B ITAM, which enables full activation of SYK and downstream BCR signalling (63). The significance of mutations in CD79B was described in a mouse model, in which the signalling tails of CD79B were mutated. These mice were able to develop mature B lymphocytes, but in contrast to normal mice, enhanced BCR signalling was detected in mutant cells. This may be due to reduced BCR internalisation as well as the elevated expression of surface IgM. Moreover, stimulation of B cells with CD79B mutation evokes longer and stronger activation of AKT signalling pathway (64). Similar results were observed in human B lymphocytes (29, 65–67). In contrast to normal cells, the cells with mutated CD79B are more resistant to the negative effects of protein tyrosine kinase LYN, for example, BCR internalisation (29, 66–69). Thus, mutations in CD79A and B probably might positively affect the amplitude and duration of BCR signalling and/or allow a premalignant B cell to escape anergy via impairing LYN kinase activity (66, 67). In CLL, the balance of BCR signalling shifts through a permanent activation of LYN. This is responsible for the anomalously high cellular tyrosine phosphorylation in CLL. Because CSK, a negative regulator of LYN, is similarly expressed in normal and CLL cells, the constitutive activity of LYN could be due to the fact that the amount of CSK is insufficient to inactivate LYN. As Ig expression is anomalous in CLL cells, a possible explanation of this observation might also be that ongoing BCR activation leads to LYN upregulation (70). As a consequence, high

levels of LYN mediate procaspase 8 dimerisation, which blocks apoptotic signalling in CLL (71).

In another subgroup of ABC DLBCL, the mutation of myeloid differentiation primary response gene 88 (MYD88) is present in ~30% of cases (72). In normal cells, MYD88 works as an adaptor protein that in response to stimuli couples Toll/interleukin-1 receptor (TIR) homology domain-containing proteins such as the toll-like receptors (TLRs) or some cytokine receptors. Subsequently, MYD88 interacts through its own death domains with interleukin-1 receptor-associated kinase (IRAK) family kinases and activates different downstream-signalling pathways such as NF- κ B and p38 or other MAPKs (72, 73). It is interesting to note that it is again a deregulation of an adaptor molecule, and this seems to be a recurrent theme in B-cell lymphomas. In the malignant cells, substitution in the TIR domain of MYD88 was described, which replaces leucine at position 265 with proline (L265P) (72). Cells with the MYD88 L265P mutation secrete greater amounts of chemokines and cytokines, for example, interleukin-6 and interleukin-10. These cells represent possible positive regulators of autocrine signalling and thereby promote the survival of malignant cells (53, 74). This mutation is rare or absent in other lymphomas such as GCB DLBCL but occurs in ~5% of CLL cases with mutated IGHV (53). Furthermore, genetic and functional analysis suggests chronic active BCR signalling and MYD88-dependent signalling cooperate in the survival of ABC DLBCL. This is supported by evidence of the fact that more ABC DLBCL has both MYD88 L265P and CD79A/B ITAM mutations than would be expected by coincidence. Besides, knockdown of MYD88 and CD79A acts in synergy in case of cell killing, indicating these two pathways are not redundant (72).

The activity of the BCR-signalling pathway and its ability to protect cells from apoptosis is dominantly dependent on PI3K. The connection of PI3K to BCR is mediated by redundantly acting adaptor molecules like GAB1. We have recently shown that GAB1 levels determine the propensity of BCR signalling in CLL (56). GAB1 serves as an adaptor protein for important components of BCR pathway (75). GAB1 is activated upon B-cell stimulation after its recruitment to the plasma cell membrane and is phosphorylated by different PTKs such as SYK, LYN or FYN (76–78). Its activation results in the recruitment and activation of PI3K, which triggers the major pro-survival pathways in the B cell as described previously. GAB1 is also involved in other pathways where it serves as a docking/scaffolding for PLC γ , CRK and CRKL proteins (79). Interestingly, GAB1 can also serve as mediator of positive feedback for PI3K activation. Non-phosphorylated GAB1 in cytoplasm is able to bind via its PH domain to the product of PI3K–PIP3 in the plasma cell membrane, which brings it to the proximity of protein tyrosine kinases (PTKs). GAB1 phosphorylation results in the stronger activation of PI3K-signalling pathways (Fig. 1)

(80). Silencing GAB1 directly downmodulates the calcium flux after BCR crosslinking on the B-cell membrane. We have shown GAB1 expression is regulated via microRNA miR-150, which represents the first case of non-coding RNAs role in BCR signalling (56). This resembles miRNA's known role in balancing TCR-signalling strength and PI3K signalling in T cells (81).

This is not the only example of BCR regulation by adaptors in lymphomas as one of the most commonly used prognostic markers in CLL, namely zeta-chain (TCR)-associated protein kinase 70 kDa (ZAP-70) is also a debated adaptor protein. This protein was originally identified in T cells, where it is involved in TCR signalling occupying a similarly important role to SYK kinase in B lymphocytes (82, 83). Normal B cells generally lack ZAP-70, but studies showed that CLL B cells expressing unmutated IGHV are often positive for ZAP-70 (84). Currently, the cause of ZAP-70 expression in these cells is unknown. Perhaps it reflects a particular stage of B-cell differentiation, and it is a consequence of signalling and/or disease progression (85). On the other hand, the clinical and physiological outcome of ZAP-70 expression in CLL is clearer: patients with higher levels of ZAP-70 displayed an enhanced signal transduction via BCR and worse survival (86, 87). CLL cells with ectopically overexpressed ZAP-70 showed a greater intracellular calcium flux and a significant increase in phosphorylated SYK, BLNK and PLC γ levels in response to BCR crosslinking with anti-IgM antibodies (88). Although the mechanism of ZAP-70 action in CLL is still debated, there is evidence that ZAP-70 may be affecting BCR signalling in several ways. Firstly, it has been reported ZAP-70 may reconstitute BCR signalling in SYK-deficient B cells, which suggests the contribution of ZAP-70 kinase activity (89). In SYK-positive B cells, ZAP-70 might mediate phosphorylation of ITAMs resulting in the recruitment of SYK (88, 90). However, studies have shown ZAP-70 has approximately a 100-fold lower kinase activity than SYK (91), as such it is conceivable that ZAP-70 may enhance BCR signalling not only via its kinase activity, but also by another mechanism, for example, it may serve as an adaptor protein (88, 90). It is also assumed ZAP-70 could compete with the SYK to bind to the E3 ubiquitin ligase c-CBL protein and thus sequester its proteosomal degradation (92–95).

Altogether, inhibiting BCR signalling seems to be a logical, and in some lymphoma cases, a true targeted therapeutic approach.

BCR inhibition and its crosstalk with other pathways

Bruton tyrosine kinase inhibitor ibrutinib received a breakthrough designation and was approved by the F.D.A. to treat MCL in 2013 and CLL in 2014. PI3K δ inhibitor idelalisib is expected to be approved soon. The clinical trials with inhibi-

tors for SYK (fosfatinib), BTK (ibrutinib) and PI3K δ (idelalisib) are likely to lead to a major shift in the treatment of B-cell malignancies. We would not review the exciting clinical activity of these molecules, as this has already been elegantly carried out by others (96–98). However, we would like to touch on some of the interesting consequences of BCR inhibition in malignant B cells.

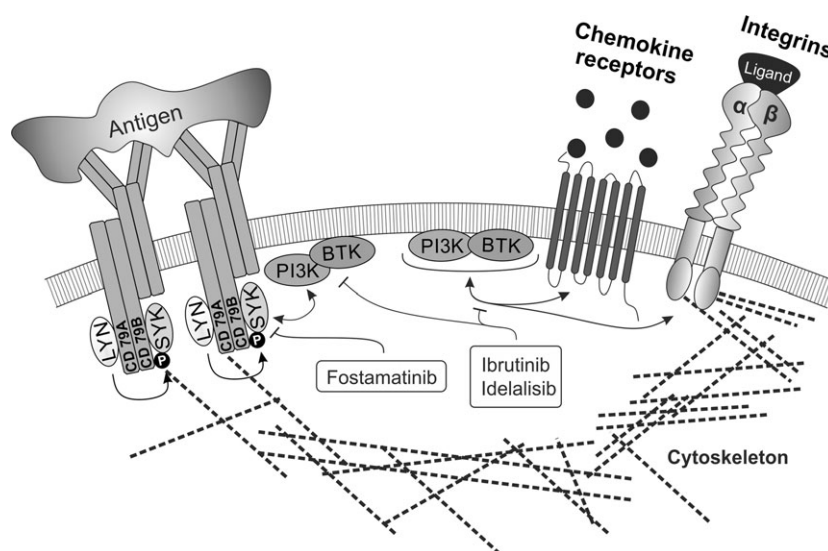
First of all, none of the targeted molecules (BTK, PI3K δ , SYK) are exclusively involved in BCR signalling, and none of the inhibitors are exclusively targeting one kinase. One of the hallmarks of CLL patients treated with BCR inhibitor like ibrutinib is a prolonged lymphocytosis (99). This is believed to be a consequence of the redistribution of leukaemic cells from lymph node compartment to circulation. A straight forward explanation of this could be that BTK inhibition interferes with malignant B cells' ability to reside in the microenvironment or recirculate from the periphery to the lymph nodes. Indeed, BTK is known to be expressed in many haematopoietic cell types and participate in multiple pathways in B cells (100). BCR signalling happens in the context of microenvironmental interactions, and this involves B-cell adhesion, migration and TLR signalling. It is intuitive that a B cell should be prepared for BCR signalling and costimulatory interactions once it enters the lymph node compartment.

Migration and *in vivo* homing to lymphoid organs was impaired in BTK-deficient B cells in mice. The chemokine C-X-C motif ligand (CXCL)12 (also known as SDF-1) stimulates the migration of B cells to the lymph nodes by interacting with its receptor CXCR4, which induces BTK activation (101). It is the SYK kinase that was postulated to activate BTK after CXCL12 ligation (101). PI3K inhibition has a less prominent effect on CXCL12-1-induced B-cell migration (102–104). BTK inhibition can disrupt the adhesion and migration of malignant B cells controlled by stimulated BCR signalling (105). Another interesting aspect is

related to the chemokines CCL3 and CCL4, which are secreted by CLL B cells in response to BCR activation, presumably to promote interactions with T cells. Ibrutinib inhibits CCL3/4 expression by CLL cells and rapidly reduces CCL3 and CCL4 plasma levels in ibrutinib-treated patients (105). Therefore, BCR inhibition also targets chemokine signalling, which is known to be very important in drug resistance and survival of malignant B cells (Fig. 2) (97, 106).

The activation of BTK by BCR signalling also affects adhesion through integrins, especially the integrin VLA-4 (also known as: α 4- β 1), which is expressed on B cells (101). BTK-deficient mice have been reported to display not only a pre-B cell block in proliferation, but also a defect in the retention of immature B cells in the bone marrow (107). BTK mediates adhesion through cytoskeletal re-organisation (Fig. 2) (108). Interestingly, many of the B-cell defects in mice deficient in CXCL12-CXCR4, CXCL13-CXCR5, vascular cell adhesion molecule 1 (VCAM-1) or α 4 integrins, such as impaired development and retention of B cell precursors in the bone marrow (109–116), are similar to the defects observed in the immunodeficiency diseases caused by loss-of-function mutations in BTK (107, 117–122). Despite the important role of chemokine-controlled integrin-mediated migration in B-cell development the underlying signal transduction mechanisms are as yet poorly defined. Interestingly, α 4 integrin-deficient pre-B cells also display a proliferation defect, and CXCL12 was originally identified as a pre-B-cell growth-stimulating factor (111). This intimate molecular connection between BCR and cell adhesion regulation allows B cells to interact more strongly with other immune cells, marrow stromal cells and receive additional stimulatory signals. Indeed, VLA-4 facilitates B-cell activation following the recognition of an antigen (123). VCAM1, the major ligand for VLA-4, is highly expressed on the surface of follicular dendritic cells and their interaction pro-

Figure 2 B-cell receptor (BCR) crosstalks with chemokine- and integrin- signalling. Activation of BTK and/or PI3K via chemokine receptors signalling contribute to migration of the B cell to microenvironmental niches. Integrin-dependent adhesion is affected through cytoskeletal reorganisation, which occurs after BCR and BTK activation and vice versa. 'BCR inhibitors' affect BCR signalling and also its crosstalk with cell migration and adhesion of B cells.



motes tight adhesion of B cells to dendritic cells. In lymphomas, such signalling can protect cells from chemotherapy and immunotherapy, providing yet another therapeutic target (106, 124). It is tempting to speculate that 'BCR inhibitors' are so successful thanks to their lack of specificity to inhibit solely BCR signalling. Additionally, the previously observed activity of adhesion/migration inhibitors like CXCR4 inhibitor plerixafor or VLA-4 inhibitor natalizumab could be an effect on BCR pathway (124–126).

The off-target effects of ibrutinib could also potentially affect the functions of T cells in the immune microenvironment. In T cells, ibrutinib inhibits ITK kinase, which mediates TCR-controlled actin polymerisation and the activation of beta-1 integrins (127, 128). ITK binding by ibrutinib in T cells leads to the inhibition of T_{H2} -cell activation after TCR stimulation, while T_{H1} - and CD8 T-cell activation seems to be retained (128). In CLL, T cells provide IL-4, IL-6 and CD40L stimulation, driving the expansion of the disease, and the immunomodulatory potential of ibrutinib might also affect this component of the microenvironment (129). The effects of ibrutinib on natural killer T (NKT) cells and mast cells, both of which express ITK, remain unclear. One could envision the potential interference of ibrutinib effect on the NKT functions for the antibody-induced cellular cytotoxicity (ADCC), which is an important component of the mechanism of action for therapeutic antibodies like rituximab.

It is not clear if clinically used BCR inhibitors fully block BCR signalling *in vivo*. B cells taken from ibrutinib-treated patients can still be activated by BCR and TLR stimulation, although the response is weaker than CD40L stimulation which bypasses BTK (130). Ibrutinib treatment also leads to AKT upregulation, which is probably compensatory to the inhibition of BCR signalling and supports cell survival. It was shown that in mice, total depletion of BCR signalling in mature B cells leads to a relatively rapid apoptosis, while BCR inhibition using ibrutinib in patients with CLL allows high B-cell counts for many months. Why are malignant B cells able to tolerate its inhibition for so long? It is tempting to speculate BTK inhibition still allows some level of BCR signalling which might be based on its 'tonic' component. Moreover, the presence of a large number of circulating malignant B cells under the constant selective pressure of ibrutinib is a window of opportunity for cells with a pre-existing or novel mutation which interferes with ibrutinib binding. Indeed, a recent analysis of patients that relapsed on ibrutinib revealed the presence of mutations in BTK which impair ibrutinib binding and the development of activating mutations in PLC γ 2 that overcome BTK inhibition (131). These observations suggest for combination of BCR inhibitors with cytoreductive therapy like monoclonal antibodies or classical chemotherapy. Although acquired resistance to ibrutinib has developed in only a small proportion of patients with CLL, data suggest that patients with

increased genomic instability, including those with P53 and ataxia-telangiectasia-mutated (ATM) aberrations, may be at risk of relapse (99). Such patients seem to be the most rational choice for combination therapies designed to avoid the development of resistance.

The use of ibrutinib or idelalisib alone has great single agent activity; however, it does not cure patients (99, 132). Correlative studies in patients with CLL demonstrate that similar to ibrutinib, idelalisib impairs BCR signalling, cell interactions and chemokine production (Fig. 2) (133). It would be very interesting to know whether some lymphoma/CLL patients have malignant B cells that are more addicted to the PI3K- vs. BTK signalling. Currently, it is not clear which patients would benefit more from PI3K inhibition or BTK inhibition or their combination. There is an emerging clinical need to identify predictive markers for these novel agents. The combination of idelalisib and anti-CD20 monoclonal antibody rituximab in CLL limits the disease for only a relative short period (~40% of patients progressed during the first 12 months of treatment) (132). This is somehow disappointing and underscores the need for a better understanding of molecular pathways in malignant B cells which could be targeted in combination to obtain durable disease control or cure with low risk of disease transformation and/or drug resistance.

Concluding remarks

The encouraging results of recent clinical trials with 'BCR inhibitors' are boosting BCR-signalling studies in lymphomas. BCR signalling represents an integration of multiple pathways, which are balanced and tuned, and this needs to be studied in detail. The clinical use of drugs targeting different members of the BCR signalosome will require the understanding of compensatory pathways activated in response in malignant B cells to design smart combinatorial treatment. Additionally, the application of next generation sequencing tools is providing an overwhelming amount of new information. It is clear that novel mutations will be identified in lymphomas, and their functional significance and implication for therapy (and BCR signalling) needs to be tested.

Author contributions

M.M. and V.S. wrote the paper.

Conflict of interest disclosure

The authors declare no competing financial interests.

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References

- Reth M, Nielsen P. Signaling circuits in early B-cell development. *Adv Immunol* 2014;**122**:129–75.
- Dal Porto JM, Gauld SB, Merrell KT, Mills D, Pugh-Bernard AE, Cambier J. B cell antigen receptor signaling 101. *Mol Immunol* 2004;**41**:599–613.
- Monroe JG. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat Rev Immunol* 2006;**6**:283–94.
- Rawlings DJ, Saffran DC, Tsukada S, *et al.* Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science* 1993;**261**:358–61.
- Fruman DA. Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling. *Curr Opin Immunol* 2004;**16**:314–20.
- Okada T, Maeda A, Iwamatsu A, Gotoh K, Kurosaki T. BCAP: the tyrosine kinase substrate that connects B cell receptor to phosphoinositide 3-kinase activation. *Immunity* 2000;**13**:817–27.
- Kurosaki T. Regulation of B-cell signal transduction by adaptor proteins. *Nat Rev Immunol* 2002;**2**:354–63.
- Craxton A, Jiang A, Kurosaki T, Clark EA. Syk and Bruton's tyrosine kinase are required for B cell antigen receptor-mediated activation of the kinase Akt. *J Biol Chem* 1999;**274**:30644–50.
- Cambier JC, Pleiman CM, Clark MR. Signal transduction by the B cell antigen receptor and its coreceptors. *Annu Rev Immunol* 1994;**12**:457–86.
- Hodson DJ, Turner M. The role of PI3K signalling in the B cell response to antigen. *Adv Exp Med Biol* 2009;**633**:43–53.
- Kurosaki T, Hikida M. Tyrosine kinases and their substrates in B lymphocytes. *Immunol Rev* 2009;**228**:132–48.
- Hibbs ML, Tarlinton DM, Armes J, Grail D, Hodgson G, Maglitt R, Stacker SA, Dunn AR. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell* 1995;**83**:301–11.
- Hata A, Sabe H, Kurosaki T, Takata M, Hanafusa H. Functional analysis of Csk in signal transduction through the B-cell antigen receptor. *Mol Cell Biol* 1994;**14**:7306–13.
- Pao LI, Cambier JC. Syk, but not Lyn, recruitment to B cell antigen receptor and activation following stimulation of CD45- B cells. *J Immunol* 1997;**158**:2663–9.
- Mayo LD, Donner DB. The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. *Trends Biochem Sci* 2002;**27**:462–7.
- Brauweiler A, Tamir I, Dal Porto J, Benschop RJ, Helgason CD, Humphries RK, Freed JH, Cambier JC. Differential regulation of B cell development, activation, and death by the src homology 2 domain-containing 5' inositol phosphatase (SHIP). *J Exp Med* 2000;**191**:1545–54.
- Vivier E, Daeron M. Immunoreceptor tyrosine-based inhibition motifs. *Immunol Today* 1997;**18**:286–91.
- Nitschke L, Floyd H, Crocker PR. New functions for the sialic acid-binding adhesion molecule CD22, a member of the growing family of Siglecs. *Scand J Immunol* 2001;**53**:227–34.
- Liu W, Meckel T, Tolar P, Sohn HW, Pierce SK. Intrinsic properties of immunoglobulin IgG1 isotype-switched B cell receptors promote microclustering and the initiation of signaling. *Immunity* 2010;**32**:778–89.
- Treanor B, Depoil D, Gonzalez-Granja A, Barral P, Weber M, Dushek O, Bruckbauer A, Batista FD. The membrane skeleton controls diffusion dynamics and signaling through the B cell receptor. *Immunity* 2010;**32**:187–99.
- Treanor B, Batista FD. Organisation and dynamics of antigen receptors: implications for lymphocyte signalling. *Curr Opin Immunol* 2010;**22**:299–307.
- Lam KP, Kuhn R, Rajewsky K. *In vivo* ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* 1997;**90**:1073–83.
- Srinivasan L, Sasaki Y, Calado DP, Zhang B, Paik JH, DePinho RA, Kutok JL, Kearney JF, Otipoby KL, Rajewsky K. PI3 kinase signals BCR-dependent mature B cell survival. *Cell* 2009;**139**:573–86.
- Cambier JC, Johnson SA. Differential binding activity of ARH1/TAM motifs. *Immunol Lett* 1995;**44**:77–80.
- Delgado P, Cubelos B, Calleja E, Martinez-Martin N, Cipres A, Merida I, Bellas C, Bustelo XR, Alarcon B. Essential function for the GTPase TC21 in homeostatic antigen receptor signaling. *Nat Immunol* 2009;**10**:880–8.
- Schmedt C, Saijo K, Niidome T, Kuhn R, Aizawa S, Tarakhovsky A. Csk controls antigen receptor-mediated development and selection of T-lineage cells. *Nature* 1998;**394**:901–4.
- Wienands J, Larbolette O, Reth M. Evidence for a pre-formed transducer complex organized by the B cell antigen receptor. *Proc Natl Acad Sci USA* 1996;**93**:7865–70.
- Wright G, Tan B, Rosenwald A, Hurt EH, Wiestner A, Staudt LM. A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. *Proc Natl Acad Sci USA* 2003;**100**:9991–6.
- Davis RE, Ngo VN, Lenz G, *et al.* Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. *Nature* 2010;**463**:88–92.
- Duhren-von Minden M, Ubelhart R, Schneider D, *et al.* Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature* 2012;**489**:309–12.
- Lenz G, Staudt LM. Aggressive lymphomas. *N Engl J Med* 2010;**362**:1417–29.
- Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer* 2005;**5**:251–62.

33. Lenz G, Nagel I, Siebert R, *et al.* Aberrant immunoglobulin class switch recombination and switch translocations in activated B cell-like diffuse large B cell lymphoma. *J Exp Med* 2007;**204**:633–43.
34. Engels N, König LM, Heemann C, Lutz J, Tsubata T, Griep S, Schrader V, Wienands J. Recruitment of the cytoplasmic adaptor Grb2 to surface IgG and IgE provides antigen receptor-intrinsic costimulation to class-switched B cells. *Nat Immunol* 2009;**10**:1018–25.
35. Wakabayashi C, Adachi T, Wienands J, Tsubata T. A distinct signaling pathway used by the IgG-containing B cell antigen receptor. *Science* 2002;**298**:2392–5.
36. Horikawa K, Martin SW, Pogue SL, Silver K, Peng K, Takatsu K, Goodnow CC. Enhancement and suppression of signaling by the conserved tail of IgG memory-type B cell antigen receptors. *J Exp Med* 2007;**204**:759–69.
37. Martin SW, Goodnow CC. Burst-enhancing role of the IgG membrane tail as a molecular determinant of memory. *Nat Immunol* 2002;**3**:182–8.
38. Rosenwald A, Wright G, Chan WC, *et al.*, Lymphoma/Leukemia Molecular Profiling Project. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N Engl J Med* 2002;**346**:1937–47.
39. Lenz G, Davis RE, Ngo VN, *et al.* Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science* 2008;**319**:1676–9.
40. Ngo VN, Davis RE, Lamy L, *et al.* A loss-of-function RNA interference screen for molecular targets in cancer. *Nature* 2006;**441**:106–10.
41. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 1999;**94**:1848–54.
42. Stamatopoulos K, Belessi C, Moreno C, *et al.* Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: pathogenetic implications and clinical correlations. *Blood* 2007;**109**:259–70.
43. Ghiotto F, Fais F, Valetto A, *et al.* Remarkably similar antigen receptors among a subset of patients with chronic lymphocytic leukemia. *J Clin Invest* 2004;**113**:1008–16.
44. Hadzidimitriou A, Agathangelidis A, Darzentas N, *et al.* Is there a role for antigen selection in mantle cell lymphoma? Immunogenetic support from a series of 807 cases. *Blood* 2011;**118**:3088–95.
45. Herve M, Xu K, Ng YS, Wardemann H, Albesiano E, Messmer BT, Chiorazzi N, Meffre E. Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. *J Clin Invest* 2005;**115**:1636–43.
46. CATERA R, Silverman GJ, Hatzi K, *et al.* Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. *Mol Med* 2008;**14**:665–74.
47. Chu CC, CATERA R, Hatzi K, *et al.* Chronic lymphocytic leukemia antibodies with a common stereotypic rearrangement recognize nonmuscle myosin heavy chain IIA. *Blood* 2008;**112**:5122–9.
48. Lanemo Myhrinder A, Hellqvist E, Sidorova E, *et al.* A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. *Blood* 2008;**111**:3838–48.
49. Binder M, Lechenne B, Ummanni R, Scharf C, Balabanov S, Trusch M, Schluter H, Braren I, Spillner E, Trepel M. Stereotypical chronic lymphocytic leukemia B-cell receptors recognize survival promoting antigens on stromal cells. *PLoS ONE* 2010;**5**:e15992.
50. Hoogetboom R, van Kessel KP, Hochstenbach F, Wormhoudt TA, Reinten RJ, Wagner K, Kater AP, Guikema JE, Bende RJ, van Noesel CJ. A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. *J Exp Med* 2013;**210**:59–70.
51. Que X, Widhopf GF 2nd, Amir S, Hartvigsen K, Hansen LF, Woelkers D, Tsimikas S, Binder CJ, Kipps TJ, Witzum JL.IGHV1-69-encoded antibodies expressed in chronic lymphocytic leukemia react with malondialdehyde-acetaldehyde adduct, an immunodominant oxidation-specific epitope. *PLoS ONE* 2013;**8**:e65203.
52. Wang L, Lawrence MS, Wan Y, *et al.* SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. *N Engl J Med* 2011;**365**:2497–506.
53. Puente XS, Pinyol M, Quesada V, *et al.* Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature* 2011;**475**:101–5.
54. Blix ES, Irish JM, Husebekk A, Delabie J, Tierens AM, Myklebust JH, Kolstad A. Altered BCR and CD40 signaling are associated with clinical outcome in small lymphocytic lymphoma/chronic lymphocytic leukaemia and marginal zone lymphoma patients. *Br J Haematol* 2012;**159**:604–8.
55. Palomba ML, Piersanti K, Ziegler CG, *et al.* Multidimensional single-cell analysis of BCR signaling reveals proximal activation defect as a hallmark of chronic lymphocytic leukemia B cells. *PLoS ONE* 2014;**9**:e79987.
56. Mraz M, Chen L, Rassenti LZ, Ghia EM, Li H, Jepsen K, Smith EN, Messer K, Frazer KA, Kipps TJ. miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1. *Blood* 2014;**124**:84–95.
57. Mraz M, Kipps TJ. MicroRNAs and B cell receptor signaling in chronic lymphocytic leukemia. *Leuk Lymphoma* 2013;**54**:1836–9.
58. Cui B, Chen L, Zhang S, *et al.* MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia. *Blood* 2014;**124**:546–54.
59. Leseux L, Hamdi SM, Al Saati T, Capilla F, Recher C, Laurent G, Bezombes C. Syk-dependent mTOR activation in follicular lymphoma cells. *Blood* 2006;**108**:4156–62.
60. Gulmann C, Espina V, Petricoin E 3rd, Longo DL, Santi M, Knutsen T, Raffeld M, Jaffe ES, Liotta LA, Feldman AL. Proteomic analysis of apoptotic pathways reveals prognostic factors in follicular lymphoma. *Clin Cancer Res* 2005;**11**:5847–55.

61. Rinaldi A, Kwee I, Tadorelli M, *et al.* Genomic and expression profiling identifies the B-cell associated tyrosine kinase Syk as a possible therapeutic target in mantle cell lymphoma. *Br J Haematol* 2006;**132**:303–16.
62. Jares P, Colomer D, Campo E. Molecular pathogenesis of mantle cell lymphoma. *J Clin Invest* 2012;**122**:3416–23.
63. Rolli V, Gallwitz M, Wossning T, Flemming A, Schamel WW, Zurn C, Reth M. Amplification of B cell antigen receptor signaling by a Syk/ITAM positive feedback loop. *Mol Cell* 2002;**10**:1057–69.
64. Gazumyan A, Reichlin A, Nussenzweig MC. Ig beta tyrosine residues contribute to the control of B cell receptor signaling by regulating receptor internalization. *J Exp Med* 2006;**203**:1785–94.
65. Ma H, Yankee TM, Hu J, Asai DJ, Harrison ML, Geahlen RL. Visualization of Syk-antigen receptor interactions using green fluorescent protein: differential roles for Syk and Lyn in the regulation of receptor capping and internalization. *J Immunol* 2001;**166**:1507–16.
66. Gross AJ, Lyandres JR, Panigrahi AK, Prak ET, DeFranco AL. Developmental acquisition of the Lyn-CD22-SHP-1 inhibitory pathway promotes B cell tolerance. *J Immunol* 2009;**182**:5382–92.
67. Gauld SB, Benschof RJ, Merrell KT, Cambier JC. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol* 2005;**6**:1160–7.
68. Xu Y, Harder KW, Huntington ND, Hibbs ML, Tarlinton DM. Lyn tyrosine kinase: accentuating the positive and the negative. *Immunity* 2005;**22**:9–18.
69. Yarkoni Y, Gethahun A, Cambier JC. Molecular underpinning of B-cell anergy. *Immunol Rev* 2010;**237**:249–63.
70. Contri A, Brunati AM, Trentin L, Cabrelle A, Miorin M, Cesaro L, Pinna LA, Zambello R, Semenzato G, Donella-Deana A. Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. *J Clin Invest* 2005;**115**:369–78.
71. Zonta F, Pagano MA, Trentin L, *et al.* Lyn-mediated procaspase 8 dimerization blocks apoptotic signaling in B-cell chronic lymphocytic leukemia. *Blood* 2014;**123**:875–83.
72. Ngo VN, Young RM, Schmitz R, *et al.* Oncogenically active MYD88 mutations in human lymphoma. *Nature* 2011;**470**:115–9.
73. Lin SC, Lo YC, Wu H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature* 2010;**465**:885–90.
74. Ding BB, Yu JJ, Yu RY, Mendez LM, Shaknovich R, Zhang Y, Cattoretti G, Ye BH. Constitutively activated STAT3 promotes cell proliferation and survival in the activated B-cell subtype of diffuse large B-cell lymphomas. *Blood* 2008;**111**:1515–23.
75. Nishida K, Yoshida Y, Itoh M, *et al.* Gab-family adapter proteins act downstream of cytokine and growth factor receptors and T- and B-cell antigen receptors. *Blood* 1999;**93**:1809–16.
76. Gu H, Neel BG. The “Gab” in signal transduction. *Trends Cell Biol* 2003;**13**:122–30.
77. Schaeper U, Gehring NH, Fuchs KP, Sachs M, Kempkes B, Birchmeier W. Coupling of Gab1 to c-Met, Grb2, and Shp2 mediates biological responses. *J Cell Biol* 2000;**149**:1419–32.
78. Ingham RJ, Holgado-Madruga M, Siu C, Wong AJ, Gold MR. The Gab1 protein is a docking site for multiple proteins involved in signaling by the B cell antigen receptor. *J Biol Chem* 1998;**273**:30630–7.
79. Sarmay G, Angyal A, Kertesz A, Maus M, Medgyesi D. The multiple function of Grb2 associated binder (Gab) adaptor/scaffolding protein in immune cell signaling. *Immunol Lett* 2006;**104**:76–82.
80. Ingham RJ, Santos L, Dang-Lawson M, Holgado-Madruga M, Dudek P, Maroun CR, Wong AJ, Matsuuchi L, Gold MR. The Gab1 docking protein links the b cell antigen receptor to the phosphatidylinositol 3-kinase/Akt signaling pathway and to the SHP2 tyrosine phosphatase. *J Biol Chem* 2001;**276**:12257–65.
81. Henao-Mejia J, Williams A, Goff LA, Staron M, Licona-Limon P, Kaech SM, Nakayama M, Rinn JL, Flavell RA. The microRNA miR-181 is a critical cellular metabolic rheostat essential for NKT cell ontogenesis and lymphocyte development and homeostasis. *Immunity* 2013;**38**:984–97.
82. Qian D, Weiss A. T cell antigen receptor signal transduction. *Curr Opin Cell Biol* 1997;**9**:205–12.
83. Scielzo C, Camporeale A, Geuna M, Alessio M, Poggi A, Zocchi MR, Chilosi M, Caligaris-Cappio F, Ghia P. ZAP-70 is expressed by normal and malignant human B-cell subsets of different maturational stage. *Leukemia* 2006;**20**:689–95.
84. Kipps TJ. The B-cell receptor and ZAP-70 in chronic lymphocytic leukemia. *Best Pract Res Clin Haematol* 2007;**20**:415–24.
85. Wiestner A, Rosenwald A, Barry TS, *et al.* ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood* 2003;**101**:4944–51.
86. Chen L, Apgar J, Huynh L, Dicker F, Giago-McGahan T, Rassenti L, Weiss A, Kipps TJ. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. *Blood* 2005;**105**:2036–41.
87. Rassenti LZ, Huynh L, Toy TL, *et al.* ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med* 2004;**351**:893–901.
88. Chen L, Huynh L, Apgar J, Tang L, Rassenti L, Weiss A, Kipps TJ. ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukemia. *Blood* 2008;**111**:2685–92.
89. Kong GH, Bu JY, Kurosaki T, Shaw AS, Chan AC. Reconstitution of Syk function by the ZAP-70 protein tyrosine kinase. *Immunity* 1995;**2**:485–92.
90. Ashe JM, Wiest DL, Abe R, Singer A. ZAP-70 protein promotes tyrosine phosphorylation of T cell receptor signaling motifs (ITAMs) in immature CD4(+)8(+) thymocytes with limiting p56(lck). *J Exp Med* 1999;**189**:1163–8.

91. Latour S, Chow LM, Veillette A. Differential intrinsic enzymatic activity of Syk and Zap-70 protein-tyrosine kinases. *J Biol Chem* 1996;**271**:22782–90.
92. Rao N, Ghosh AK, Ota S, Zhou P, Reddi AL, Hakezi K, Druker BK, Wu J, Band H. The non-receptor tyrosine kinase Syk is a target of Cbl-mediated ubiquitylation upon B-cell receptor stimulation. *EMBO J* 2001;**20**:7085–95.
93. Zhang Z, Elly C, Qiu L, Altman A, Liu YC. A direct interaction between the adaptor protein Cbl-b and the kinase zap-70 induces a positive signal in T cells. *Curr Biol* 1999;**9**:203–6.
94. Lupher ML Jr, Rao N, Lill NL, Andoniou CE, Miyake S, Clark EA, Druker B, Band H. Cbl-mediated negative regulation of the Syk tyrosine kinase. A critical role for Cbl phosphotyrosine-binding domain binding to Syk phosphotyrosine 323. *J Biol Chem* 1998;**273**:35273–81.
95. Mankai A, Eveillard JR, Buhe V, Le Ster K, Loisel S, Ghedira I, Youinou P, Berthou C, Bordron A. Is the c-Cbl proto-oncogene involved in chronic lymphocytic leukemia? *Ann N Y Acad Sci* 2007;**1107**:193–205.
96. Hendriks RW, Yuvaraj S, Kil LP. Targeting Bruton's tyrosine kinase in B cell malignancies. *Nat Rev Cancer* 2014;**14**:219–32.
97. Burger JA. Targeting the microenvironment in chronic lymphocytic leukemia is changing the therapeutic landscape. *Curr Opin Oncol* 2012;**24**:643–9.
98. Fruman DA, Rommel C. PI3Kdelta inhibitors in cancer: rationale and serendipity merge in the clinic. *Cancer Discov* 2011;**1**:562–72.
99. Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med* 2013;**369**:32–42.
100. de Weers M, Verschuren MC, Kraakman ME, Mensink RG, Schuurman RK, van Dongen JJ, Hendriks RW. The Bruton's tyrosine kinase gene is expressed throughout B cell differentiation, from early precursor B cell stages preceding immunoglobulin gene rearrangement up to mature B cell stages. *Eur J Immunol* 1993;**23**:3109–14.
101. de Gorter DJ, Beuling EA, Kersseboom R, Middendorp S, van Gils JM, Hendriks RW, Pals ST, Spaargaren M. Bruton's tyrosine kinase and phospholipase Cgamma2 mediate chemokine-controlled B cell migration and homing. *Immunity* 2007;**26**:93–104.
102. Nombela-Arrieta C, Lacalle RA, Montoya MC, et al. Differential requirements for DOCK2 and phosphoinositide-3-kinase gamma during T and B lymphocyte homing. *Immunity* 2004;**21**:429–41.
103. Smit MJ, Verdijk P, van der Raaij-Helmer EM, Navis M, Hensbergen PJ, Leurs R, Tensen CP. CXCR3-mediated chemotaxis of human T cells is regulated by a Gi- and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. *Blood* 2003;**102**:1959–65.
104. Ward SG. Do phosphoinositide 3-kinases direct lymphocyte navigation? *Trends Immunol* 2004;**25**:67–74.
105. Ponader S, Chen SS, Buggy JJ, Balakrishnan K, Gandhi V, Wierda WG, Keating MJ, O'Brien S, Chiorazzi N, Burger JA. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing *in vitro* and *in vivo*. *Blood* 2012;**119**:1182–9.
106. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood* 2009;**114**:3367–75.
107. Cariappa A, Kim TJ, Pillai S. Accelerated emigration of B lymphocytes in the Xid mouse. *J Immunol* 1999;**162**:4417–23.
108. Spaargaren M, Beuling EA, Rurup ML, Meijer HP, Klok MD, Middendorp S, Hendriks RW, Pals ST. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J Exp Med* 2003;**198**:1539–50.
109. Allen CD, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, Cyster JG. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat Immunol* 2004;**5**:943–52.
110. Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, Browning JL, Lipp M, Cyster JG. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 2000;**406**:309–14.
111. Arroyo AG, Yang JT, Rayburn H, Hynes RO. Alpha4 integrins regulate the proliferation/differentiation balance of multilineage hematopoietic progenitors *in vivo*. *Immunity* 1999;**11**:555–66.
112. Leuker CE, Labow M, Muller W, Wagner N. Neonatally induced inactivation of the vascular cell adhesion molecule 1 gene impairs B cell localization and T cell-dependent humoral immune response. *J Exp Med* 2001;**193**:755–68.
113. Ma Q, Jones D, Springer TA. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* 1999;**10**:463–71.
114. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 1996;**382**:635–8.
115. Nie Y, Waite J, Brewer F, Sunshine MJ, Littman DR, Zou YR. The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. *J Exp Med* 2004;**200**:1145–56.
116. Okada T, Ngo VN, Ekland EH, Forster R, Lipp M, Littman DR, Cyster JG. Chemokine requirements for B cell entry to lymph nodes and Peyer's patches. *J Exp Med* 2002;**196**:65–75.
117. Conley ME, Partain JD, Norland SM, Shurtleff SA, Kazazian HH Jr. Two independent retrotransposon insertions at the same site within the coding region of BTK. *Hum Mutat* 2005;**25**:324–5.
118. Ellmeier W, Jung S, Sunshine MJ, Hatam F, Xu Y, Baltimore D, Mano H, Littman DR. Severe B cell deficiency in mice lacking the tec kinase family members Tec and Btk. *J Exp Med* 2000;**192**:1611–24.

119. Hendriks RW, de Bruijn MF, Maas A, Dingjan GM, Karis A, Grosveld F. Inactivation of Btk by insertion of lacZ reveals defects in B cell development only past the pre-B cell stage. *EMBO J* 1996;**15**:4862–72.
120. Khan WN, Alt FW, Gerstein RM, *et al.* Defective B cell development and function in Btk-deficient mice. *Immunity* 1995;**3**:283–99.
121. Middendorp S, Dingjan GM, Hendriks RW. Impaired precursor B cell differentiation in Bruton's tyrosine kinase-deficient mice. *J Immunol* 2002;**168**:2695–703.
122. Nomura K, Kanegane H, Karasuyama H, *et al.* Genetic defect in human X-linked agammaglobulinemia impedes a maturational evolution of pro-B cells into a later stage of pre-B cells in the B-cell differentiation pathway. *Blood* 2000;**96**:610–7.
123. Carrasco YR, Batista FD. B-cell activation by membrane-bound antigens is facilitated by the interaction of VLA-4 with VCAM-1. *EMBO J* 2006;**25**:889–99.
124. Mraz M, Zent CS, Church AK, *et al.* Bone marrow stromal cells protect lymphoma B-cells from rituximab-induced apoptosis and targeting integrin alpha-4-beta-1 (VLA-4) with natalizumab can overcome this resistance. *Br J Haematol* 2011;**155**:53–64.
125. Burger JA. Nurture versus nature: the microenvironment in chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program* 2011;**2011**:96–103.
126. Kurtova AV, Tamayo AT, Ford RJ, Burger JA. Mantle cell lymphoma cells express high levels of CXCR4, CXCR5, and VLA-4 (CD49d): importance for interactions with the stromal microenvironment and specific targeting. *Blood* 2009;**113**:4604–13.
127. Berg LJ, Finkelstein LD, Lucas JA, Schwartzberg PL. Tec family kinases in T lymphocyte development and function. *Annu Rev Immunol* 2005;**23**:549–600.
128. Dubovsky JA, Beckwith KA, Natarajan G, *et al.* Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. *Blood* 2013;**122**:2539–49.
129. Gorgun G, Holderried TA, Zahrieh D, Neuberg D, Gribben JG. Chronic lymphocytic leukemia cells induce changes in gene expression of CD4 and CD8 T cells. *J Clin Invest* 2005;**115**:1797–805.
130. Woyach JA, Smucker K, Smith LL, *et al.* Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. *Blood* 2014;**123**:1810–7.
131. Byrd JC, Brown JR, O'Brien S, *et al.*, RESONATE Investigators. Ibrutinib versus ofatumumab in previously treated chronic lymphoid leukemia. *N Engl J Med* 2014;**371**:213–23.
132. Furman RR, Sharman JP, Coutre SE, *et al.* Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. *N Engl J Med* 2014;**370**:997–1007.
133. Hoellenriegel J, Meadows SA, Sivina M, *et al.* The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood* 2011;**118**:3603–12.

LYMPHOID NEOPLASIA

FoxO1-GAB1 axis regulates homing capacity and tonic AKT activity in chronic lymphocytic leukemia

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KEY POINTS

- GAB1 upregulation in an intraclonal subpopulation of peripheral blood CLL cells leads to tonic AKT activity and increased homing capacity.
- FoxO1-GAB1-pAKT axis can be targeted therapeutically by novel GAB1 inhibitors, alone or in combination with BTK inhibition.

Recirculation of chronic lymphocytic leukemia (CLL) cells between the peripheral blood and lymphoid niches plays a critical role in disease pathophysiology, and inhibiting this process is one of the major mechanisms of action for B-cell receptor (BCR) inhibitors such as ibrutinib and idelalisib. Migration is a complex process guided by chemokine receptors and integrins. However, it remains largely unknown how CLL cells integrate multiple migratory signals while balancing survival in the peripheral blood and the decision to return to immune niches. Our study provided evidence that CXCR4/CD5 intraclonal subpopulations can be used to study the regulation of migration of CLL cells. We performed RNA profiling of CXCR4^{dim}CD5^{bright} vs CXCR4^{bright}CD5^{dim} CLL cells and identified differential expression of dozens of molecules with a putative function in cell migration. GRB2-associated binding protein 1 (GAB1) positively regulated CLL cell homing capacity of CXCR4^{bright}CD5^{dim} cells. Gradual GAB1 accumulation in CLL cells outside immune niches was mediated by FoxO1-induced transcriptional GAB1 activation. Upregulation of GAB1 also played an important role in maintaining basal phosphatidylinositol 3-kinase (PI3K) activity and the “tonic” AKT phosphorylation required to sustain the survival of resting CLL B cells. This finding is

important during ibrutinib therapy, because CLL cells induce the FoxO1-GAB1-pAKT axis, which represents an adaptation mechanism to the inability to home to immune niches. We have demonstrated that GAB1 can be targeted therapeutically by novel GAB1 inhibitors, alone or in combination with BTK inhibition. GAB1 inhibitors induce CLL cell apoptosis, impair cell migration, inhibit tonic or BCR-induced AKT phosphorylation, and block compensatory AKT activity during ibrutinib therapy. (*Blood*. 2021;00(00):1-15)

Introduction

Chronic lymphocytic leukemia (CLL) cell recirculation to and from immune niches has been proposed as a feature of CLL biology,¹⁻⁷ although it has not been entirely experimentally proven. Inside the immune niches, CLL cells obtain various prosurvival and proliferative signals leading to activation of the B-cell receptor (BCR), phosphatidylinositol 3-kinase (PI3K)/AKT, and NFκB pathways.^{2,8-11} Inhibiting CLL cell homing to immune niches represents an important mechanism of action for BCR inhibitors and a possible therapeutic strategy.¹²⁻¹⁴ One proposed model for the study of CLL cell recirculation is to use CXCR4/CD5 intraclonal subpopulations. It has been postulated that CXCR4^{dim}CD5^{bright} cells represent a proliferative fraction of cells that have recently exited the microenvironment, whereas

CXCR4^{bright}CD5^{dim} cells correspond to resting cells circulating in peripheral blood for an extended time before they return back to the immune niches.¹⁻⁷ CLL cell migration to immune niches is known to be regulated by selectin CD62L^{15,16}; integrin VLA4^{17,18}; and chemokine receptors, such as CXCR4, CXCR5, and CCR7 that guide cells toward gradients of ligands.^{10,17,19-23} Migration is a highly complex process, and CXCR4 levels in CLL subpopulations were shown to reflect the history of microenvironmental interactions, since binding of SDF1 chemokine to CXCR4 leads to its lower levels on the CLL cell surface, whereas CXCR4 upregulation sensitizes cells to SDF1-guided migration.^{1-7,20} However, it remains largely unknown how precisely CLL cells integrate multiple migratory signals while balancing survival in peripheral blood and the decision to return to the immune

niches. It has been described that CLL cells or normal B cells outside the immune niches can use “tonic” PI3K activation²⁴⁻²⁸ or autonomous BCR signaling to sustain their survival²⁹; however, factors regulating this or their interplay with homing capacity are largely unclear. It is also unclear whether PI3K activation plays a role in adaptation/resistance to BCR signaling inhibition and impaired homing during ibrutinib therapy.³⁰ We hypothesized that analyzing the “resting” nonproliferative CXCR4^{bright}CD5^{dim} cells could help to understand the factors determining the return of these cells to immune niches and/or factors that balance the survival of peripheral blood CLL cells. This may have implications for CLL cell adaption to therapeutic inhibition of homing by BCR inhibitors.

In this study, we have shown that the CXCR4^{bright}CD5^{dim} cell subpopulation has a higher capacity to home into immune niches. Analysis of this subpopulation revealed differential expression of dozens of migration-related genes, including up-regulation of GAB1, which is known to act as a migration regulator in nonimmune cells.^{31,32} However, its role in lymphocyte migration is unknown. It has been demonstrated that GAB1 serves as an adaptor protein that integrates signals from different receptors and affects signaling cascades, such as PI3K/AKT,³³⁻³⁵ MAPK/extracellular signal-regulated kinase (ERK),³⁶⁻³⁸ PLC γ ,³⁹ and Crk/CrkL.⁴⁰ The results of our study show that GAB1 positively affects CLL cell migration in response to various chemoattractants. Gradual GAB1 accumulation in CLL cells outside immune niches is mediated by FoxO1-induced transcriptional activation of the *GAB1* gene. We also found that GAB1 up-regulation has a role in maintaining the basal tonic AKT phosphorylation required to sustain CLL cell survival. This is important during ibrutinib therapy since CLL cells induce the FoxO1-GAB1-pAKT axis in this context, which can be targeted therapeutically by the GAB1 inhibitors tested in this study.

Methods

CLL samples and cell lines

The study was approved by the institutional review board, and samples were obtained with written informed consent. CLL samples were purified to contain $\geq 95\%$ of CD5⁺19⁺ cells (see supplemental Methods, available on the *Blood* Web site). MEC1 and HS5 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures GmbH, and American Type Culture Collection, respectively, and the OSU-CLL was a gift from John Byrd (The Ohio State University, Columbus, OH).⁴¹

Gene expression

For RNAseq analysis, freshly isolated CLL cells were sorted according to CXCR4/CD5 expression (purity $\geq 99.9\%$), as described previously.⁷ Libraries were prepared using the TruSeq Stranded messenger mRNA (mRNA) LT Sample Prep Kit (Illumina) and sequenced with the NextSeq 500/550 High Output v2.5 Kit (supplemental Methods).

Migration assays and cell treatments

Cells transfected with siRNA against *GAB1*, or *GAB1*/*FoxO1* knockout (KO) cells, or control cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) or FarRed CellTrace dye (Thermo Fisher Scientific) or were traced with a plasmid encoding GFP or Azurite, and an equal number of the cells was mixed in a 1:1 ratio (validated by flow cytometry) and injected

into the tail vein of NSG mice.⁴² For a description of the in vitro competitive migration assay, see the supplemental Methods. Cells were treated with ibrutinib (1 μ M), MK-2206 2HCl (1 μ M), AS1842856 (0.5 μ M), plerixafor (5 μ g/mL; all from Selleckchem), or GAB1 pleckstrin homology (PH) domain-targeting inhibitors (GAB-001, GAB-004).⁴³ The cells were treated by goat F(ab')₂ anti-human IgM (10 μ g/mL; Southern Biotech), SDF1, or CXCL13 (Peprotech), as described previously.^{2,44,45}

Statistical analysis

Apart from NGS analysis (supplemental Methods), all statistical analyses were performed with Prism v8.0.1 (GraphPad). Values of $P < .05$ were considered significant.

Results

GAB1 is accumulated in CXCR4^{bright}CD5^{dim} intracolonial CLL cell subpopulation

CXCR4/CD5 cell subpopulations have been suggested as a model distinguishing cells ready to reenter immune niches (CXCR4^{bright}CD5^{dim}) vs recent emigrants from lymph nodes (CXCR4^{dim}CD5^{bright}).¹⁻⁷ We aimed to first test each subpopulation's migration potential in NSG mice; however, labeling cells with an anti-CXCR4 antibody could affect their responsiveness to the CXCR4 ligand SDF1. To overcome this problem, we used CD5 and forward scatter area (FSC-A reflecting cell size) for cell sorting, because lower FSC-A is typical for CXCR4^{bright}CD5^{dim} and higher FSC-A for the CXCR4^{dim}CD5^{bright} subpopulation (supplemental Figure 1). Subsequently, the sorted subpopulations were stained with 2 cell-tracing dyes, and their 1:1 mixture was injected into the tail vein of NSG mice for a 4-hour competitive migration analysis. In line with the model, the CXCR4^{bright}CD5^{dim} CLL cell subpopulation migrated significantly more into the spleen from the peripheral blood than the CXCR4^{dim}CD5^{bright} cells (Figure 1A). CLL cell migration into bone marrow or the liver was the same, and the number of CLL cells infiltrating these organs was low (supplemental Figure 2), as reported previously.⁴⁶ The higher CXCR4^{bright}CD5^{dim} cell migration was also confirmed in an in vitro Transwell cell migration assay toward SDF1 (CXCL12), CXCL13, or conditioned medium (CM) derived from primary human mesenchymal stromal cells (CM^{hMSC}; supplemental Figure 2). This finding suggested that it is possible to identify homing-related genes by analyzing CXCR4/CD5 subpopulations. We further performed transcription analysis (RNA sequencing [RNAseq]) of the CXCR4^{dim}CD5^{bright} vs the CXCR4^{bright}CD5^{dim} cell subpopulations (a sorting strategy for CXCR4/CD5 subpopulation in Figure 1B, as detailed previously^{2,7}). This process identified 1372 differentially expressed genes in CXCR4/CD5 subpopulations (fold change, >1.5 ; $P < .001$). The CXCR4^{bright}CD5^{dim} cells were characterized by BCR/NF κ B signature gene downregulation (eg, *MYC*, *CD83*, *FOS*, *CCND2*, *BCL2A1*, *DUSP2*, *CCL3*, and *CLL4*), corresponding to a resting nonactivated B-cell phenotype (for the list of genes and principal component analysis, see supplemental Figure 3 and 4; supplemental Table 2). The differentially expressed mRNAs included 147 genes annotated in databases as migration-related (Figure 1C; fold change, >1.5 ; $P < .001$; supplemental Table 3), and Gene Ontology analysis confirmed a significant enrichment of migration/adhesion-related genes (supplemental Table 4). Several such randomly selected genes were validated by real-time quantitative polymerase chain reaction

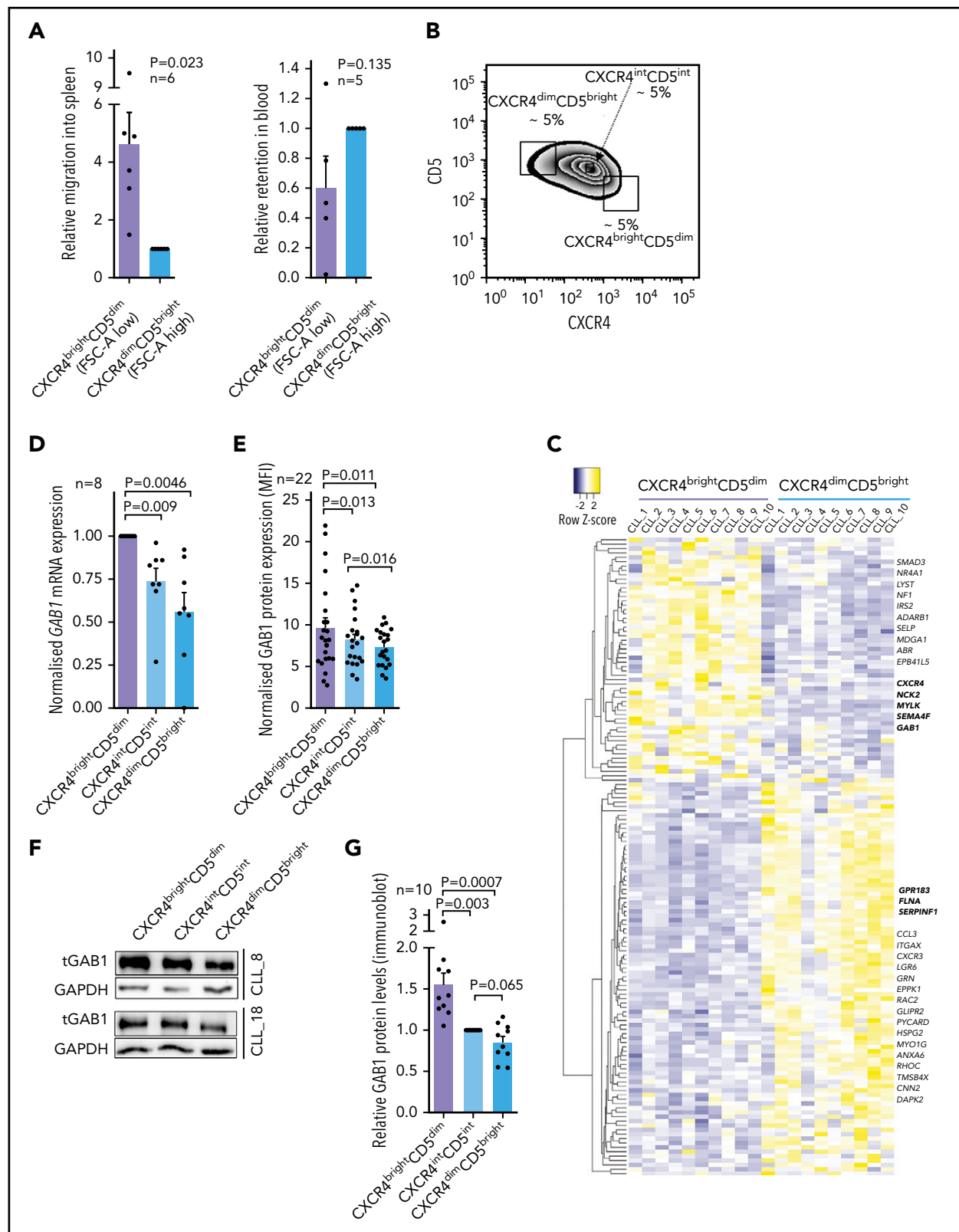


Figure 1. Accumulation of GAB1 in CXCR4^{bright}CD5^{dim} intraclonal CLL cell subpopulation. (A) Competitive migration of CXCR4/CD5 CLL cell subpopulation into the spleen of NSG mice and their relative retention in the blood (migration continued for 4 hours, $n = 6$). Cells were sorted according to FCS-A and CD5 (FCS^{dim}CD5^{dim} correspond to CXCR4^{bright}CD5^{dim} cells and FCS^{bright}CD5^{bright} correspond to CXCR4^{dim}CD5^{bright} cells; for cell-sorting strategy, see supplemental Figure 1). CXCR4 labeling was not used for cell sorting because it would block CXCR4 interaction with SDF1. One blood sample was excluded from the analysis for technical reasons. (B) Representative gating strategy for CLL cells sorted according to CXCR4/CD5 expression (each subpopulation gated as ~5% of cells). This sorting, directly based on CXCR4/CD5 labeling, was used for RNAseq profiling and all experiments other than migration assays. (C) Heat map of differentially expressed genes in CXCR4/CD5 subpopulations (fold change, >1.5 ; $P < .001$; $n = 10$), subsequently overlapped with a database of migration-related genes (GO:0016477).⁶⁷ Lower expression is indicated in blue, higher expression in yellow. Gene names marked in

(qPCR; supplemental Figure 5). Among these putative migration-related genes, we noticed a *GAB1* upregulation (~1.8-fold) in CXCR4^{bright}CD5^{dim} cells compared with CXCR4^{dim}CD5^{bright} cells. In a previous study, we showed that higher *GAB1* levels are associated with shorter overall survival in CLL,³⁵ and *GAB1* was identified as a migration regulator in epithelial and muscle cells^{31,32} and regulator of BCR responsiveness.³³⁻³⁵ The higher *GAB1* levels in CXCR4^{bright}CD5^{dim} were confirmed by real-time qPCR at the mRNA level (Figure 1D) and by flow cytometry and immunoblot analysis at the protein level (Figure 1E-G; supplemental Figure 6). On the contrary, *GAB1* was downmodulated in CLL cells exposed to microenvironmental signals, such as stromal cell coculture or BCR activation (see below). Altogether, this shows that *GAB1* expression is induced in CXCR4^{bright}CD5^{dim} CLL cells that have a relatively increased homing capacity.

GAB1 increases CLL cell migration

We hypothesized that *GAB1* upregulation in a CXCR4^{bright}CD5^{dim} subpopulation may contribute to the return of the CLL cells to the immune niches. To examine cell homing, we created a *GAB1* KO MEC1 cell line, using CRISPR-Cas9 technology (Figure 2A). The wild-type MEC1 (MEC1^{WT}) and MEC1^{GAB1-KO} cell lines were labeled with 2 cell-tracing dyes mixed in a 1:1 ratio, and injected into the tail vein of NSG mice (Figure 2B). The result revealed that MEC1^{GAB1-KO} cells were significantly retained in peripheral blood and were relatively less able (~30% reduction) to migrate to the spleen than were the control cells (Figure 2C). Bone marrow or liver infiltration by MEC1^{WT} vs MEC1^{GAB1-KO} did not differ, and the number of MEC1 cells infiltrating these organs was low (Figure 2C; immunohistology in supplemental Figure 7). Similarly, silencing *GAB1* by siRNA in CLL cells impaired their migration to the spleens of NSG mice (Figure 2D; immunohistology in supplemental Figure 8; silencing efficiency in supplemental Figure 9). B-cell migration is known to be regulated by several chemokines, such as SDF1 and CXCL13, produced by cells in immune niches.^{10,21-23} Indeed, we confirmed their presence in the bone marrow of patients with CLL, production by primary human bone marrow mesenchymal stromal cells, or the human stromal cell line HS5, and in the tissues of NSG mice (supplemental Figure 10). Treating MEC1^{WT} cells with SDF1, CXCL13, or CM^{hMSC} led to rapid (within minutes) *GAB1* phosphorylation at activating tyrosines Y307 and Y627 (Figure 2E), suggesting that *GAB1* is involved in response to multiple soluble factors. Indeed, in vitro MEC1^{GAB1-KO} cell migration toward SDF1, CXCL13, or CM^{hMSC} was impaired compared with MEC1^{WT} cells (Figure 2F). Similar data were obtained when *GAB1* was silenced by siRNA in MEC1 cells, and CM from HS5 cells was used as an attractant (supplemental Figure 11). The specific CXCR4 inhibitor plerixafor largely blocked the *GAB1* phosphorylation induced by recombinant SDF1 (CXCR4 ligand) but did not block the phosphorylation induced by the CM, also confirming that signaling by multiple soluble factors involves *GAB1* activation

(supplemental Figure 11). In primary CLL cells, silencing *GAB1* by small interfering RNA (siRNA) also reduced phosphorylation of kinases activated after cell stimulation with SDF1, CXCL13, or CM^{hMSC} (Figure 2G; supplemental Figure 12A) and impaired migration toward SDF1, CXCL13, or CM^{hMSC} in vitro, compared with cells transfected with the negative control (Figure 2H). Cell viability and CXCR4 levels were not affected by siRNA against *GAB1* (supplemental Figure 12B-C). Moreover, MEC1^{GAB1-KO} cells failed to properly polymerize F-actin (a process required for effective migration) after stimulation with CM from HS5 cells (supplemental Figure 13A). MEC1^{GAB1-KO} cell motility speed was significantly ($P < .001$) lower than MEC1^{WT} (supplemental Figure 13B). *GAB1* KO also impaired other migration-related characteristics of the cells, as assessed by holographic microscopy (supplemental Table 5). Altogether, findings suggest that *GAB1* sensitizes CLL cells to homing signals and influences the related cytoskeletal organization.

FoxO1 serves as a transcriptional activator of GAB1

To reveal potential *GAB1* expression regulators, we identified 20 annotated⁴⁷ transcription factors significantly differentially expressed in CXCR4/CD5 subpopulations (Figure 3A; supplemental Table 6). We further studied FoxO1 as a potential *GAB1* regulator because of the presence of multiple putative evolutionary conserved binding sites upstream from the *GAB1* promoter (supplemental Figures 14 and 15; supplemental Table 7). We also focused on FoxO1 because it has been identified as a regulator of T-cell homing⁴⁸ and normal B-cell migration within the germinal centers^{49,50} and is involved in malignant B-cell biology.^{51,52} Chromatin immunoprecipitation identified FoxO1 binding at 2 sites upstream from the *GAB1* transcription start sites (Figure 3B; supplemental Table 8). In addition, FoxO1 downmodulation by siRNA or treatment with FoxO1 inhibitor significantly repressed *GAB1* at the mRNA and protein levels (Figure 3C-D; supplemental Figure 16A-B). The existence of a FoxO1-*GAB1* axis is supported by a general positive correlation of *GAB1* and FoxO1 mRNA levels ($n = 42$) and protein levels ($n = 22$) in CLL cells (Figure 3E-F), and the higher levels of both molecules in CXCR4^{bright}CD5^{dim} cells (Figure 3G-I; supplemental Figure 6). Moreover, MEC1 cells without FoxO1 (MEC1^{FoxO1-KO}) had lower *GAB1* levels (supplemental Figure 16C) and had impaired migration to immune niches in NSG mice in comparison with MEC1^{WT} cells (supplemental Figure 17A). The migration of MEC1^{FoxO1-KO} cells or CLL cells with silenced FoxO1 toward SDF1, CXCL13, or CM^{hMSC} was also impaired in vitro (supplemental Figure 17B-C), which mimics the MEC1^{GAB1-KO} cell phenotype.

To understand the *GAB1* regulation via FoxO1, we further analyzed the mechanisms resulting in their relatively higher levels in CXCR4^{bright}CD5^{dim} CLL cells and lower levels in CXCR4^{dim}CD5^{bright} cells. In normal B cells, FoxO1 is known to be negatively regulated by its phosphorylation at position S256 in the context of BCR activation. This regulatory process is

Figure 1 (continued) bold were validated by real-time qPCR (see Figure 1D and supplemental Figure 5). Lists of top differentially expressed genes, differentially expressed migration-related genes, and Gene Ontology Term analysis are provided in supplemental Tables 2-4. For the volcano plot, sample correlation, and principal component analysis, see supplemental Figures 3 and 4. For patient characteristics, see supplemental Table 1. (D) Validation of *GAB1* mRNA expression (real-time qPCR) in primary CLL samples sorted according to CXCR4/CD5 expression ($n = 8$). (E) Intracellular flow cytometry staining for *GAB1* protein in CXCR4/CD5 subpopulations ($n = 22$). Normalized mean fluorescence intensity (MFI) was calculated as the ratio between the sample and control sample stained only with a secondary antibody. (F) Representative immunoblot from primary CLL samples sorted according to CXCR4/CD5 expression (purity >99.9%). (G) Densitometric quantification of relative *GAB1* protein level analyzed by immunoblot (for individual immunoblots, see Figure 3G and supplemental Figure 6A; for densitometric quantification of other proteins, see supplemental Figure 6B-C).

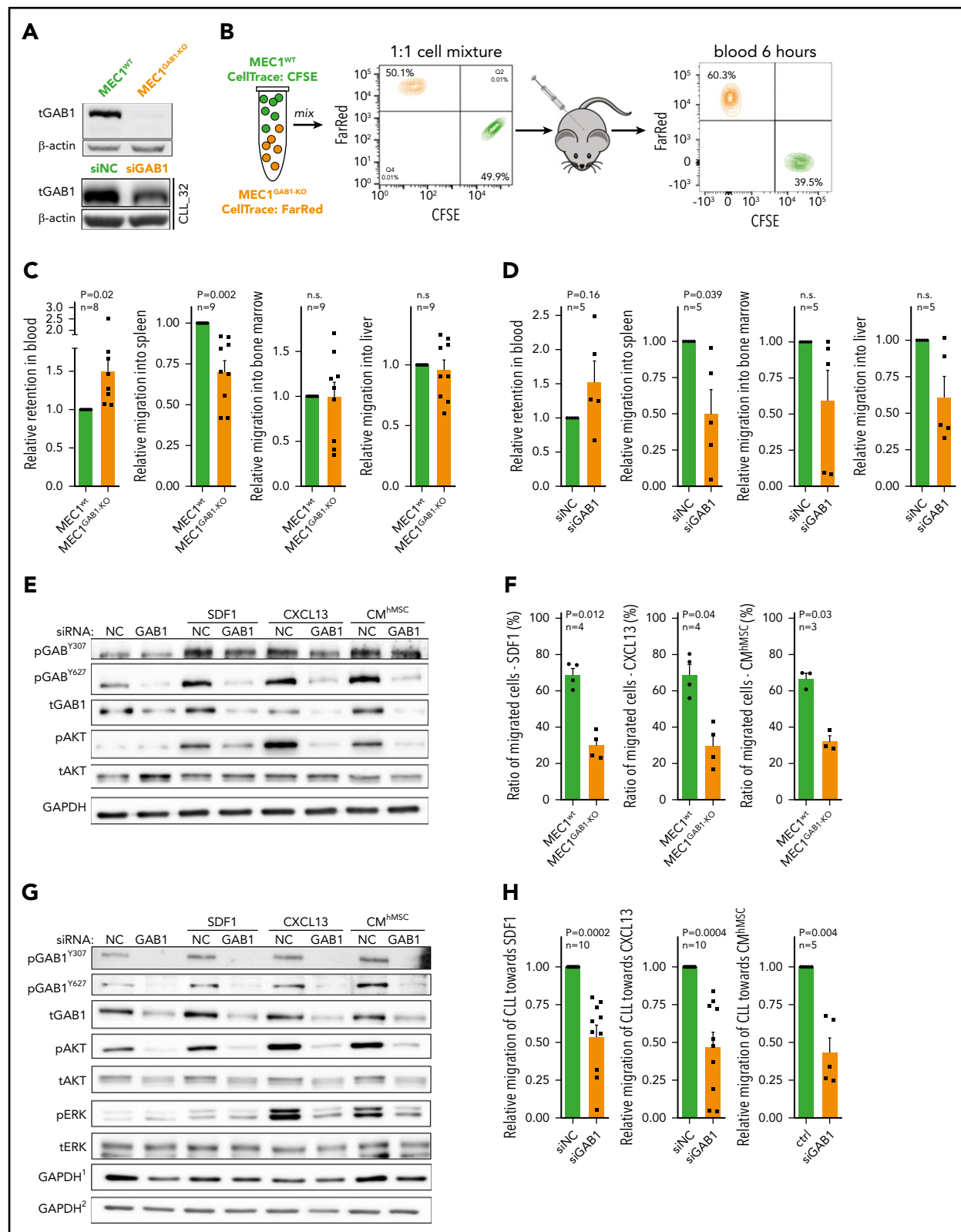


Figure 2. GAB1 increases CLL cell homing capacity. (A) Representative immunoblot for MEC1^{WT} and MEC1 cells genetically engineered for GAB1 KO, using CRISPR/Cas9 (MEC1^{GAB1-KO}) cells (top); primary CLL cells transfected with siRNA against GAB1 (siGAB1) or with the control (siNC; bottom), cells were harvested after 48 hours posttransfection. (B) In vivo competitive migration assay of MEC1^{WT} vs MEC1^{GAB1-KO}. Cells were stained with CFSE or FarRed CellTrace dye, and incubated overnight. The next day, an equal number of CFSE- and FarRed-stained cells (each 50 × 10⁶) were mixed in 1:1 ratio (validated by flow cytometry), and injected into the NSG mice via the tail vein. Organ infiltration was analyzed by gating on human CFSE/FarRed⁺ cells. The plot depicts the CFSE/FarRed⁺ MEC1 cells present in peripheral blood of NSG mice. (C) Mice (n = 9) from the experiment described in panel B were euthanized 6 hours after cell transplantation, and blood, spleen, bone marrow, and liver were analyzed by flow cytometry to detect the presence of viable CFSE⁺ or FarRed⁺ cells. The amount of MEC1 cells in each examined organ site is presented as relative migration of CFSE⁺ vs FarRed⁺ cells. (D) An in vivo

mediated by active AKT, leading to loss of FoxO1 interaction with DNA, translocation to cytoplasm, and subsequent degradation.^{53,54} Indeed, in CLL cells, strong AKT activation by BCR cross-linking or stromal cell coculture induced inhibitory FoxO1 phosphorylation with its subsequent degradation, and concurrently, GAB1 mRNA and protein levels were decreased (supplemental Figure 18). On the contrary, selective AKT inhibitor induced dephosphorylation of FoxO1, and upregulation of GAB1 mRNA and protein levels (~twofold increase; Figure 3J). The expression of other selected BCR/microenvironment-related proteins such as SYK, PLC γ , or CD79 α , was not affected by AKT inhibition (Figure 3J; supplemental Figure 19). These data suggest that AKT is a crucial, negative GAB1 regulator acting via reducing its transcriptional activator levels, FoxO1. On the other hand, relatively lower AKT activity in CXCR4^{bright}CD5^{dim} cells (supplemental Figure 21) allows for an increase in FoxO1 and induction of GAB1. Altogether, the data show that FoxO1 transcriptionally regulates GAB1, and both proteins are upregulated in the CXCR4^{bright}CD5^{dim} intraclonal cell subpopulation contributing to increased cell migration capacity.

GAB1 increases tonic AKT phosphorylation

We and others have shown that GAB1 is phosphorylated after BCR activation and serves as an important docking protein, allowing for propagation of the signal to PI3K and AKT.^{33,35} In line with this, we observed that silencing of GAB1 impaired AKT^{S473} phosphorylation after BCR cross-linking (Figure 4A). In this respect, we noted that modulation of the GAB1 level also affected basal AKT phosphorylation levels, irrespective of BCR cross-linking (Figure 4A), hinting at a possible role in tonic AKT activity. Indeed, GAB1 overexpression by a plasmid construct induced AKT phosphorylation (Figure 4B). Analogically, silencing endogenous GAB1 by siRNA reduced AKT phosphorylation (Figure 4C). The effect of GAB1 on AKT activation was validated on MEC1 and OSU-CLL cells with tetracycline-inducible shRNA against GAB1 (Figure 4D). GAB1 overexpression led to the induction of phosphorylation of the PI3K subunit p85, whereas GAB1 silencing by siRNA had the opposite effect (Figure 4B-C), which indicates that a GAB1-induced increase in p85 phosphorylation is a possible mechanism behind the observed increase in AKT phosphorylation. We have also tested MEC1^{GAB1-KO} vs MEC1^{WT} cell growth capacity after engraftment in mice for 3 weeks. Similar to the short-term migration assays, the MEC1^{WT} and MEC1^{GAB1-KO} cell lines (each traced with GFP/Azurite) were mixed in a 1:1 ratio and injected into the tail vein of NSG mice. This revealed approximately twice as many MEC1^{WT} cells as MEC1^{GAB1-KO} cells in the spleen, bone marrow, and liver, a difference slightly larger than in the migration experiments (Figure 4E; immunohistology in supplemental Figure 22), suggesting that GAB1 KO also negatively affects general cell fitness.

To further investigate the FoxO1-GAB1-pAKT axis, we analyzed MEC1^{FoxO1-KO} cells and observed diminished GAB1 levels and reduced AKT phosphorylation compared with MEC1^{WT} cells (Figure 4F). A similar phenotype was obtained after FoxO1 silencing by siRNA in primary CLL cells, after MEC1 cells, or CLL-cell treatment with FoxO1 inhibitor (Figure 4F; supplemental Figure 16). Altogether, this suggests that increased FoxO1/GAB1 levels in CXCR4^{bright}CD5^{dim} CLL cells contribute to tonic pAKT activity.

Ibrutinib leads to compensatory GAB1-mediated AKT activation

Ibrutinib therapy inhibits CLL cell homing,^{6,55} and we hypothesized that this could affect the FoxO1-GAB1-pAKT axis. Indeed, treating MEC1 cells with ibrutinib *in vitro* led to FoxO1 and GAB1 protein and mRNA accumulation (Figure 5A-C), albeit the kinetics of mRNA and protein induction were not identical, and the GAB1 protein levels were sustained for longer. Similarly, treating CLL patients with ibrutinib *in vivo* leads to increased FoxO1 and GAB1 protein and mRNA levels (Figure 5D-E). Notably, after an initial drop in AKT phosphorylation, we observed restoration of AKT^{S473} phosphorylation after several days of continuous MEC1 cell exposure to ibrutinib *in vitro* (Figure 5A) or after several days or weeks of ibrutinib therapy in CLL patients *in vivo* (Figure 5D; supplemental Figure 23). Idelalisib, as a single agent, also increased GAB1 and FoxO1 protein levels, but the induction of pAKT was less prominent (supplemental Figure 24). This result corresponds to the PI3K involvement in inducing AKT phosphorylation after GAB1 upregulation (Figures 4B-C and Figure 5D). Importantly, the AKT phosphorylation in response to ibrutinib did not lead to FoxO1 localization into the cytoplasm, and FoxO1 remained in the nucleus, enabling it to function as a transcription factor (supplemental Figure 25). This finding is in line with the data of another study suggesting that transport of FoxO1 from the nucleus upon AKT activation is impaired in B-cell lymphomas,⁵¹ and the FoxO1 activity is not mutually exclusive with low-level pAKT. Importantly, the GAB1 knockdown prevented the restoration of AKT phosphorylation in ibrutinib-treated cells (Figure 5F). Altogether, this result suggests that increasing GAB1 levels in CLL cells during ibrutinib therapy provides tonic pAKT activity and may compensate for the lack of AKT activation via BCR/microenvironmental interactions during ibrutinib therapy.

GAB1 inhibitors reduce migration and induce cell apoptosis alone and in combination with ibrutinib

We hypothesize that GAB1 upregulation during ibrutinib therapy acts as a mechanism to compensate for the lack of pAKT levels and that this effect can be targeted therapeutically. Recently, the first GAB1 inhibitors targeting the PH-domain

Figure 2 (continued) competitive migration assay of primary CLL cells ($n = 5$) transfected with siRNA against GAB1 (siGAB1) or negative control (siNC). After 48 hours, cells were stained with Sytox Blue and sorted for viable cells, and 10×10^6 of cells from each condition were processed as described in panel B. *In vivo* migration continued for 4 hours, and blood, spleen, bone marrow, and liver were analyzed by flow cytometry to detect the presence of viable CFSE⁺ or FarRed⁺ cells. A small aliquot of sorted cells was assayed by immunoblot to confirm GAB1 silencing (supplemental Figure 9). (E) Representative immunoblots of MEC1 cells transfected with siRNA against GAB1 (siGAB1) or the control (siNC). After 48 hours, the cells were treated for 10 minutes with SDF1 (250 ng/mL), CXCL13 (500 ng/mL), or CM (100%) produced by primary human mesenchymal stromal cells (CM^{hMSC}). (F) An *in vitro* competitive migration assay of MEC1^{WT} vs MEC1^{GAB1-KO} cells ($n = 4$) stained as described in panel B. Migration toward SDF1 (250 ng/mL), CXCL13 (500 ng/mL), and CM^{hMSC} continued for 6 hours. (G) Representative immunoblots of primary CLL cells treated for 5 minutes with SDF1 (100 ng/mL), CXCL13 (250 ng/mL), or CM^{hMSC}. The immunoblot contained 2 endogenous controls (GAPDH) marked by the index at top, because for technical reasons, pGAB1^{Y627}, tGAB1, tAKT, and tERK (loading control GAPDH¹) were analyzed on the first gel and the remaining proteins (loading control GAPDH²) on the second gel (identical protein loading and conditions). (H) *In vitro* competitive migration assay of primary CLL cells transfected by siRNA against GAB1 (siGAB1) or control siRNA (siNC). Cells were stained as described in panel B and loaded onto a Transwell 48 hours after transfection. Migration continued for 6 hours toward SDF1 (100 ng/mL), CXCL13 (250 ng/mL), or CM^{hMSC} ($n = 10$ for SDF1 and CXCL13; $n = 5$ for CM^{hMSC}).

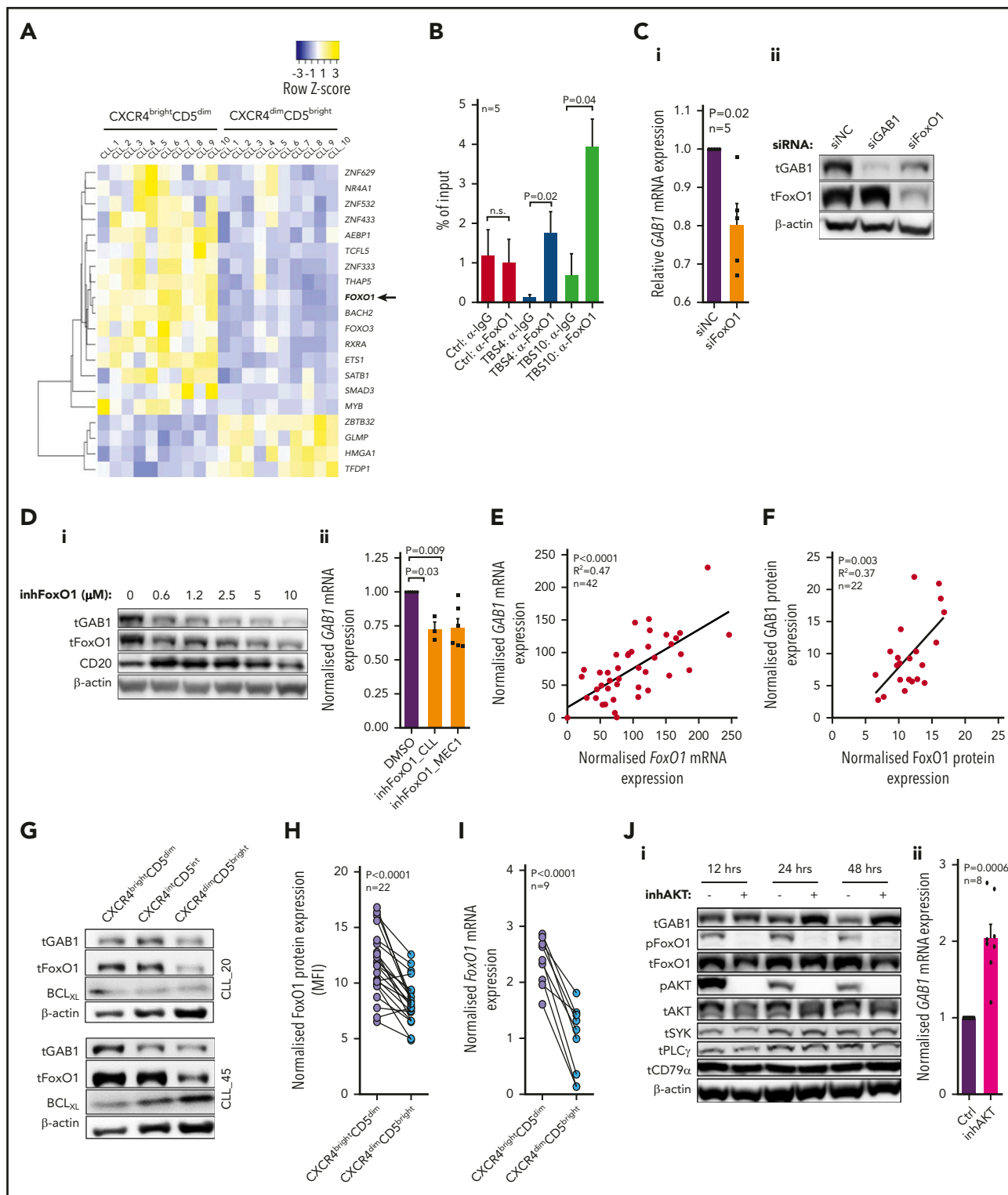


Figure 3. FoxO1 serves as a transcriptional activator of GAB1. (A) Heat map of differentially expressed transcription factors (RNAseq; Illumina) in 10 primary CLL samples sorted according to CXCR4/CD5 expression (fold change, ≥ 2 ; $P < .001$). The list of all known human transcriptional factors was obtained from Lambert et al.⁴⁷ (B) Chromatin immunoprecipitation analysis of FoxO1 binding upstream of the transcription start site of GAB1 gene ($n = 5$; binding sites are numbered as in supplemental Figure 14). The results for transcription binding sites (TBS) 4 and 10 are represented as the DNA input percentage, and error bars indicate the standard error of the mean. Control samples (Ctrl, α -IgG; Ctrl, α -FoxO1) were analyzed by primers that amplified a region in the GAB1 second intron, which does not contain any predicted binding site for FoxO1. Data for other predicted TBSs were not analyzed or were inconclusive (supplemental Table 8). (C) The effect of siRNA against FoxO1 (siFoxO1) on GAB1 mRNA expression in MEC1 cells compared with cell transfection with a control scrambled siRNA (siNC; 48 hours after transfection, $n = 5$). (Cii) Representative immunoblot of FoxO1 silencing by siRNA (siFoxO1) and its impact on GAB1 protein level in MEC1 cells (48 hours). The siRNA against GAB1 (siGAB1) serves as a positive control and control scrambled siRNA (siNC) as a negative control. (D) Representative immunoblot of GAB1 levels in primary CLL cells treated in vitro with various doses of FoxO1 inhibitor (inhFoxO1, 0.6–10 μ M; 48 hours). CD20 induction was used as a positive control, because FoxO1 acts as a transcriptional repressor of CD20.⁶⁸ (Dii) Statistical analysis of FoxO1 inhibitor's effect (inhFoxO1; 1 μ M) on

necessary for GAB1's docking function have been developed.⁴³ We observed that these compounds led to a dose-dependent inhibition of GAB1 phosphorylation, AKT inhibition (Figure 6A; supplemental Figure 26) and decreased CLL cell viability at higher micromolar concentrations after 48 hours (Figure 6B). To test GAB1 inhibitor specificity, MEC1^{WT} and MEC1^{GAB1-KO} cells were treated with various doses of the inhibitors. This demonstrated a greater induction of apoptosis and pERK/pAKT repression in MEC1^{WT} cells by GAB1 inhibitor than in MEC1^{GAB1-KO} cells (supplemental Figure 27). GAB1 inhibitors also inhibited CLL cell migration toward HS5 CM, SDF1, CXCL13, or CM^{hMSC} (Figure 6C-E). GAB1 inhibition impaired Ca²⁺ influx induced by SDF1 chemokine (Figure 6F) and BCR-induced (anti-IgM) AKT phosphorylation (Figure 6G). Importantly, the GAB1 inhibitor blocked the "compensatory" AKT phosphorylation induced by ibrutinib (Figure 6Hi) and increased the apoptosis of MEC1 cells exposed to ibrutinib for an extended time (Figure 6Hii). To test this in primary CLL cells undergoing spontaneous apoptosis within days, CLL cells were cultured in medium with interleukin 4 (IL4), which partially prevented their spontaneous apoptosis, and were simultaneously treated with ibrutinib or vehicle for 6 days, to allow time for FoxO1-GAB1 induction (supplemental Figure 28A). Subsequently, these cells were treated with GAB1 inhibitors (or vehicle) for an additional 48 hours. CLL cell killing was increased by GAB1 inhibitors combined with ibrutinib, compared with ibrutinib or GAB1 inhibitor alone (Figure 6I-J; *P* < .001). In culture conditions without IL4, allowing for only a short-term experiment, GAB1 inhibitors showed an ability to kill primary CLL cells within 48 hours, but this was not synergistic with ibrutinib (supplemental Figure 28B). This result was expected because in a brief time of ibrutinib exposure, the GAB1 inhibitor acts similarly to a BCR inhibitor in inhibiting tonic pAKT levels, and ibrutinib does not yet induce compensatory AKT phosphorylation via GAB1. Altogether, GAB1 inhibition can be used to inhibit CLL migration, basal or BCR-induced AKT activity, and adaptive AKT activation during ibrutinib therapy.

Discussion

In this study, FoxO1 transcriptionally induced GAB1 in CLL cells, which increased their homing capacity by influencing chemokine responsiveness and F-actin polymerization. FoxO1-induced GAB1 also increased the basal/tonic AKT activity, which is underscored in the context of ibrutinib therapy. Ibrutinib leads to FoxO1-GAB1 axis induction and adaptive restoration of AKT phosphorylation. We have demonstrated that GAB1 can be targeted therapeutically by novel GAB1 inhibitors, alone or in combination with BTK inhibition.

It has been hypothesized that CLL cell recirculation can be studied by analyzing CXCR4/CD5 intracolon CLL subpopulations, given that CXCR4^{bright}CD5^{dim} cells should be more

responsive to SDF1 because of higher CXCR4 levels.¹⁻⁷ Indeed, the CXCR4^{bright}CD5^{dim} subpopulation showed a significantly higher tendency to migrate into the spleen from the peripheral blood of NSG mice than did CXCR4^{dim}CD5^{bright} cells and, in vitro, toward chemokines. We have previously analyzed CXCR4^{dim}CD5^{bright} cells to understand the pathways activated in the microenvironment.^{2,3,7,56} In the current study, we hypothesized that analyzing the "resting" nonproliferative CXCR4^{bright}CD5^{dim} cells could help to understand the factors influencing their return to immune niches. RNA sequencing identified 147 genes differentially expressed in CXCR4/CD5 subpopulations and annotated in databases as migration related. We further demonstrated that one of the genes upregulated in CXCR4^{bright}CD5^{dim} cells (namely, GAB1) is a regulator of CLL cell migration. The genetic ablation of GAB1 in malignant MEC1 cells largely inhibited their migration capacity toward SDF1, CXCL13, or CM produced by bone marrow stromal cells, and a similar result was obtained with siRNA against GAB1 in primary CLL cells. This inhibitory action was confirmed by an in vivo competitive migration assay in mice, showing that GAB1 KO or GAB1 downmodulation by siRNA leads to impaired migration of malignant B cells to the spleen. This result is in line with data from muscle progenitor cells,³² where genetic ablation of either GAB1 or CXCR4 leads to highly similar (but not identical) defects in migration to specific tissue locations, suggesting that GAB1 has a nonredundant role in the CXCR4 pathway, but also in other cell-migration mechanisms. In line with this notion, we observed that cytokines produced by cells in immune niches, including CXCR4 ligand SDF1 and CXCR5 ligand CXCL13, induced GAB1 phosphorylation within minutes, and GAB1 positively regulated F-actin polymerization and cell migration in response to chemokines. This effect could be functionally linked to GAB1's acting as a cell-membrane-docking site for multiple proteins involved in migration such as PI3K,^{34,57} ERK,³⁶ PLCγ, Crk, or CrkL proteins.⁵⁸ Indeed, we have observed that GAB1 positively affects the activity of PI3K, a known regulator of F-actin polymerization, and CLL/T lymphocyte migration.^{57,59,60} Altogether, the data show that GAB1 accumulation in CLL cells increases their homing capacity by influencing responsiveness to chemokines and integrating signals, resulting in actin reorganization.

We further studied the mechanism that leads to GAB1 accumulation in CXCR4^{bright}CD5^{dim} cells, showing that the transcription factor FoxO1 accumulates in these cells and transcriptionally activates GAB1. It is known that AKT activity negatively regulates FoxO1 by mediating inhibitory FoxO1^{S256} phosphorylation, resulting in its subsequent translocation from the nucleus to the cytoplasm and degradation.^{61,62} Indeed, a strong AKT phosphorylation induction in CLL cells after adhesion or BCR activation leads to FoxO1 degradation and concurrent GAB1 downmodulation. We also tested whether *miR-150*, a

Figure 3 (continued) GAB1 mRNA expression in primary CLL (n = 3; 48 hours) and MEC1 (n = 6; 48 hours) cells. GAB1 mRNA expression in each control sample (dimethyl sulfoxide [DMSO] treated) was set at 1. (E) GAB1 and FoxO1 mRNA expression correlation analysis in primary CLL cells (n = 42; real-time qPCR). (F) Correlation of GAB1 and FoxO1 intracellular protein levels assessed by flow cytometry (n = 22). (G) Representative immunoblot from intracolon CLL cell subpopulations sorted according to CXCR4/CD5 expression (purity >99.9%). Subpopulation sorting was performed according to the gating strategy in Figure 1B. BCL_{XL} levels served as a positive control for microenvironmental interactions. For other immunoblots and densitometric quantification, see supplemental Figure 6. (H) FoxO1 expression in CXCR4/CD5 subpopulations was assessed by intracellular staining (n = 22; flow cytometry). As a control, an identical CXCR4/CD5-stained sample was incubated with a secondary antibody only. (I) Validation of FoxO1 mRNA expression in primary CLL samples. CLL cells were sorted according to CXCR4/CD5 expression and analyzed by real-time qPCR (n = 9). (Ji) Representative immunoblot from MEC1 cells treated with a selective inhibitor of AKT (inhAKT; MK-2206, 2HCl; 1 μM) or vehicle (DMSO). The primary antibody against pFoxO1 detected phosphorylation on S256, which led to inhibition of FoxO1 activity. (Jii) GAB1 mRNA expression in MEC1 cells treated with a selective AKT inhibitor (as in Ji) for 24 hours (n = 8).

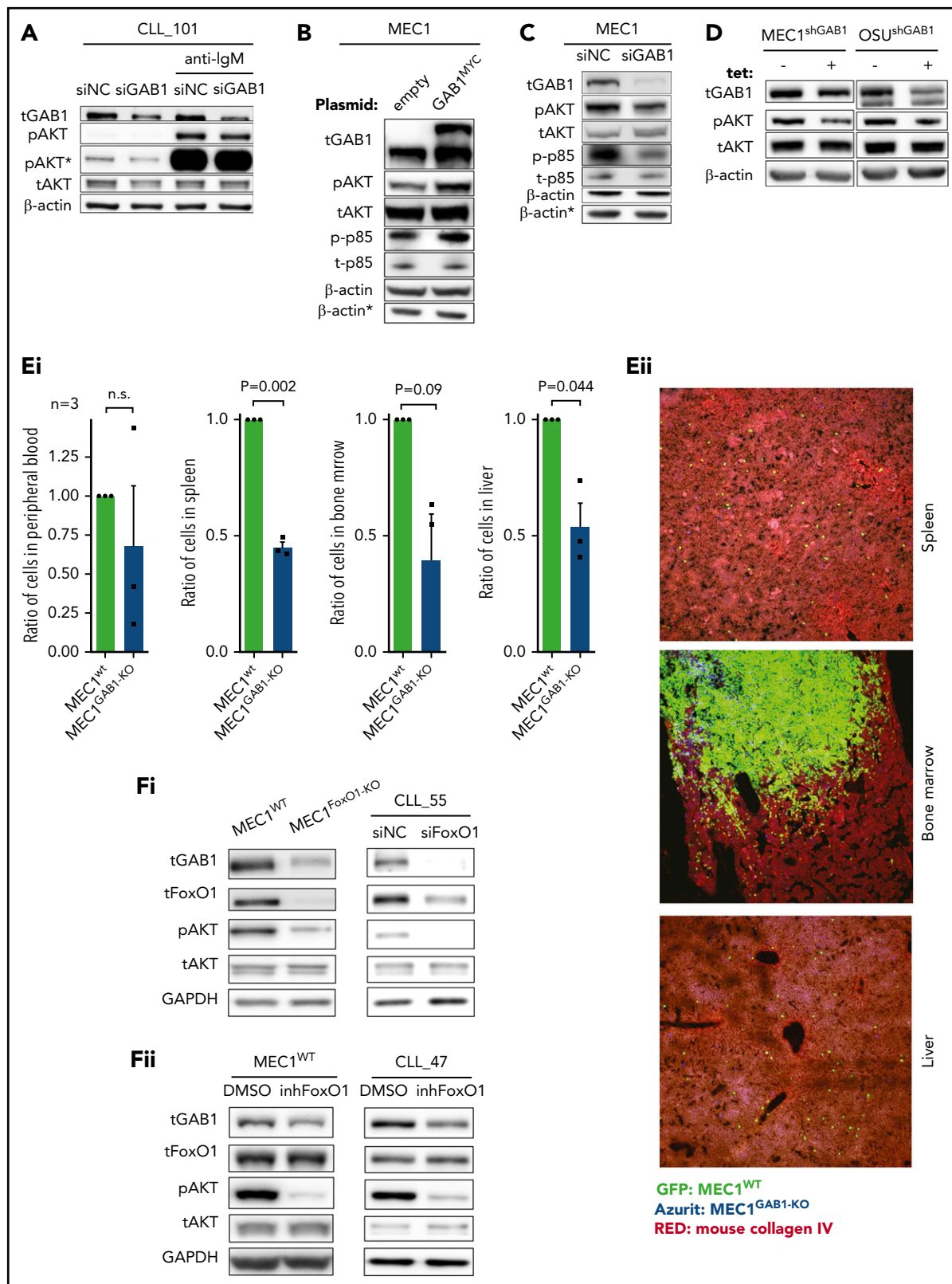


Figure 4. GAB1 increases tonic AKT phosphorylation. (A) Representative immunoblots of primary CLL cells transfected with siRNA against GAB1 (siGAB1) or the negative control (siNC). Cells were harvested after 48 hours, divided into equal portions, and exposed to vehicle (control samples) or anti-IgM for 5 minutes (10 µg/mL). *pAKT (S473) is the same immunoblot as pAKT but was developed for a prolonged time. (B) Representative immunoblot of MEC1 cells transfected with a plasmid for GAB1 protein overexpression tagged with MYC sequence (GAB1^{MYC}, top band) or with an empty vector (empty), and cultured for 48 hours. *β-Actin was the endogenous control for p-p85 and t-p85 assayed on a separate gel with identical loading. (C) Representative immunoblot of MEC1 cells transfected with siRNA against GAB1 (siGAB1) or the negative control (siNC) and harvested 48 hours after transfection. *β-Actin, as in panel B. (D) Representative immunoblot of MEC1 and OSU-CLL cells (CLL-derived cell line) with downmodulated GAB1 levels. MEC1 and OSU-CLL cells were infected with a lentiviral construct (permanent cell line) for tetracycline-inducible shRNA against GAB1 (shGAB1). shRNA expression was induced by

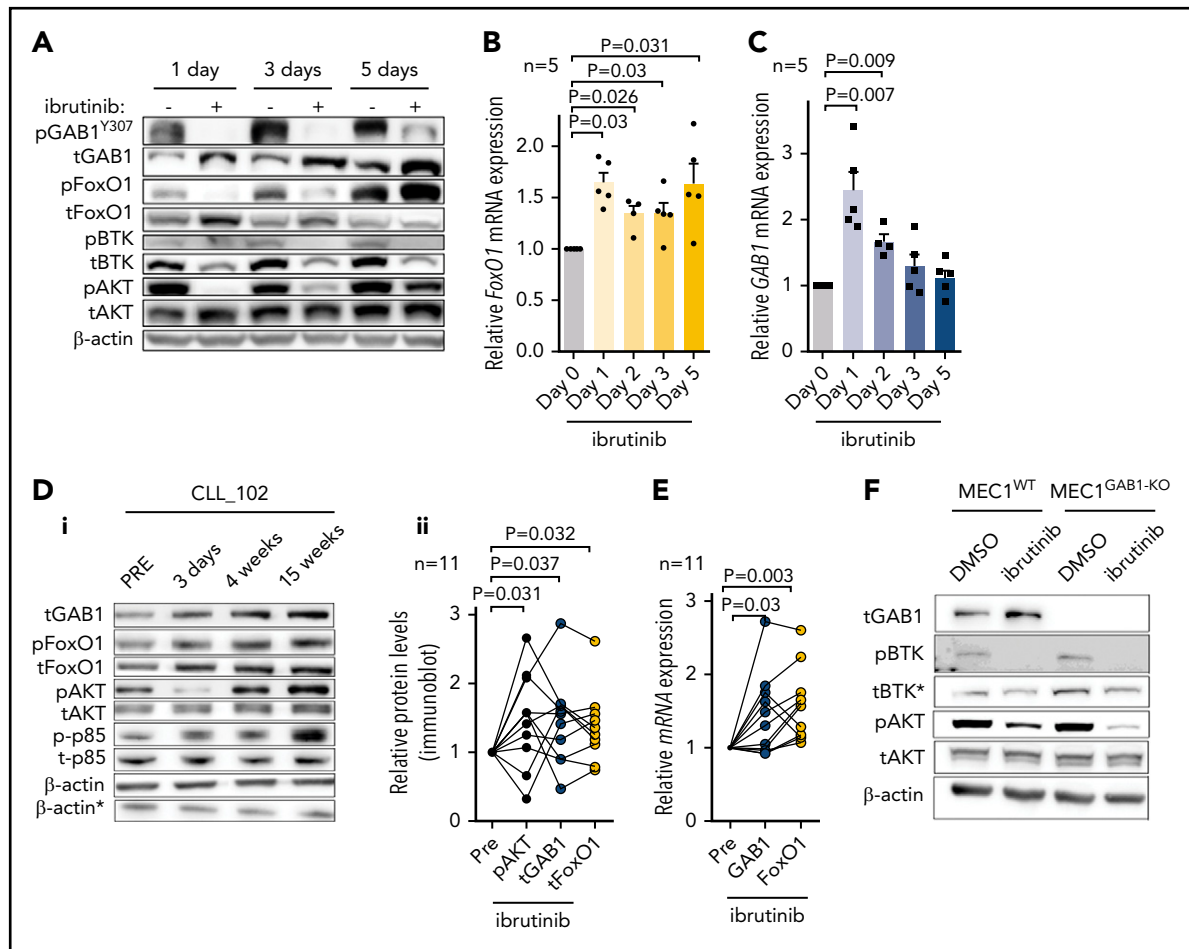


Figure 5. GAB1 levels and AKT phosphorylation is increased during ibrutinib therapy. (A) Representative immunoblot of MEC1 cells treated with ibrutinib (2 μ M) or vehicle (dimethyl sulfoxide [DMSO]) for the indicated time points. Ibrutinib and DMSO were freshly supplemented every 24 hours, and the cells were sampled at the indicated time points for immunoblot analysis. (B-C) FoxO1 and GAB1 mRNA expression in MEC1 cells treated with ibrutinib or DMSO, as described in panel A (n = 5). (Di) Representative immunoblot for primary CLL cells isolated from peripheral blood of patient before (Pre) and during ibrutinib therapy. For the patients' characteristics, see supplemental Table 1. * β -Actin was the endogenous control for p-p85 and t-p85, assayed on a separate gel with identical loading. (Dii) Statistical analysis of normalized densitometric data from immunoblots of CLL samples (n = 11) obtained from patients before (Pre) and during ibrutinib therapy. The data are relative to expression before ibrutinib administration (set at 1). For additional immunoblots, see supplemental Figure 23. (E) GAB1 and FoxO1 mRNA expression in primary samples isolated from patients with CLL before (Pre) and during ibrutinib therapy (n = 11; for patients' characteristics, see supplemental Table 1). The data are relative to expression before ibrutinib (set at 1). (F) Representative immunoblot of MEC1^{WT} and MEC1^{GAB1-KO} cells treated with ibrutinib (1 μ M) or vehicle (DMSO) for 7 days. *Decrease in total BTK protein level is a common effect of ibrutinib, as described previously.⁶⁹

known regulator of GAB1, affects GAB1 levels, but we did not find anticorrelation of the *miR-150* levels with GAB1 levels in CXCR4/CD5 subpopulations (supplemental Figure 29), suggesting that posttranscriptional regulation by *miR-150* is not relevant in this context, but more likely contributes to the interpatient differences, as proposed previously.³⁵ Altogether, the data indicate that FoxO1 upregulation in the CXCR4^{bright}CD5^{dim} CLL subpopulation is permitted by relatively low-level AKT activity (related to the absence of BCR/adhesion-mediated signals) and leads to transcriptional GAB1

activation and supports cell homing back to "signal-rich" niches. Similar to GAB1 KO, FoxO1's genetic ablation or its silencing by siRNA also resulted in decreased B-cell migration capacity. FoxO1's role in influencing genes involved in cell migration corresponds to observations describing its involvement in dendritic cell homing,⁶³ T-cell homing,⁴⁸ and normal B-cell migration within the germinal centers.^{49,50} In our data, several migration-related genes (such as *ITGB2*, *PIK3CA*, *NF1*, *ETS1*, *ST14*, *LRRK2*, and *APBB2*), which are putative FoxO1 targets, were differentially expressed in CXCR4/CD5 subpopulations.

Figure 4 (continued) adding tetracycline to medium (tet⁺) for 48 hours, and control cells were treated with vehicle (tet⁻). (Ei) Effect of GAB1 KO on MEC1 cell growth in vivo. MEC1^{WT} and MEC1^{GAB1-KO} cells were transduced with vectors expressing GFP or Azurite, mixed 1:1, and injected via tail vein into NSG mice (n = 3). Mice were euthanized 3 weeks after injection, and organs were analyzed by flow cytometry for the presence of MEC1^{WT} and MEC1^{GAB1-KO} cells labeled by GFP or Azurite in blood, spleen, bone marrow, and liver. We also performed a switch of fluorescent labels to exclude any potential effect of a specific fluorescent proteins on the cells (not shown). (Eii) Representative images from fluorescence microscopy of the spleen, bone marrow, and liver of NSG transplant recipients, as described in panel Ei (original magnification, $\times 20$). (Fi) Representative immunoblot for the effect of FoxO1 attenuation by KO in MEC1 cells (MEC1^{WT} vs MEC1^{FoxO1-KO} cells) or by siRNA in primary CLL cells. Primary CLL cells were transfected with siRNA against FoxO1 (siFoxO1) or with control (siNC) and analyzed by immunoblot 48 hours later. (Fii) Representative immunoblots for MEC1 cells and primary CLL cells treated with FoxO1 inhibitor (inhFoxO1; 0.5 μ M, 24 hours).

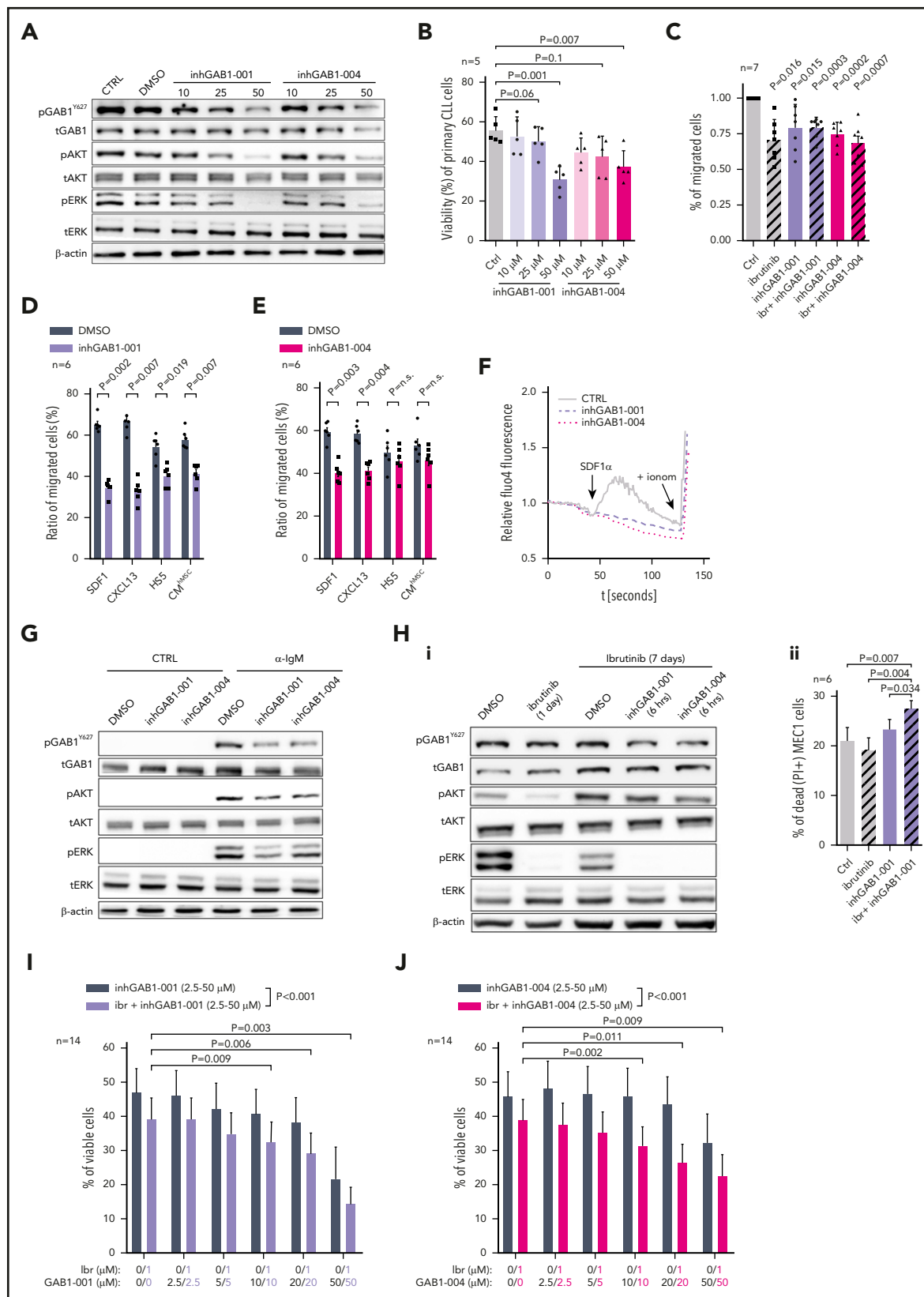


Figure 6. GAB1 inhibitors impair CLL migration and induce cell apoptosis, alone and in combination with ibrutinib. (A) Representative immunoblots from primary CLL cells treated for 6 hours with various doses (10, 25, and 50 μ M) of 2 GAB1 inhibitors or with the same volume of vehicle (dimethyl sulfoxide [DMSO]) or with culture medium only (CTRL). This short-term treatment (6 hours) did not significantly impact cell viability (supplemental Figure 26). (B) The effect of various doses of GAB1 inhibitors on CLL cell viability after 48 hours (n = 5). The primary CLL cells were treated as described in panel A. (C) GAB1 inhibitor effect on primary CLL cell migration (n = 7). Cells were treated with inhGAB1-001 (25 μ M) or inhGAB1-004 (25 μ M) or ibrutinib (1 μ M) or their combination for 12 hours. Cells were subsequently stained with CellTrace dye and loaded into a Transwell, and

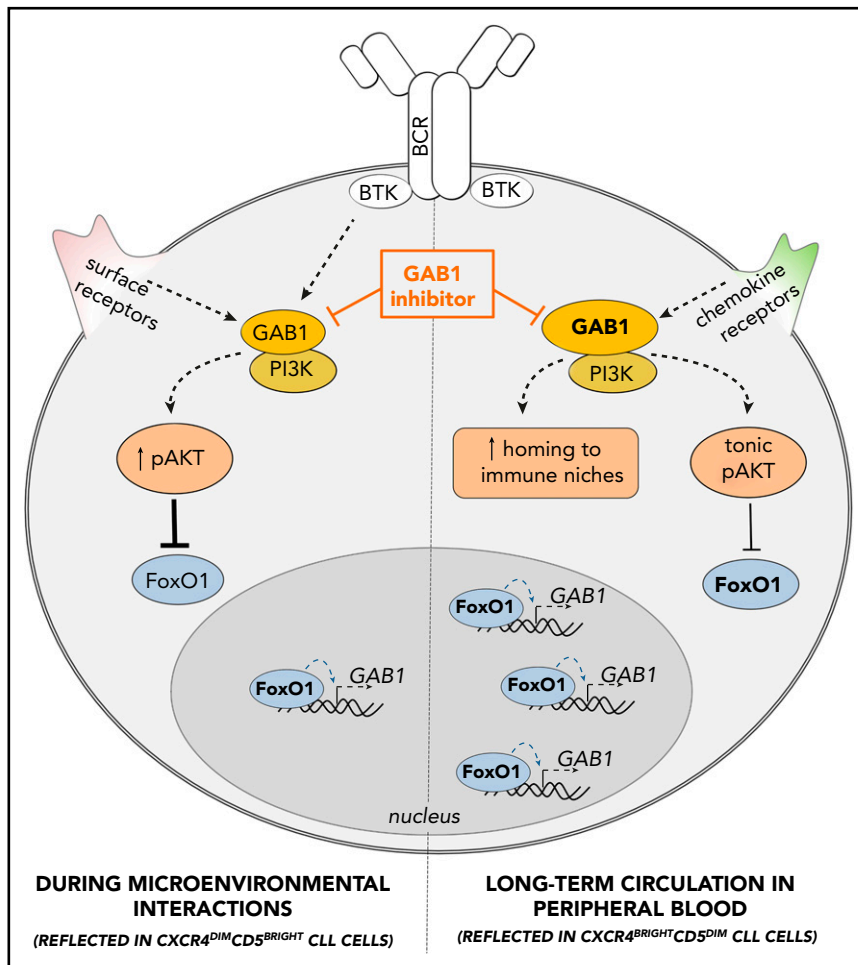


Figure 7. GAB1 regulation by FoxO1 and its role in CLL migration and tonic AKT activity: novel therapeutic target. GAB1 is upregulated by FoxO1 during the transition of intraclonal CLL cell subpopulation from CXCR4^{dim}CD5^{bright} into CXCR4^{bright}CD5^{dim} cells. This increases homing capacity of CXCR4^{bright}CD5^{dim} cells and also sustains prosurvival tonic AKT activity. During microenvironmental interactions (reflected in CXCR4^{dim}CD5^{bright} cells) GAB1 is involved in signaling from BCR and other cell surface receptors, and subsequent AKT activation represses GAB1 transcription via FoxO1. GAB1 inhibitors impair cell migration, inhibit tonic or BCR-induced AKT phosphorylation, and block compensatory AKT activity during ibrutinib therapy.

This finding suggests that FoxO1 contributes to regulating CLL migration by inducing GAB1 and other genes. Notably, the Fox family has been suggested to be one of the 3 transcription factors responsible for CLL cell identity.⁵² In addition, FoxO1 is known to be aberrantly transcriptionally active in B-cell lymphomas⁵¹ and to be activated by mutations in some cases.⁶⁴

We also identified another GAB1 function by describing its role in increasing the so-called tonic PI3K/AKT signaling by affecting the phosphorylation of the PI3K subunit p85 and AKT^{S473}. It has been shown that GAB1 contains multiple binding sites for SH2 domain-containing proteins³³ and has a crucial role in PI3K docking to the B-cell membrane connecting BCR-activation with

AKT phosphorylation.³⁴ However, the role of GAB1 in tonic AKT activity was not anticipated. It is known that normal B cells depend on constant tonic signaling to maintain their basal PI3K/AKT for their survival²⁵; however, the proteins that mediate this tonic signaling remain poorly understood. Constitutive PI3K activity was observed in most peripheral blood CLL samples.^{24,26-28} Our data show that upregulated GAB1 in CXCR4^{bright}CD5^{dim} CLL cells in peripheral blood contributes to their higher migration capacity and also supports tonic PI3K/AKT activity, which may help to explain the previously noted association of higher GAB1 levels with unfavorable prognosis in CLL.³⁵ The data suggest an interdependent balance of AKT activity and the FoxO1-GAB1 axis, which maintains tonic pAKT

Figure 6 (continued) migration continued for 4 hours toward 50% CM (produced by HS5 stroma cells). (D-E) Effect of a GAB1 inhibitors on primary CLL cell migration (n = 6). Cells were pretreated, stained, and loaded into a Transwell as described in panel C. Cell migration continued for 6 hours toward SDF1 (100 ng/mL), CXCL13 (250 ng/mL), or 50% CM produced by HS5 cells or 100% CM produced by primary human bone marrow MSCs (CM^{HMSC}). (F) Effect of GAB1 inhibitors (6 hours pretreatment; 25 μ M) on calcium influx in primary CLL cells after SDF1 stimulation (100 μ g/mL; arrow). Ionomycin (indicated by + ionomycin added every 48 hours) was added as a positive control. (G) Representative immunoblot from primary CLL cells treated with GAB1 inhibitors (inhGAB1-001 or inhGAB1-004; 25 μ M) and stimulated with anti-IgM. Primary CLL cells were treated as described in panel A, and cells were divided into equal portions and exposed to vehicle (CTRL) or anti-IgM (10 μ g/mL, 5 minutes). (Hi) Representative immunoblot of MEC1 cells pretreated with ibrutinib for 24 hours or 7 days and subsequently exposed to vehicle (DMSO) or GAB1 inhibitors for 6 hours (inhGAB1-001 or inhGAB1-004; 25 μ M). (Hii) Statistical analysis of MEC1 cells' viability (n = 6). Cells were pretreated for 7 days with ibrutinib (1 μ M; fresh ibrutinib added every 48 hours) or vehicle (DMSO; added every 48 hours), and subsequently exposed to GAB1 inhibitor (inhGAB1-001; 25 μ M) for another 48 hours. (I-J) Effect of GAB1 inhibitors GAB1-001 (I) or GAB1-004 (J), alone or in combination with ibrutinib on the viability of primary CLL cells (n = 14). CLL cells were cultured in medium with IL4 (5 ng/mL) and simultaneously treated with ibrutinib (ibr; 1 μ M) or vehicle (DMSO) for 6 days. Subsequently, the cells were treated with a combination of GAB1 inhibitors (2.5-50 μ M) and ibrutinib (1 μ M), vehicle (DMSO), ibrutinib alone (1 μ M), or GAB1 inhibitor alone for an additional 48 hours. The color of the numbers below the graphs indicates the combination of drugs for each column. Viability was assessed by DiOC6/PI staining. The statistical significance of differences between the escalating doses of GAB1 inhibitor and GAB1 inhibitor plus ibrutinib was evaluated by 2-way analysis of variance with the Tukey test (both $P < .001$); the combination for individual columns was evaluated by paired Student t test (all $P < .05$). Ctrl, negative control treated only with equal volume of DMSO.

above a certain minimal level in CLL cells that do not have access to microenvironmental interactions. This balance is important during ibrutinib therapy, because CLL cells induce the FoxO1-GAB1-pAKT axis and thus partially compensate for the lack of microenvironmental interactions that normally strongly induce pAKT levels. FoxO1's role in maintaining tonic pAKT^{S473} levels is surprising, given that FoxO1 activity was shown to be mutually exclusive with PI3K activity, and FoxO1 was shown to inhibit B-cell proliferation by inducing genes such as p27.⁶⁵ However, we have shown that moderate pAKT activity is not mutually exclusive with FoxO1 nuclear localization in CLL. This finding is in line with data from some B-cell lymphomas that tend to gain an oncogenic constitutive FoxO1 nuclear activity to circumvent negative feedback regulation by strong pAKT activity.⁵¹ It is plausible that in the nonproliferative CXCR4^{bright}CD5^{dim} subpopulation, FoxO1 induces genes involved in cell cycle arrest (indeed, these cells have higher p27 levels), but also upregulates GAB1 to increase homing of CLL cells and sustain their survival by maintaining tonic pAKT levels.

We have further shown that an inhibitor targeting GAB1 PH domain suppresses BCR-induced and tonic AKT phosphorylation during ibrutinib treatment, and GAB1 inhibition combined with ibrutinib increases cell killing in vitro. Moreover, GAB1 inhibitors also impaired ERK phosphorylation, which is in line with GAB1's role in ERK activation.^{36,66} In addition, GAB1 inhibitors impaired CLL cell migration to an extent similar to ibrutinib. GAB1 may represent a promising therapeutic target the inhibition of which may have a different toxicity profile compared with those of PI3K inhibitors or the CXCR4 agonists such as plerixafor. However, development of this inhibitory therapy will require more potent and selective GAB1 PH-domain inhibitor(s) because their effect on CLL migration and viability was observed only at higher concentrations. It is likely that the tested inhibitors also have off-target effects by inhibiting other PH-domain-containing proteins.⁴³ We have, for example, noted that these GAB1 inhibitors have an effect on cell migration similar to that of GAB1 silencing or KO, but a stronger impact on cell viability. This similarity could be caused by incomplete GAB1 silencing by siRNA or selection of single-cell clones tolerating GAB1 KO during its genomic editing, but could also be caused by the off-target effects of the inhibitors.

In summary, we have described a novel FoxO1/GAB1-dependent regulatory mechanism of CLL homing and maintenance mechanism for tonic AKT signaling (summarized in Figure 7). We have shown that during ibrutinib therapy, CLL cells sustain AKT activity via the FoxO1-GAB1 axis and this can be therapeutically targeted with novel GAB1 inhibitors.

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Authorship

Contribution: V.S. designed the study, performed experiments, analyzed the data, and wrote the article; L.O., L. Kostalova, K.M.L., E.V., J.V., and S.S. performed experiments; G.M.P., K.A.C., and V.B. performed the NGS experiments; J.O. analyzed the NGS data; D.Z. performed the holographic microscopy for the cell migration; T.L. and M.B. performed the cell sorting; M.K.P. and J.K. performed the fluorescence microscopy; K.L. and L. Kren performed the immunohistology; Z.T. and S.Z. provided and discussed the use of GAB1 inhibitors; S.P., Y.B., M.D., A.P., and J.M. provided samples and clinical data; M.M. designed the study, interpreted the data, and wrote the article; and all authors edited and approved the article for submission.

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Footnotes

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The results of the RNAseq analysis and data deposition may be found in a data supplement available with the online version of this article.

The online version of this article contains a data supplement.

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REFERENCES

- Calissano C, Damle RN, Marsilio S, et al. Intracanal complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. *Mol Med*. 2011;17(11-12):1374-1382.
- Pavlasova G, Borsky M, Seda V, et al. Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis. *Blood*. 2016;128(12):1609-1613.
- Pavlasova G, Borsky M, Svobodova V, et al. Rituximab primarily targets an intra-clonal BCR signaling proficient CLL subpopulation characterized by high CD20 levels. *Leukemia*. 2018;32(9):2028-2031.
- Herndon TM, Chen SS, Saba NS, et al. Direct in vivo evidence for increased proliferation of CLL cells in lymph nodes compared to bone marrow and peripheral blood. *Leukemia*. 2017;31(6):1340-1347.
- Bartholdy BA, Wang X, Yan XJ, et al. CLL intracanal fractions exhibit established and recently acquired patterns of DNA methylation. *Blood Adv*. 2020;4(5):893-905.
- Chen SS, Chang BY, Chang S, et al. BTK inhibition results in impaired CXCR4 chemokine receptor surface expression, signaling and function in chronic lymphocytic leukemia. *Leukemia*. 2016;30(4):833-843.

7. Sharma S, Pavlasova GM, Seda V, et al. miR-29 modulates CD40 signaling in chronic lymphocytic leukemia by targeting TRAF4: an axis affected by BCR inhibitors [published online ahead of print 10 November 2020]. *Blood*. doi:10.1182/blood.202005627.
8. Panayiotidis P, Jones D, Ganeshaguru K, Foroni L, Hoffbrand AV. Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro. *Br J Haematol*. 1996;92(1):97-103.
9. Herishanu Y, Pérez-Galán P, Liu D, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood*. 2011;117(2):563-574.
10. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood*. 2000;96(8):2655-2663.
11. Mraz M, Zent CS, Church AK, et al. Bone marrow stromal cells protect lymphoma B-cells from rituximab-induced apoptosis and targeting integrin α -4- β -1 (VLA-4) with natalizumab can overcome this resistance. *Br J Haematol*. 2011;155(1):53-64.
12. Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2013;369(1):32-42.
13. Furman RR, Sharman JP, Coutre SE, et al. Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2014;370(11):997-1007.
14. Ponader S, Chen SS, Buggy JJ, et al. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood*. 2012;119(5):1182-1189.
15. Arbonés ML, Ord DC, Ley K, et al. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity*. 1994;1(4):247-260.
16. Lafouresse F, Bellard E, Laurent C, et al. L-selectin controls trafficking of chronic lymphocytic leukemia cells in lymph node high endothelial venules in vivo. *Blood*. 2015;126(11):1336-1345.
17. Till KJ, Lin K, Zuzel M, Cawley JC. The chemokine receptor CCR7 and alpha4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes. *Blood*. 2002;99(8):2977-2984.
18. Tissino E, Benedetti D, Herman SEM, et al. Functional and clinical relevance of VLA-4 (CD49d/CD29) in ibrutinib-treated chronic lymphocytic leukemia. *J Exp Med*. 2018;215(2):681-697.
19. Ghobrial IM, Bone ND, Stenson MJ, et al. Expression of the chemokine receptors CXCR4 and CCR7 and disease progression in B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma. *Mayo Clin Proc*. 2004;79(3):318-325.
20. Burger JA, Burger M, Kipps TJ. Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. *Blood*. 1999;94(11):3658-3667.
21. Bürkle A, Niedermeier M, Schmitt-Gräff A, Wierda WG, Keating MJ, Burger JA. Overexpression of the CXCR5 chemokine receptor, and its ligand, CXCL13 in B-cell chronic lymphocytic leukemia. *Blood*. 2007;110(9):3316-3325.
22. Niemann CU, Herman SE, Maric I, et al. Disruption of in vivo Chronic Lymphocytic Leukemia Tumor-Microenvironment Interactions by Ibrutinib—Findings from an Investigator-Initiated Phase II Study. *Clin Cancer Res*. 2016;22(7):1572-1582.
23. Sivina M, Xiao L, Kim E, et al. CXCL13 plasma levels function as a biomarker for disease activity in patients with chronic lymphocytic leukemia [published online ahead of print 21 Oct 2020]. *Leukemia*. doi:10.1038/s41375-020-01063-7
24. Ringshausen I, Schneller F, Bogner C, et al. Constitutively activated phosphatidylinositol-3 kinase (PI-3K) is involved in the defect of apoptosis in B-CLL: association with protein kinase Cdelta. *Blood*. 2002;100(10):3741-3748.
25. Srinivasan L, Sasaki Y, Calado DP, et al. PI3 kinase signals BCR-dependent mature B cell survival. *Cell*. 2009;139(3):573-586.
26. Zhuang J, Hawkins SF, Glenn MA, et al. Akt is activated in chronic lymphocytic leukemia cells and delivers a pro-survival signal: the therapeutic potential of Akt inhibition. *Haematologica*. 2010;95(1):110-118.
27. Gobessi S, Laurenti L, Longo PG, et al. Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukemia B cells. *Leukemia*. 2009;23(4):686-697.
28. Herman SE, Sun X, McAuley EM, et al. Modeling tumor-host interactions of chronic lymphocytic leukemia in xenografted mice to study tumor biology and evaluate targeted therapy. *Leukemia*. 2013;27(12):2311-2321.
29. Lam KP, Kühn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell*. 1997;90(6):1073-1083.
30. Ondrisova L, Mraz M. Genetic and Non-Genetic Mechanisms of Resistance to BCR Signaling Inhibitors in B Cell Malignancies. *Front Oncol*. 2020;10:591577.
31. Sang H, Li T, Li H, Liu J. Down-regulation of Gab1 inhibits cell proliferation and migration in hilar cholangiocarcinoma. *PLoS One*. 2013;8(11):e81347.
32. Vasyutina E, Stebler J, Brand-Saberi B, Schulz S, Raz E, Birchmeier C. CXCR4 and Gab1 cooperate to control the development of migrating muscle progenitor cells. *Genes Dev*. 2005;19(18):2187-2198.
33. Ingham RJ, Holgado-Madruga M, Siu C, Wong AJ, Gold MR. The Gab1 protein is a docking site for multiple proteins involved in signaling by the B cell antigen receptor. *J Biol Chem*. 1998;273(46):30630-30637.
34. Ingham RJ, Santos L, Dang-Lawson M, et al. The Gab1 docking protein links the b cell antigen receptor to the phosphatidylinositol 3-kinase/Akt signaling pathway and to the SHP2 tyrosine phosphatase. *J Biol Chem*. 2001;276(15):12257-12265.
35. Mraz M, Chen L, Rassenti LZ, et al. miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1. *Blood*. 2014;124(1):84-95.
36. Meng S, Chen Z, Munoz-Antonia T, Wu J. Participation of both Gab1 and Gab2 in the activation of the ERK/MAPK pathway by epidermal growth factor. *Biochem J*. 2005;391(pt 1):143-151.
37. Cai T, Nishida K, Hirano T, Khavari PA. Gab1 and SHP-2 promote Ras/MAPK regulation of epidermal growth and differentiation. *J Cell Biol*. 2002;159(1):103-112.
38. Takahashi-Tezuka M, Yoshida Y, Fukada T, et al. Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK mitogen-activated protein kinase. *Mol Cell Biol*. 1998;18(7):4109-4117.
39. Holgado-Madruga M, Emlet DR, Moscatello DK, Godwin AK, Wong AJ. A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature*. 1996;379(6565):560-564.
40. Sakkab D, Lewitzky M, Posern G, et al. Signaling of hepatocyte growth factor/scatter factor (HGF) to the small GTPase Rap1 via the large docking protein Gab1 and the adapter protein CRKL. *J Biol Chem*. 2000;275(15):10772-10778.
41. Hertlein E, Beckwith KA, Lozanski G, et al. Characterization of a new chronic lymphocytic leukemia cell line for mechanistic in vitro and in vivo studies relevant to disease. *PLoS One*. 2013;8(10):e76607.
42. Shultz LD, Lyons BL, Burzenski LM, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol*. 2005;174(10):6477-6489.
43. Chen L, Du-Cuny L, Moses S, et al. Novel inhibitors induce large conformational changes of GAB1 pleckstrin homology domain and kill breast cancer cells. *PLoS Comput Biol*. 2015;11(1):e1004021.
44. Cerna K, Oppelt J, Chochola V, et al. MicroRNA miR-34a downregulates FOXP1 during DNA damage response to limit BCR signalling in chronic lymphocytic leukaemia B cells. *Leukemia*. 2019;33(2):403-414.
45. Chen L, Widhopf G, Huynh L, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. 2002;100(13):4609-4614.
46. Bagnara D, Kaufman MS, Calissano C, et al. A novel adoptive transfer model of chronic lymphocytic leukemia suggests a key role for T lymphocytes in the disease. *Blood*. 2011;117(20):5463-5472.
47. Lambert SA, Jolma A, Campitelli LF, et al. The Human Transcription Factors [published correction appears in *Cell*. 2018;175(2):598-599]. *Cell*. 2018;172(4):650-665.
48. Kerdiles YM, Beisner DR, Tinoco R, et al. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and

- interleukin 7 receptor. *Nat Immunol.* 2009; 10(2):176-184.
49. Dengler HS, Baracho GV, Omori SA, et al. Distinct functions for the transcription factor Foxo1 at various stages of B cell differentiation. *Nat Immunol.* 2008;9(12):1388-1398.
 50. Sander S, Chu VT, Yasuda T, et al. PI3 Kinase and FOXO1 Transcription Factor Activity Differentially Control B Cells in the Germinal Center Light and Dark Zones. *Immunity.* 2015; 43(6):1075-1086.
 51. Kabrani E, Chu VT, Tasouri E, et al. Nuclear FOXO1 promotes lymphomagenesis in germinal center B cells. *Blood.* 2018;132(25): 2670-2683.
 52. Beekman R, Chapaprieta V, Russiñol N, et al. The reference epigenome and regulatory chromatin landscape of chronic lymphocytic leukemia. *Nat Med.* 2018;24(6):868-880.
 53. Zhang X, Tang N, Hadden TJ, Rishi AK. Akt, FoxO and regulation of apoptosis. *Biochim Biophys Acta.* 2011;1813(11):1978-1986.
 54. Deng Y, Wang F, Hughes T, Yu J. FOXOs in cancer immunity: Knowns and unknowns. *Semin Cancer Biol.* 2018;50:53-64.
 55. Byrd JC, Brown JR, O'Brien S, et al; RESONATE Investigators. Ibrutinib versus ofatumumab in previously treated chronic lymphoid leukemia. *N Engl J Med.* 2014; 371(3):213-223.
 56. Cui B, Chen L, Zhang S, et al. MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia. *Blood.* 2014;124(4): 546-554.
 57. Ali AY, Wu X, Eissa N, et al. Distinct roles for phosphoinositide 3-kinases γ and δ in malignant B cell migration. *Leukemia.* 2018;32(9): 1958-1969.
 58. Roy NH, MacKay JL, Robertson TF, Hammer DA, Burkhardt JK. Crk adaptor proteins mediate actin-dependent T cell migration and mechanosensing induced by the integrin LFA-1. *Sci Signal.* 2018;11(560):eaat3178.
 59. Thomas MS, Mitchell JS, DeNucci CC, Martin AL, Shimizu Y. The p110gamma isoform of phosphatidylinositol 3-kinase regulates migration of effector CD4 T lymphocytes into peripheral inflammatory sites. *J Leukoc Biol.* 2008;84(3):814-823.
 60. Martin AL, Schwartz MD, Jameson SC, Shimizu Y. Selective regulation of CD8 effector T cell migration by the p110 gamma isoform of phosphatidylinositol 3-kinase. *J Immunol.* 2008;180(4):2081-2088.
 61. Zhang X, Gan L, Pan H, et al. Phosphorylation of serine 256 suppresses transactivation by FKHR (FOXO1) by multiple mechanisms. Direct and indirect effects on nuclear/cytoplasmic shuttling and DNA binding. *J Biol Chem.* 2002;277(47):45276-45284.
 62. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell.* 1999;96(6):857-868.
 63. Xiao W, Dong G, Pacios S, et al. FOXO1 deletion reduces dendritic cell function and enhances susceptibility to periodontitis. *Am J Pathol.* 2015;185(4):1085-1093.
 64. Trinh DL, Scott DW, Morin RD, et al. Analysis of FOXO1 mutations in diffuse large B-cell lymphoma. *Blood.* 2013;121(18):3666-3674.
 65. Szydłowski M, Kiliszek P, Sewastianik T, et al. FOXO1 activation is an effector of SYK and AKT inhibition in tonic BCR signal-dependent diffuse large B-cell lymphomas. *Blood.* 2016; 127(6):739-748.
 66. Aasrum M, Ødegård J, Sandnes D, Christoffersen T. The involvement of the docking protein Gab1 in mitogenic signalling induced by EGF and HGF in rat hepatocytes. *Biochim Biophys Acta.* 2013;1833(12): 3286-3294.
 67. Ashburner M, Ball CA, Blake JA, et al; The Gene Ontology Consortium. Gene ontology: tool for the unification of biology. *Nat Genet.* 2000;25(1):25-29.
 68. Pyrzynska B, Dwojak M, Zerrouqi A, et al. FOXO1 promotes resistance of non-Hodgkin lymphomas to anti-CD20-based therapy. *Oncol Immunology.* 2018;7(5):e1423183.
 69. Cervantes-Gomez F, Kumar Patel V, Bose P, Keating MJ, Gandhi V. Decrease in total protein level of Bruton's tyrosine kinase during ibrutinib therapy in chronic lymphocytic leukemia lymphocytes. *Leukemia.* 2016;30(8): 1803-1804.

Supplementary material 3

Supplemental Materials

Seda et al. FoxO1-GAB1 Axis Regulates Homing Capacity and Tonic AKT Activity in Chronic Lymphocytic Leukemia

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1) Supplemental Methods

CLL samples

Primary CLL samples were collected with written informed consent, and the Institutional Review Board approved the study. Peripheral blood samples were obtained from patients who did not receive any therapy for at least six months (except the ibrutinib/idelalisib treated patients). The samples from ibrutinib/idelalisib-treated patients were collected at a day before the drug administration and during therapy at the time points indicated in the figure legend. All primary CLL samples were purified by negative selection with RosetteSep Human B Cell Enrichment Cocktail (Stemcell Technologies) and RosetteSep Human CD3 Depletion Cocktail (Stemcell Technologies) and Ficoll-Paque PLUS (Sigma Aldrich) according to manufacturer's protocol and the final purity was >95% of CD5+19+ cells. In some cases PBMCs (Fig. 5E, samples CLL_113-118) were isolated and subsequently purified by magnetic separation using anti-CD3 MicroBeads (Miltenyi Biotec). Final purity was ≥95% of CD5+19+ cells in all cases (evaluated by flow cytometry). Purified primary CLL cells were cultivated in RPMI-1640

(Sigma-Aldrich) supplemented with 10% fetal bovine serum (Biosera) and 100 U·ml⁻¹ / 100 µg·ml⁻¹ of penicillin/streptomycin (Sigma Aldrich) and in 5% CO₂ at 37°C.

Cell culture

MEC1 cell line was obtained from the German Collection of Microorganisms and was cultivated in Iscove's Modified Dulbecco's Medium (IMDM, Biosera) supplemented with 10% fetal bovine serum (Biosera) and 100 U·ml⁻¹ / 100 µg·ml⁻¹ penicillin/streptomycin (Sigma Aldrich) and further cultivated in 5% CO₂ at 37°C. OSU-CLL cell line was a kind gift from Dr. Byrd (The Ohio State University) and was cultivated in RPMI-1640 media (Biosera) supplemented with 10% fetal bovine serum (Biosera) and 100 U·ml⁻¹ / 100 µg·ml⁻¹ penicillin/streptomycin (Sigma Aldrich) and further cultivated in 5% CO₂ at 37°C. HS5 cell line as well as HEK293-FT cell line were obtained from the German Collection of Microorganisms and both were cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Biosera) supplemented with 10% fetal bovine serum (Biosera) and 100 U·ml⁻¹ / 100 µg·ml⁻¹ penicillin/streptomycin (Sigma Aldrich) and further cultivated in 5% CO₂ at 37°C.

Gene expression analysis

Total RNA was isolated by TRIzol (Molecular Research Center) as described previously.¹ Protein-coding gene expression analyses was performed using TaqMan Gene Expression Assays (Thermo Fisher Scientific) and normalized to an endogenous control (*GAPDH*, *HPRT*), as described previously.² Probes used in this study: *GAB1* (Hs00157646_m1), *FoxO1* (Hs00231106_m1), *NCK2* (Hs02561903_s1), *MYLK* (Hs00364926_m1), *GPR183* (Hs00270639_s1), *FLNA* (Hs00924645_m1), *SERPINF1* (Hs01106937_m1), *SEMA4F* (Hs01586592_m1), *HPRT1* (Hs02800695_m1), *GAPDH* (4333764F), mouse *CXCL12* (Mm00445553_m1), mouse *CXCL13* (Mm00444534_m1), mouse *HPRT* (Mm03024075_m1).

mRNA-seq and data analysis

The sequencing of poly(A) transcriptome of CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} CLL intraclonal subpopulations was performed as described previously.³ Briefly, libraries were prepared using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) according to the manufacturer instructions. The final libraries were diluted to the desired concentration of 2 pM and were used to prepare a paired-end flow cell using a cBot System (Illumina) and then sequenced with Illumina NextSeq 500/550 High Output v2.5 kit (Illumina) generating 75-bp paired-end sequences.

The quality of the sequencing data was checked using the FastQC⁴ (v0.11.3) and Kraken package⁵ (v13-274). The presence of adapters was scanned using Minion and Swan (Kraken package,⁵ v13-274). Pre-processing of the raw reads was done using Trimmomatic⁶

(v0.36) in the following steps (i) *N* and very low-quality bases (Phred < 3) from both 5' and 3' ends were removed, (ii) Poly(A) and poly(T) tails were removed, (iii) Sequencing adapters were removed, (iv) Reads were trimmed to a maximal length of 75 bp, (v) Low quality ends with average Phred score < 5 of 4 consecutive bases were trimmed using the sliding window approach, (vi) Reads shorter than 15 bp and without a proper pairing after the pre-processing were removed. The pre-processed reads were then mapped to the *Homo sapiens* reference genome (Ensembl⁷ release 84, primary assembly) together with corresponding Ensembl genome annotation (Ensembl release 84) using STAR⁸ (v2.5.2a) in 2-pass mapping approach. Exon-mapped reads were summarized to genes using STAR (a similar approach to the HTSeq-count⁹ 'union') . Only uniquely mapped reads were used. Strandedness of the sequencing protocol was taken into account and 'reverse' counts were used. Differential gene expression was analyzed using DESeq2¹⁰ (v1.13.8) Bioconductor¹¹ package for genes with a base mean count >200 per million. P-values were adjusted for multiple testing using the Benjamini-Hochberg method. The fold change was calculated as CXCR4^{dim}CD5^{bright}/CXCR4^{bright}CD5^{dim} cells. The heatmap visualization was created using the Heatmapper tool.¹¹

siRNA transfection

MEC1 cells or freshly isolated primary CLL cells (5x10⁶) were electroporated (Neon Transfection System, Thermo Fisher Scientific (program for MEC1 cells: 1500 V / 20 ms / 2 pulses; program for primary CLL cells: 1900V / 30ms / 1 puls) with a short interfering RNA (siRNA) against *GAB1*, or against *FoxO1* (both On-Target Plus Smartpool siRNAs, 1000 nM, Dharmacon), or by On-Target Plus Smartpool Negative Control (1000 nM, Dharmacon). The cells were harvested 48 hrs after transfection for further analyses.

Flow cytometry and cell sorting

The primary CLL cells or cell lines were labeled with one or combination of these antibodies: CD5 (FITC, UCHT2; Sony), CD184 [CXCR4] (PE, 12G5; Sony), CD19 (PECy7, JC-119, Beckman Coulter), CD45 (BV510, HI30; SONY), CD105 (A647, 43A3; SONY) and labeled with SYTOX-BLUE (Invitrogen) for viability. For intracellular staining, the cells were further fixed using 4% paraformaldehyde (PFA) and 50% methanol and stained with primary rabbit antibodies against *GAB1* (#3232, 1:100; Cell Signaling) or *FoxO1* (#2880, 1:100; Cell Signaling) and secondary anti-rabbit antibody conjugated with Alexa-647 (#4414, 1:500; Cell Signaling). Data were collected from at least 20,000 events using BD FACSVerse and analyzed using FlowJo software (version 10). Normalised expression of intracellular proteins was calculated as MFI of the protein of interest relative to MFI for secondary antibody only.

Cell viability was investigated by DiOC6 (3,3'-dihexyloxacarbocyanine iodide) together with PI (propidium iodide) staining (Thermo Fisher Scientific). All measurements were performed on FACS Verse (BD Biosciences-US) and cell sorting on BD FACS Aria Fusion (BD Biosciences-US).

Calcium flux assay

The primary CLL cells ($1 \times 10^6/\text{ml}$) were incubated in Hanks Balanced Salt Solution (HBSS) containing 2 mM Fluo-4AM (Molecular Probes) for 30 min at 37°C . We established a baseline fluorescence threshold for each sample (by acquiring samples for 60 seconds without any stimulus). We then calculated the peak Fluo-4AM signal and fluorescence intensity above baseline Fluo-4AM signal following stimulation.

HS5 coculture and conditioned media

HS5 cells were seeded at 2×10^6 in 75 cm^2 culture flasks in DMEM with 10% FBS. The next day, DMEM medium was replaced by IMDM or RPMI media containing 10% FBS and $2.5 \times 10^6/\text{ml}$ of MEC1 cells (in IMDM) or $5 \times 10^6/\text{ml}$ primary CLL cells (in RPMI). The HS5 coculture was allowed for the specified time and then harvested and stained with Sytox blue and CD105 antibody. The purity of B cells after sorting was validated by anti-CD19 antibody. The conditioned media was harvested after cultivating confluent HS5 cells in DMEM with 10% FBS for 48 hrs.

To prepare conditioned media, 3.5×10^6 of HS5 cells was seeded in T75 cultivation flask (TPP) in 12 ml of DMEM media. The next day, original media was discarded, and the cells attached to the bottom of the flask were washed once with 10 ml of PBS. Subsequently, 25 ml of RPMI media was added to the attached HS5 cells. The conditioned media was collected after 48 hrs, separated from cell-debris by 10 minutes centrifugation at 2000 RCF and diluted with fresh RPMI media containing 10% FBS and $100 \text{ U} \cdot \text{ml}^{-1}$ / $100 \text{ } \mu\text{g} \cdot \text{ml}^{-1}$ penicillin/streptomycin to the final concentration of 50% conditioned media. This media was immediately used for experiments.

Primary human mesenchymal stromal cells and conditioned media

Human bone marrow was obtained from a healthy male donor with a written consent. Cells were resuspended in osmolysis buffer and spun ($300g$ for 10 min). This step was repeated till all erythrocytes were removed. Cells were subsequently washed with PBS and plated in alphaMEM media (Sigma-Aldrich) containing 10% FBS and $100 \text{ U} \cdot \text{ml}^{-1}$ / $100 \text{ } \mu\text{g} \cdot \text{ml}^{-1}$ of penicillin/streptomycin (Sigma-Aldrich). Cells were cultured at 37°C for 48 hrs and subsequently washed (3x) with PBS in order to remove non-adherent cells. Adherent cells were resupplemented in fresh media, and incubated for another 48 hrs. For all experiments

only cells within 2nd-6th passage were used. To prepare conditioned media, 4×10^6 of the primary human bone marrow mesenchymal stromal cells were seeded in T75 cultivation flask (TPP) in 12 ml of full alpha-MEM media. The next day, original media was discarded, and the cells attached to the bottom of the flask were washed once with 10 ml of PBS and supplemented with fresh 12 ml of alpha-MEM media. The conditioned media was collected after 72 hrs, separated from cell-debris by 10 min centrifugation at 2000 RCF and immediately used for experiment or directly frozen at -80°C .

Chemokine concentration measurements by ELISA

The levels of chemokines SDF1 (CXCL12) and CXCL13 (BLC/BCA-1) were measured in conditioned media produced by HS5 or by primary human bone marrow mesenchymal stromal cells (for conditioned media preparation see above), or in serum from CLL peripheral blood or paired serum sample from sternal puncture in CLL patients. All measurements were performed according to manufacturer's protocol (Quantikine, R&D Systems, Minneapolis, MN). The absorbance at 450 and 570 nm was measured by TECAN SPARK.

***In vitro* migration assay and F-actin polymerization**

The competitive migration assay was performed by staining of MEC1 cells or primary CLL cells with CFSE or FarRed CellTrace dye according to manufacturer protocol and incubated overnight. The next day, the stained control cells and GAB1-KO cells were analyzed for viability (stained with Sytox Blue; Thermo Fisher Scientific). The paired samples with low viability (<80%) were excluded from further experiments as well as samples where the difference in viability between paired samples was higher than 5%. To compare the migration, the stained cells were mixed in ratio 1:1 and loaded to transwell inserts with 5 μm pores (Falcon) according to manufacturer protocol. The migration towards SDF1 (100 ng/ml for CLL cells or 250 ng/ml for MEC1 cells), CXCL13 (250 ng/ml for CLL cells or 500 ng/ml for MEC1 cells), 100% conditioned media produced by primary human bone marrow mesenchymal stromal cells, or 50% conditioned media produced by HS5 cells was allowed for the indicated time and subsequently analyzed by flow cytometry. The F-actin polymerization was performed by staining with Phalloidin-iFluor488 (Abcam) and visualized by EVOS Cell Imaging Systems (Thermo Fisher Scientific).

Plasmid transfections, CRISPR/Cas9, transduction of shRNAs

The GAB1^{MYC} plasmid was kindly provided by Armelle Yart¹² and empty control plasmid was a gift from Adam Antebi (Addgene plasmid #52535). pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid #48138;).

The gRNA sequences targeting the second exon of the *GAB1* gene or first exon of *FoxO1* gene were designed in Benchling (Biology Software, 2019) online tool. The sequence 5'-CTACTTGGTAGCAGACAGCG-3' for GAB1-gRNA or 5'-ATCCACATCGAGGCTCCTCG-3' for FoxO1-gRNA was cloned into pSpCas9(BB)-2A-GFP vector (#48138, Addgene). To obtain the mutant variant, the MEC1 wild-type cells (MEC1^{WT}) cells were electroporated as described above for siRNAs. After 48 hrs viable cells were sorted according to GFP-positivity, expanded, and validated for absence of GAB1 or FoxO1 .

To perform inducible shRNA expression, the sequence 5'-CCGGAGTTAACACACTCGTAGTATTCTCGAGAATACTACGAGTGTGTTAACTTTTTTG-3' was purchased from Sigma Aldrich and subsequently cloned into the empty plasmid Tet-pLKO-puro for shRNA expression.¹³ This plasmid was purchased from Addgene (Addgene plasmid #21915) as well as the envelope plasmid pCMV-VSV-G (Addgene plasmid #8454). The packaging plasmid dR8.91 was kindly provided by M. Smida (Masaryk University). Virus particles were produced in HEK-293-FT (obtained from ATCC) after plasmids transfection by Dharmacon (Horizon) and added to desired B cell lines. The transduced cells were subsequently selected by puromycin (3 µg/ml, Sigma Aldrich) for 5 days. The expression of shRNA in B cell lines was induced by adding tetracycline (1 µg/ml; Sigma Aldrich) into media (tet+) for 48 hrs, and control cells were treated with vehicle (tet-).

For *in vivo* growth assay MEC1^{WT} and MEC1^{GAB1-KO} cell lines were transduced by GFP or Azurit encoding vectors (both obtained from Addgene; #36083 and #36086, respectively) as described above. Cells were subsequently sorted for GFP or Azurit positive cells.

Immunoblotting

Cells were lysed in lysis buffer (1% SDS, 50 mM TRIS-HC pH 6.8, 10% glycerol) with phosphatase and protease inhibitors (Sigma Aldrich) and protein concentration was determined using DC Protein Assay (BioRad). Equal amounts of protein were separated by SDS-PAGE and transferred to the PVDF membrane (0.45 µm pore size, Millipore). The membranes were incubated with the Cell Signaling antibodies specific for the following immobilized proteins: GAB1 (#3232, 1:2000), pGAB1^{Y307} (#3234, 1:2000), FoxO1 (#2880, 1:1000), pFoxO1 (#9461, 1:2000), AKT (#2920, 1:3000), pAKT (#4060, 1:3000), BCL_{XL} (#2764, 1:2000), SYK (#12358, 1:2000), PLCγ (#3872, 1:2000), CD79α (#3351, 1:2000), ERK (#4696, 1:3000) pERK (#4377, 1:3000), B-actin (#4970, 1:5000), GAPDH (#2118, 1:4000), or with antibodies obtained from Abcam - pGAB1^{Y627} (ab131458, 1:2000), anti-CD20 antibody (Ab78237, 1:2000). Secondary horse-radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibodies (both Cell Signaling) were used to detect primary antibodies. Immunocomplexes were detected using ECL (BioRad), and the chemiluminescent signal was digitally detected with UVItec Alliance 4.7 (UVItec).

ChIP

Chromatine immunoprecipitation was performed as previously described by Carey *et al.*¹⁴ Briefly, 20x10⁶ MEC1 cells were fixed in 1% paraformaldehyde. The fixation was quenched by glycine after 10 min. After washing with cold PBS, the fixed cells were lysed in 10ml of ChIP-lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40) and incubated on ice for another 10 min. The cell nuclei were pelleted by centrifugation and subsequently lysed in 1ml of nucleolysis buffer (50 mM TRIS-Cl pH 8.0, 10 mM EDTA, 1% SDS) containing protease inhibitors. Sonication was performed by the Covaris S220 sonicator in original glass tubes. The instrument was set according to manufacturer protocol (PIP:140, DF: 5, CBP: 200, treatment time: 20 min, temperature min/max: 3/9°C). The cell debris was subsequently discarded by centrifugation and DNA concentration was measured by Nanodrop 2000 (Thermo Fisher Scientific). One-hundred µg of chromatin was aliquoted per antibody and diluted with dilution buffer (16.7 mM TRIS-Cl pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% TRITON X-100, containing protease inhibitors) to a final volume of 300 µl. Chromatin was precleared with 50 µl of protein A/G ChIP-grade magnetic beads (Thermo Fisher Scientific). The magnetic beads were discarded after 2 hrs of incubation, and 10 µl of anti-FoxO1A antibody (ab39670; ChIP Grade, Abcam) or 10 µl of control rabbit IgG (#2729; Cell Signaling) was added to chromatin. The samples were rotated and incubated at 4°C overnight. The next day, the immunocomplexes were pulled down by 50 µl of A/G ChIP-grade magnetic beads (Thermo Fisher Scientific) blocked by salmon sperm and BSA. The beads were washed three times in 1ml of High-salt wash buffer (50 mM HEPES pH 7.9, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% TRITON X-100, 0.1% deoxycholate). The elution was performed in 300 µl of Elution buffer (50 mM TRIS-Cl, 10 mM EDTA, 1% SDS) supplemented with 20 µg of proteinase K. Samples were incubated for 2 hrs at 55°C and then overnight at 65°C to reverse crosslinking. The DNA was purified by QIAquick PCR Purification Kit (Qiagen). The qRT-PCR was performed by using Luna Universal qPCR Master Mix (New England Biolabs), and three different sets of primers (see supplemental Table 5), which were designed using Primer3 online tool. The prediction of possible FoxO1-binding sites in the 3.5 kb promotor region of the *GAB1* gene was performed with ContraV3 online tool. The non-specific primer-set was designed into the 2nd intron of *GAB1* where there is no predicted binding site for FoxO1 (forward: 5'-CAGTCAGTCTAGTAGGAGGGACAA-3', reverse: 5'-CTGAAAAGATCACTGCTGCTGCTA-3'). Two sets of primer were designed for the promotor region with predicted binding sites for FoxO1 (TBS no. 4: forward 5'-TTTGGGGCTTTTTCACAAAT-3', reverse 5'-TGCCTACTTCCTTGTGTGTTTC-3'; TBS no.10: forward 5'-ATGCTGCCTATGGGAGAAGA-3', reverse 5'-AAACCAAAGCACTTCTTTTAAAGT-3'). The results were calculated as % of input.

Confocal microscopy

The MEC1 cells were plated on poly-L-lysine coated (0.01%) glass for 30 min. Glass-attached MEC1 cells or primary CLL cells in suspension were subsequently fixed with 1% paraformaldehyde for 15 min/room temperature and washed (2x) with TBS-T. The 1 hr long blocking step in 5% BSA was followed by overnight incubation in primary antibody against FoxO1 (#2880, 1:100; Cell Signaling). The next day cells were washed (3x) in TBS-T and incubated in a mixture of secondary antibody (anti-Rabbit, Alexa-647 #4414, 1:250; Cell Signaling) and Phalloidin-iFLuor488 (1:1000; Abcam) for 90 min/room temperature (in dark). Subsequently, three wash steps with TBS-T were followed by staining with DAPI (1:1000; 15 min; Abcam), and cells were mounted to glass slides using Vectashield (Vector laboratories), and visualized on confocal microscope Leica DM5500 Q with TCS SPE confocal laser scanning system.

Cell motility by holographic microscopy

Cell motility speed and other measurements were performed as described previously.¹⁵⁻¹⁷ Briefly, tissue culture treated μ -Slide (Ibidi) with 0.8 mm channel height was first filled with the culture medium to coat the substrate with serum proteins. After several minutes, the medium was removed and the μ -Slide channel was filled with 200 μ l of cell suspension containing 10 000 exponentially growing MEC1 cells in a conditioned medium to achieve a subconfluent culture. Cells were incubated for 1 h at 37°C in a humidified atmosphere enriched with 5% CO₂. The μ -Slide with cells was then positioned on the stage of our Multimodal Holographic Microscope Q-PHASE (Tescan/Telight) and subjected to time-lapse recording for 1 h with 10 s lapse-interval using 10 $\times$ objective lenses 0.3 NA using the holographic mode measuring the optical thickness of cells with an accuracy of 2 nm. The recording was repeated 3 times for each cell type on two different days producing 12 time-lapse sequences which were processed using in-house developed image processing procedure using Mathematica (Wolfram Research), which removes background fluctuations, reconstructs and subtracts the background, converts the optical thickness of cells into their dry mass distributions, segments the cells, calculates centroids of dry cell mass, and morphometric parameters of *Circularity*, *Elongation*, *Mass* and *Spreading* defined as:

$$Circularity = \sqrt{\frac{(4 \pi A)}{P^2}}$$

where A is cell area in μm^2 and P is the perimeter length in μm ;

$$Elongation = 1 - \left(\frac{Width}{Length}\right)$$

where *Width* and *Length* are the smallest and largest axes of the best fit ellipse respectively;

$$Spreading = A/Mass$$

where *Mass* is the total dry mass of a cell expressed in pg (picogram = 10^{-12} g).

The software also tracks the cells in time on the basis of the nearest cell with a similar dry mass in the next frame. Tracked centroids were processed in order to calculate *Displacements* and *Persistences*. *Persistence* was defined as the ratio of direct translocation in 60 s and the wiggly track distance was determined as the sum of six 10 s displacements. *The Persistence* of a cell moving in a straight line is 1, and bending of the trajectory reduces the *Persistence* towards 0.

Immunohistochemistry of paraffin-embedded tissues

Immediately after the tissue of experimental animals was dissected out, it was fixed in 4% paraformaldehyde pH 7.4 at room temperature overnight. The FFPE tissue sections (1 μ m) were automatically processed and stained in VENTANA BenchMark Ultra system. Universal DAB Detection Kit (Ventana) was used for staining in combination with UltraView Amplification Kit (Ventana). Counterstaining was achieved by using Hematoxylin II (Ventana) and post-counterstaining by using Bluing reagent (Ventana). Immunostaining was performed using the anti-CD20 (for MEC1 cells; M0701, DAKO) or anti-CD45 (for primary CLL cells; M0755, DAKO) antibody.

Immunohistochemistry of cryopreserved tissues

Immediately after the tissue of experimental animals was dissected out, it was fixed in 4% paraformaldehyde pH 7.4 at 4 °C for 5 hrs. Subsequently, liver and spleen were cryopreserved in 30% sucrose at 4 °C overnight and embedded in OCT medium (Tissue-Tek, 4583) on dry ice. Femurs were decalcified in 10% EDTA pH 7.4 for 7 days at 4 °C prior to cryopreservation and OCT embedding. Samples were sectioned on a cryostat (Leica CM1850UV) as 14- μ m thick slices and collected on SuperFrost Plus Adhesion slides (Thermo Fisher Scientific, J1800BMNZ). Staining with primary antibody anti-mouse collagen IV (COL4, AbD Serotec, 2150-1470; 1:500) was performed at 4 °C overnight, followed by incubation with Alexa-conjugated secondary antibody (Invitrogen; 1:1000) at room temperature for 2 hrs. Cell nuclei counterstaining was performed with DRAQ5 Fluorescent Probe Solution (Thermo Fisher Scientific, 62251), and slides were mounted with Fluoromount Aqueous Mounting Medium (Sigma–Aldrich; F4680). Imaging was performed using Zeiss LSM880 laser scanning confocal microscope. ZEN2.1 (ZEISS) and Imaris (Bitplane) software was used for image processing.

Statistical analysis

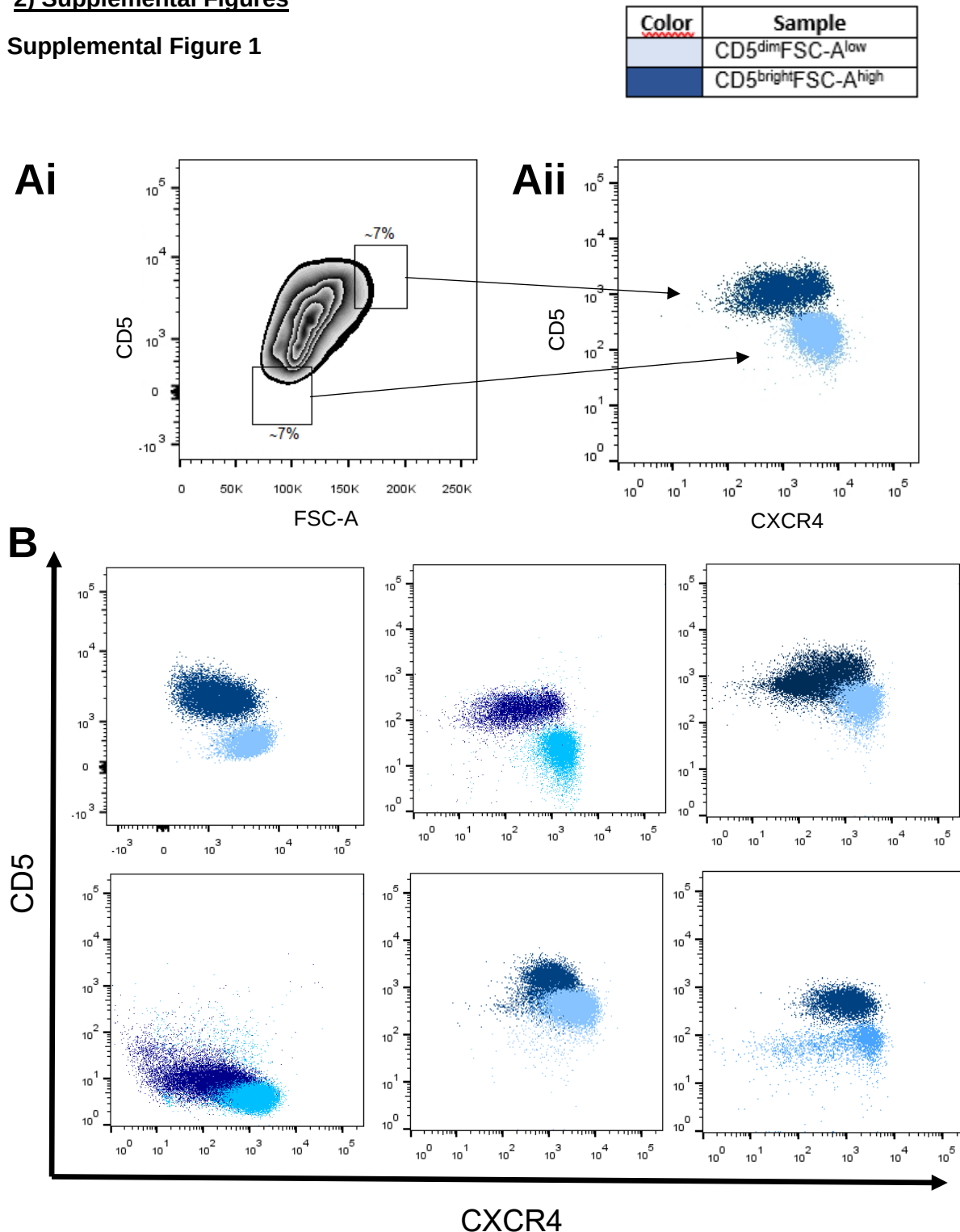
Differences in expression levels between paired samples were compared using paired t-test or Wilcoxon matched matched-pairs test and differences in non-matched groups were compared using unpaired t-test or Mann-Whitney U test. The appropriate tests were chosen based on the results of Shapiro-Wilk test of normality of input data. Statistical analysis of GAB1 inhibitors' effect on cell viability in comparison to their combination with ibrutinib was analysed by 2-way ANOVA including Tukey test. All analysis were performed in GraphPad v8.0.1.

Data Sharing Statement

The results of the RNAseq analysis and data deposition may be found above. For other original data or detailed protocols, please contact the corresponding author.

2) Supplemental Figures

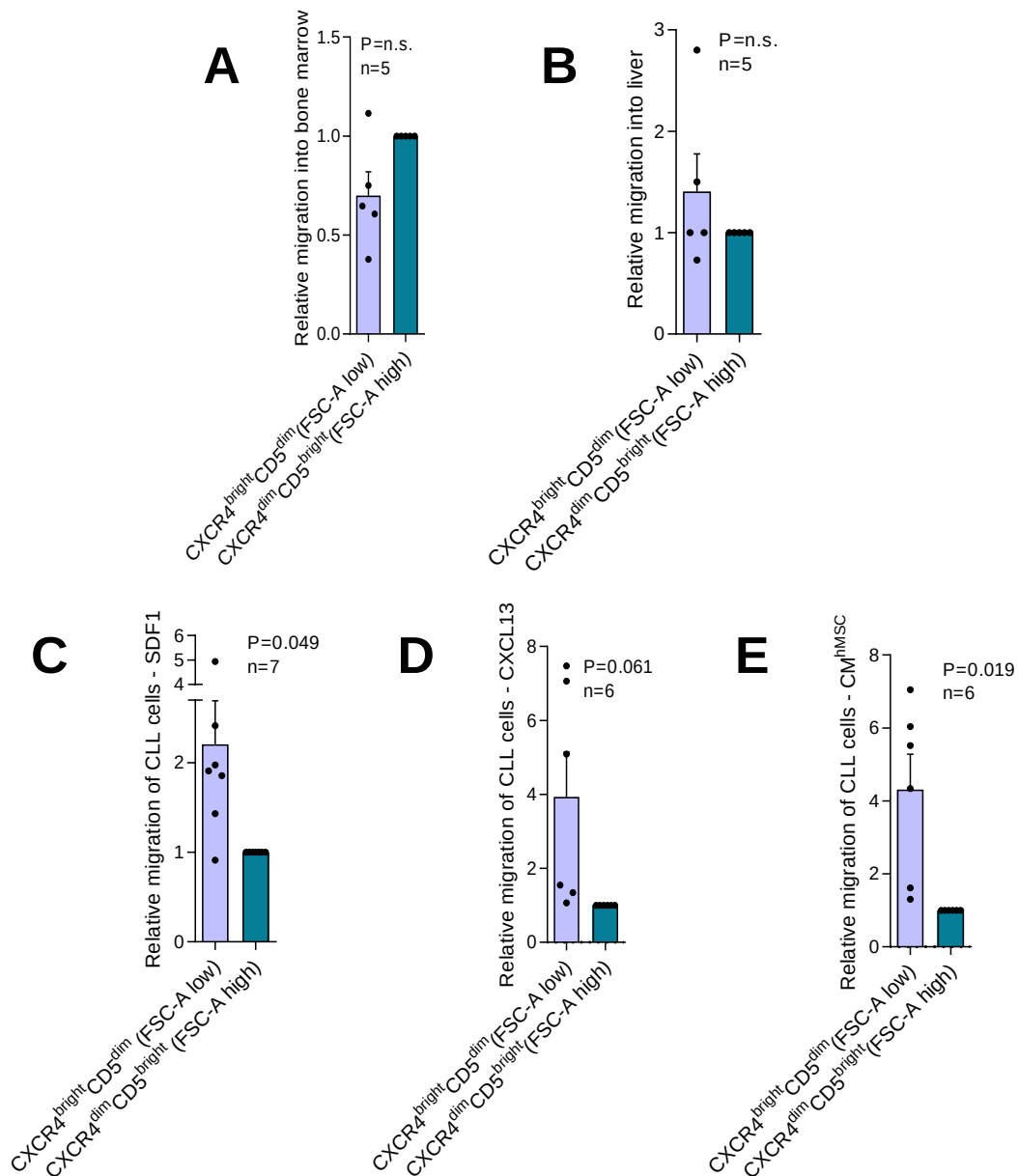
Supplemental Figure 1



Supplemental Figure 1. (Ai) Representative example of a gating strategy for sorting of CLL cells according to CD5 and FCS-A (forward scatter area). CXCR4/CD5 subpopulations were sorted according to CD5 and forward scatter area (CXCR4^{dim} cells have a higher FSC-A). The CD5/FSC-A was used to prevent labeling by an antibody binding to the CXCR4 receptor, which would interfere with the migration to SDF1 and potentially other chemokines. **(Aii)** Evaluation of CXCR4 and CD5 expression in cells sorted according to the gating strategy shown in [Ai]. Small aliquotes of sorted CD5^{dim}FSC-A^{low} and CD5^{bright}FSC-A^{high} cells

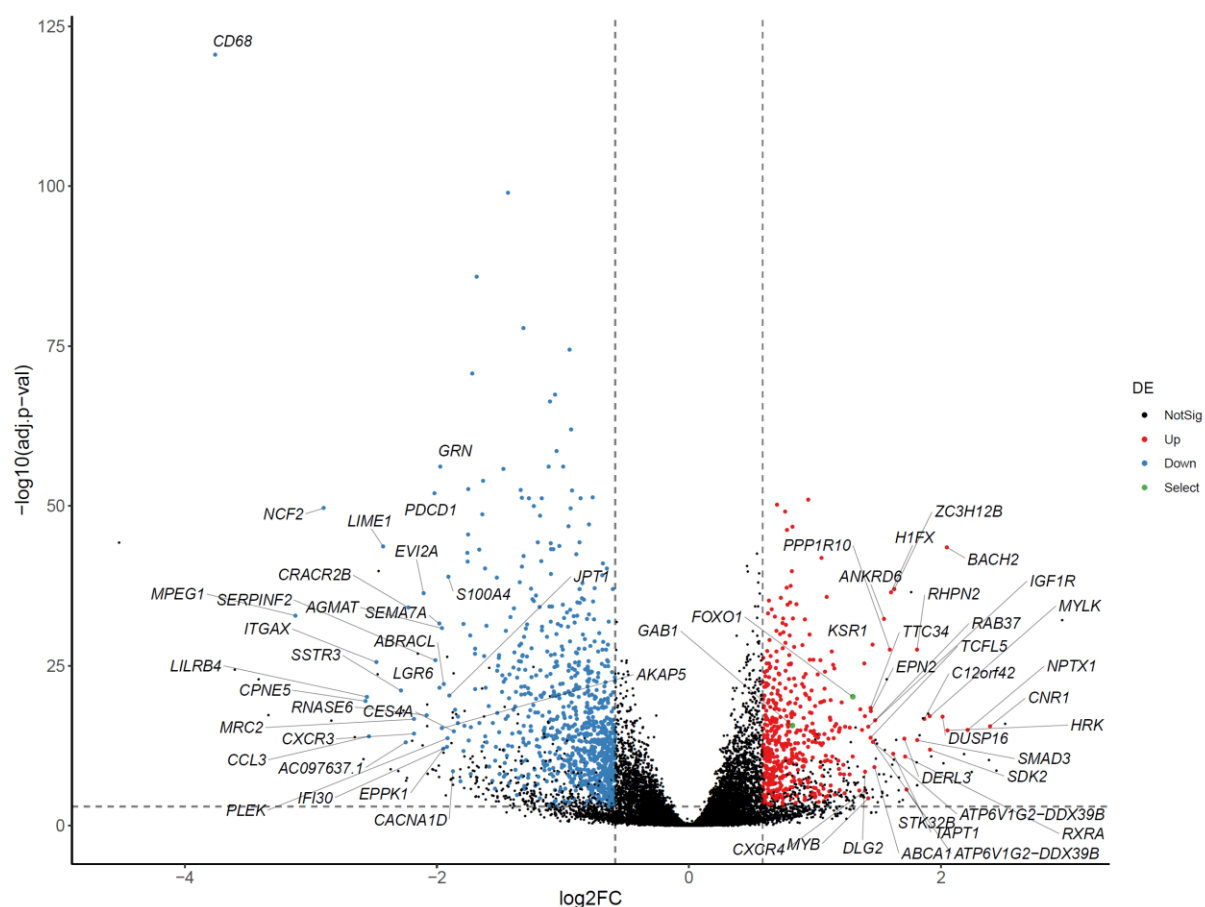
(correspond to CD5^{dim}CXCR4^{bright} and CD5^{bright}CXCR4^{dim} cells, respectively) were stained for CXCR4 and measured by flow cytometry to validate CXCR4 cell-surface levels and separation of CXCR4/CD5 subpopulations (this validation was performed in all samples used for experiments below). The sorting approach using CD5/FSC-A was successful in separating CXCR4/CD5 subpopulations in approx. 75% of CLL cases and only the samples with an acceptable profile of CXCR4/CD5 after validation by flow cytometry were used in further experiments. **(B)** Validation of CXCR4/CD5 expression in other CLL samples sorted according to FCS-A/CD5.

Supplemental Figure 2



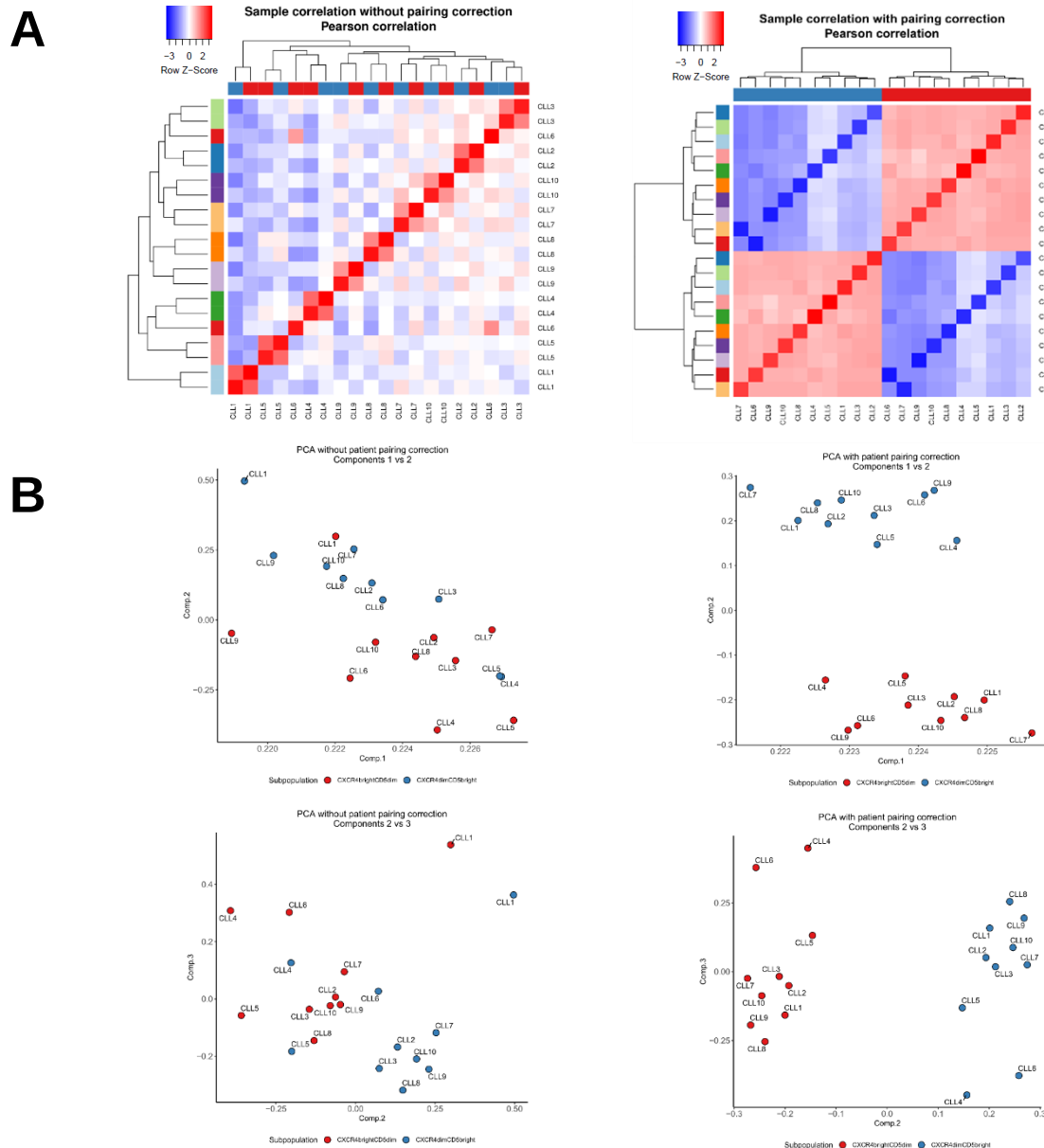
Supplemental Figure 2. Competitive migration assay of primary CLL cells sorted according to CD5/FSC-A (as surrogate markers for CD5/CXCR4 subpopulations, the cell-surface levels of CXCR4/CD5 were validated after sorting, see Supplemental Fig. 1). **(A-B)** Sorted cells were stained with two different CellTrace dyes, mixed in a 1:1 ratio, and injected into the tail vein of NGS mice (see Fig. 2A). Migration was allowed for 4 hrs, and then bone marrow **(A)** and liver **(B)** were analyzed for the presence of CLL cells ($n=5$). The numbers of CLL cells infiltrating bone marrow or liver were markedly lower in comparison to the spleen (Fig. 1A). **(C-E)** Competitive migration assay of CXCR4/CD5 CLL cell subpopulations towards SDF1 **(C)**, CXCL13 **(D)**, or conditioned media **(E)** produced by bone marrow mesenchymal stromal cells from a healthy donor (CM^{hMSC}). Sorted and validated CXCR4/CD5 subpopulations were stained with two different CellTrace dyes, mixed in a 1:1 ration, and loaded into transwells.

Supplemental Figure 3



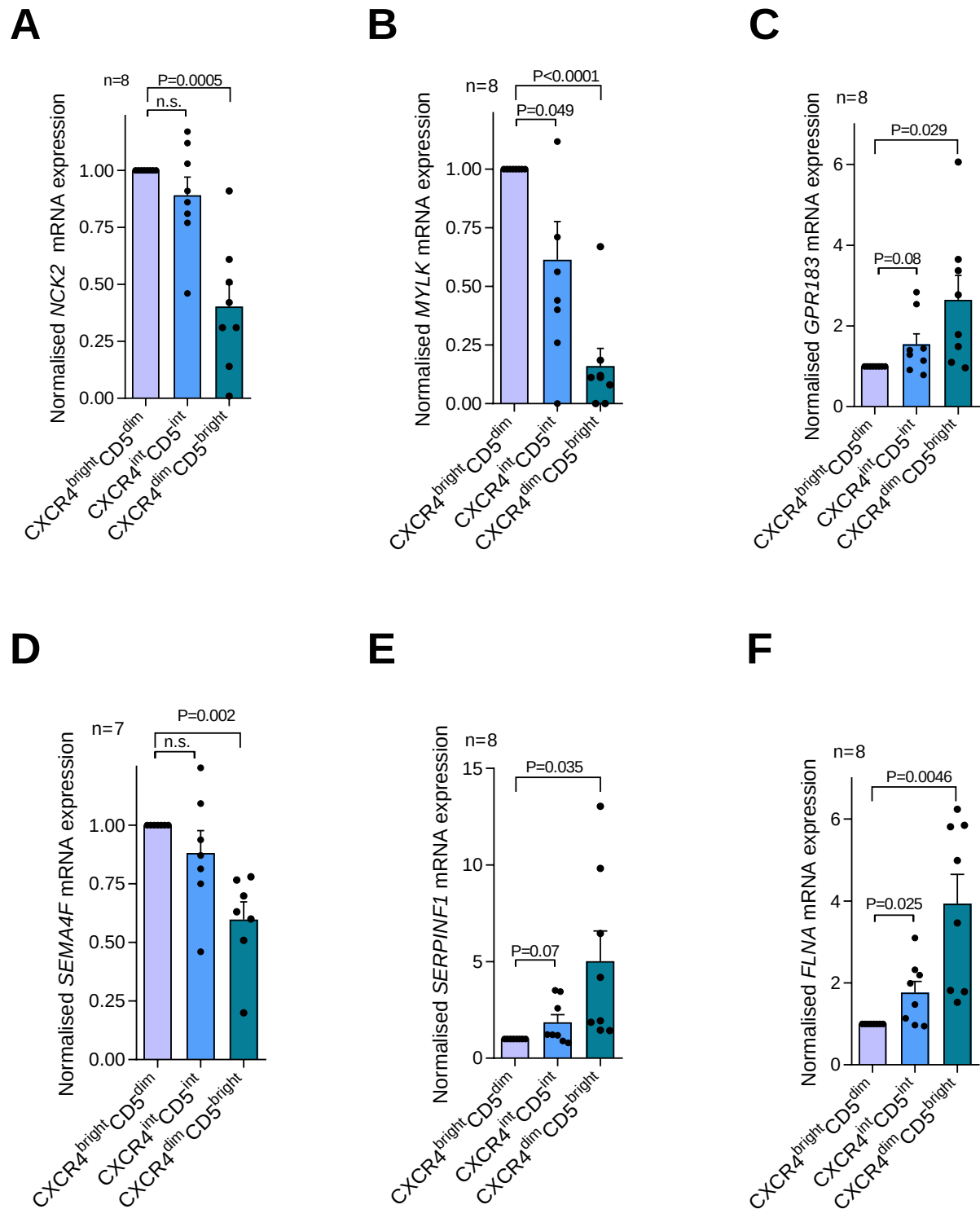
Supplemental Figure 3. Volcano plot depicting differentially expressed genes in CXCR4/CD5 subpopulations. Vertical dashed lines represent a fold-change of >1.5-fold. The horizontal dashed line represents adjusted P-value <0.001. Blue color represents genes relatively down-regulated in CXCR4^{bright}CD5^{dim} cells, and the red color represents genes relatively up-regulated in CXCR4^{bright}CD5^{dim} cells (cell sorting strategy shown in Fig. 1B). FoxO1 and GAB1 are depicted in green color. Log2FC stands for a fold-change in log2 scale, -log10(adj.p-val) stands for adjusted P-value in a log10 scale.

Supplemental Figure 4



Supplemental Figure 4. (A) Pearson correlation for gene expression between all samples used for RNAseq analysis. The correlation was calculated from log2 normalized gene counts (DESeq) + 1 to avoid logarithms of 0 counts. Correlation heatmaps for both sample pairing uncorrected (top) and corrected for paired CXCR4/CD5 subpopulations (bottom) are shown. The heatmap colors were normalized to row Z-score. Red color depicts CXCR4^{bright}CD5^{dim} cells, and blue color depicts CXCR4^{dim}CD5^{bright} cells, row colors represent individual patients. The data were corrected for pairing by limma (v3.40.6) „removeBatchEffect“ function.¹⁸ **(B)** Principal Component Analysis (PCA) of all samples used for RNAseq analysis was calculated from log2 normalized counts (DESeq1) + 1 to avoid logarithms of 0 counts. PCA components for both sample pairing uncorrected (left) and corrected for paired CXCR4/CD5 subpopulations (right) are shown. Red color depicts CXCR4^{bright}CD5^{dim} cells, and blue color depicts CXCR4^{dim}CD5^{bright} cells. The data were corrected for pairing by limma (v3.40.6) „removeBatchEffect“ function.¹⁸

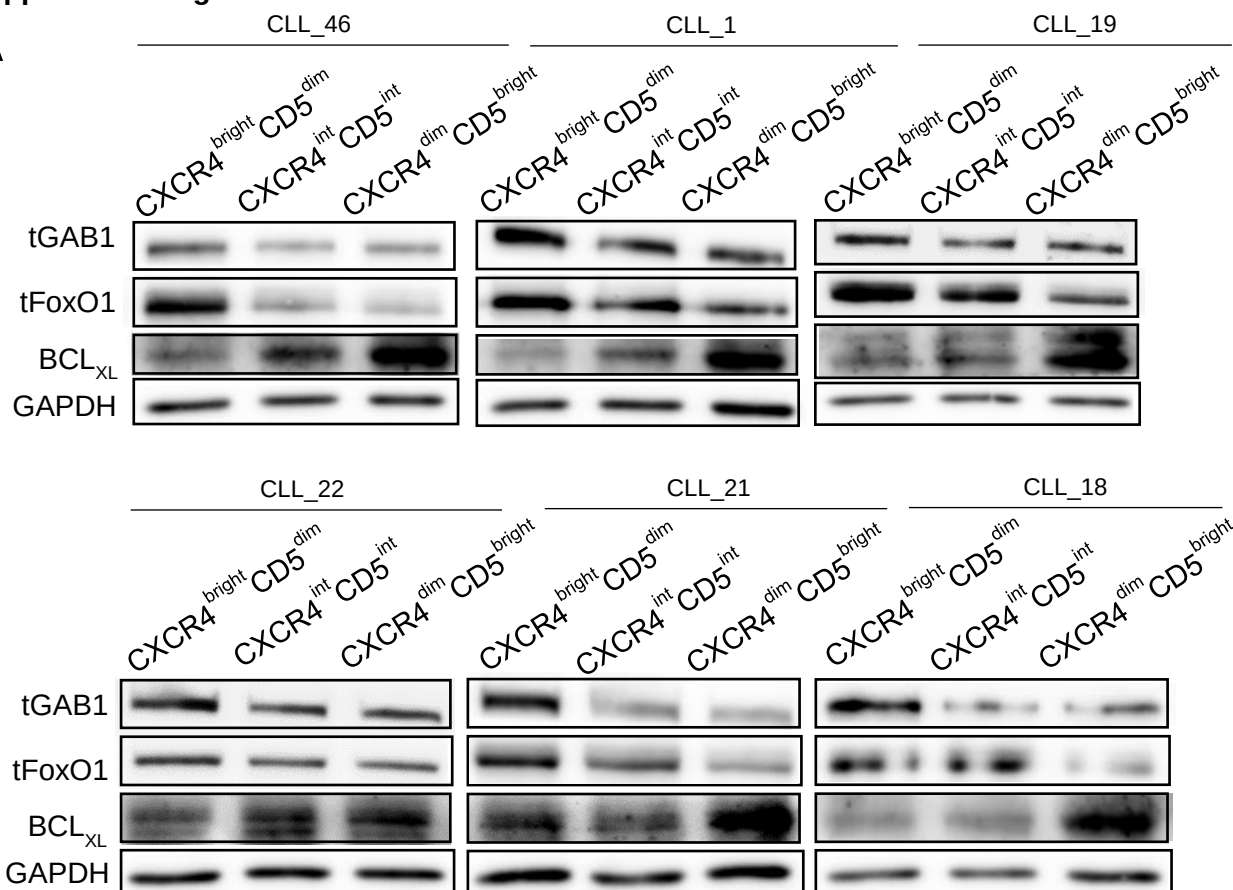
Supplemental Figure 5



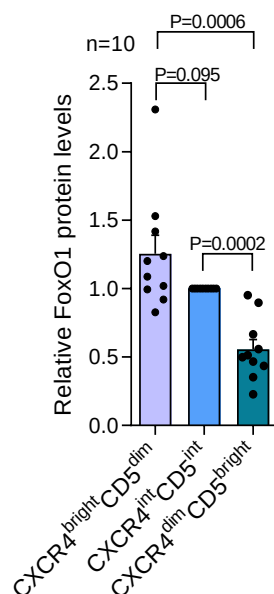
Supplemental Figure 5. Analysis of six selected migration-related genes by qRT-PCR in sorted CXCR4/CD5 subpopulations (for cell sorting strategy, see Fig. 1B). The mRNAs were randomly selected from differentially expressed migration-related genes identified by RNAseq (Fig. 1C), and their expression was validated in an independent set of sorted samples. The CXCR4^{int}CD5^{int} represents a transitional intermediate (int) subpopulation between CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} cells.

Supplemental Figure 6

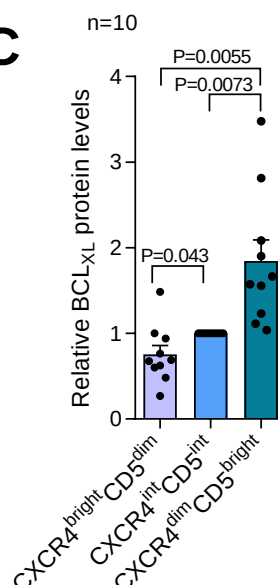
A



B

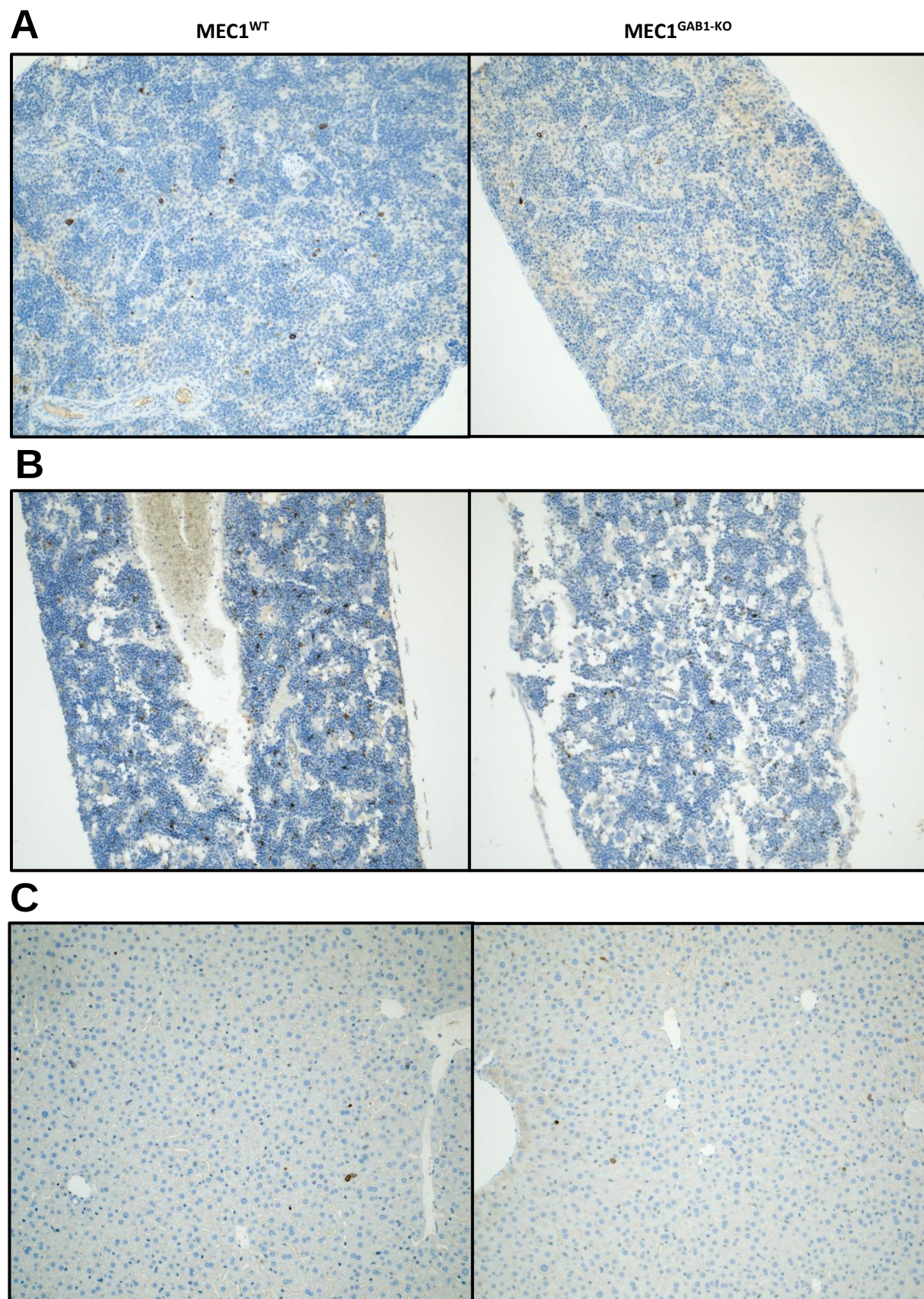


C

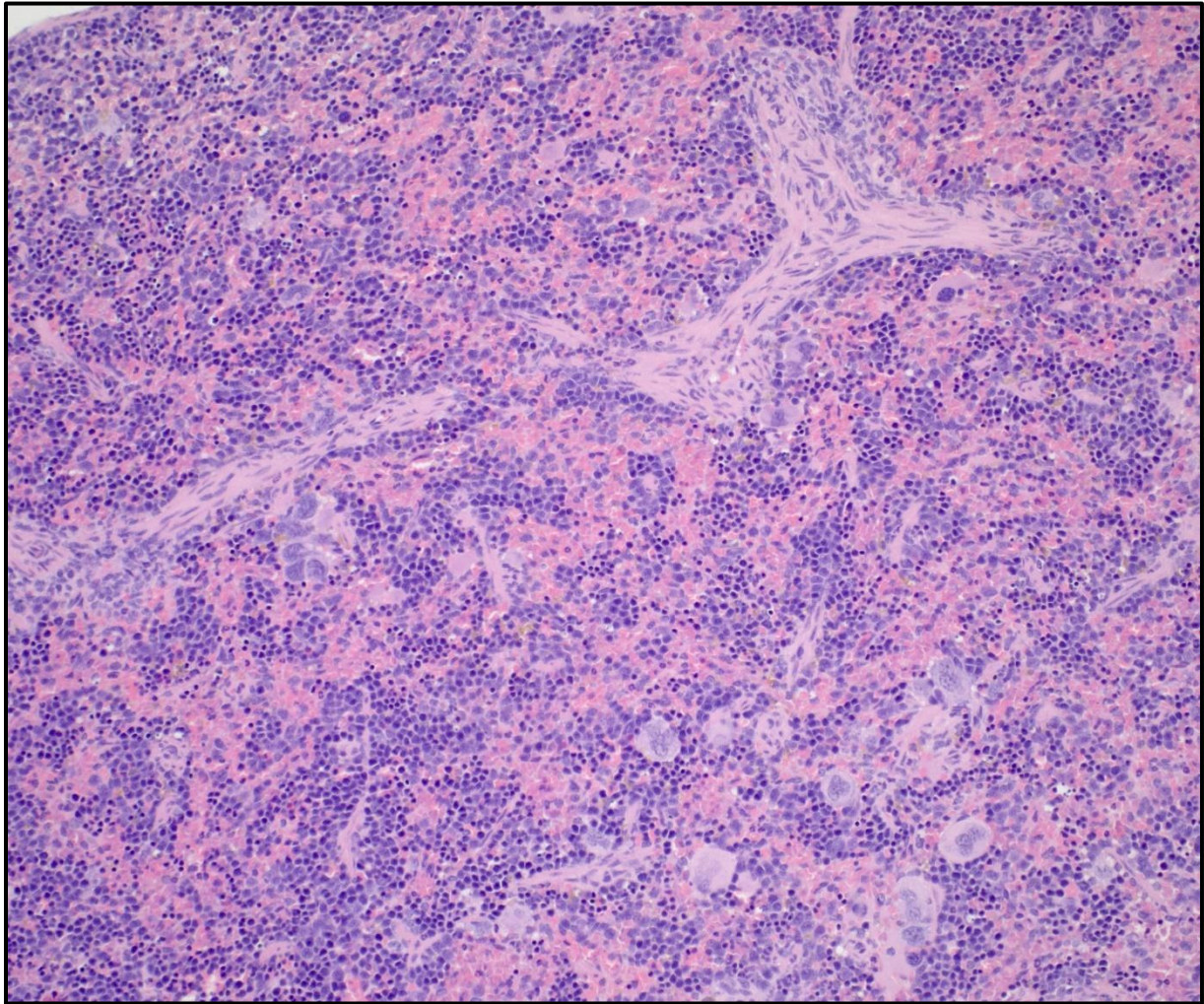


Supplemental Figure 6. (A) Additional immunoblots for proteins of interest from sorted CXCR4/CD5 subpopulations. The CXCR4^{int}CD5^{int} represents a transitional intermediate (int) subpopulation between CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} cells. **(B-C)** and Densitometric quantification of FoxO1 (B) and BCL_{XL} (C) protein levels in sorted CXCR4/CD5 subpopulations as analyzed by immunoblot. BCL_{XL}, an antiapoptotic protein, serves as a positive control for microenvironmental interactions. For cell sorting see Fig. 1B and for densitometric analysis of protein levels, see Fig. 1G

Supplemental Figure 7

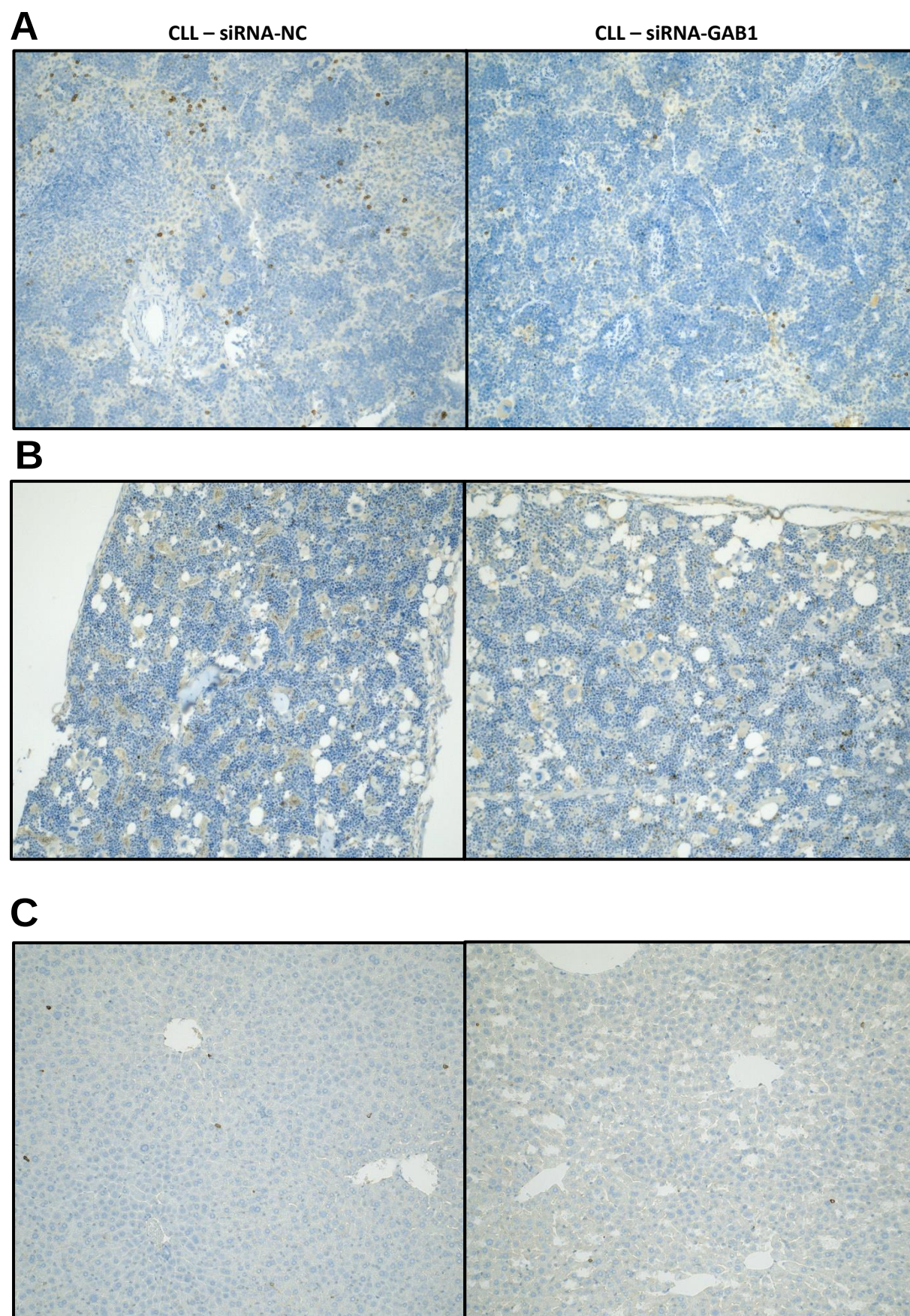


D



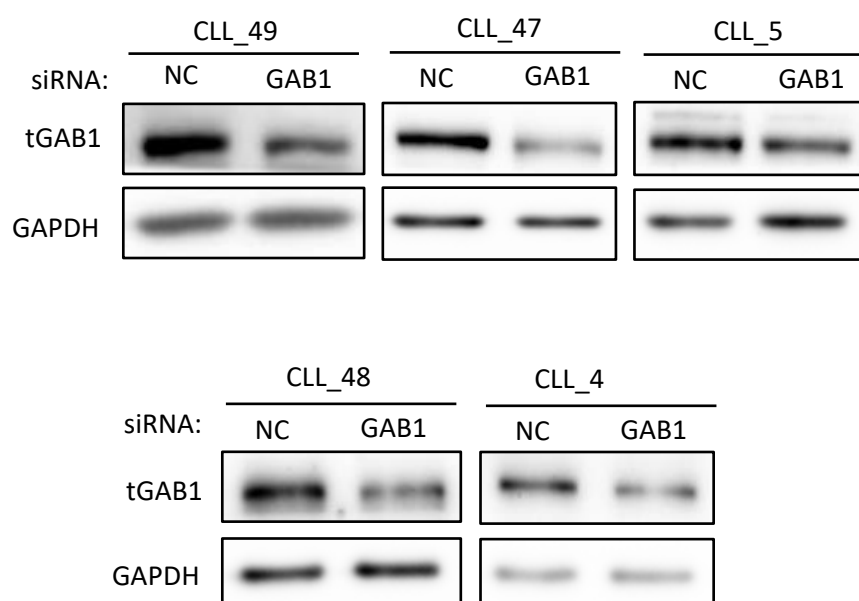
Supplemental Figure 7. Histological evaluation of spleen **(A)**, bone marrow **(B)**, and liver **(C)** of NSG mice transplanted with MEC1^{wt} (*left*) or MEC1^{GAB1-KO} (*right*) cells. In this experiment, MEC1^{wt} or MEC1^{GAB1-KO} cells were transplanted separately to individual NSG mice via a tail vein injection and sacrificed after 6 hrs. Tissue sections were stained with anti-human-CD20 antibody (brown signal) and the post-counterstaining by Bluing reagent (Ventana). The composition of the spleen structure in NSG mice is different from normal mouse spleen and reflects the absence of mature B/T/NK cells in these animals (see **D** for hematoxylin-eosin staining of the spleen, 20x). Therefore, the white pulp of the spleen is absent, and the organ is mainly formed by red pulp with a high content of overactive erythron and excessive erythropoiesis (including the presence of megakaryocyte). The migrated MEC1 cells were localized diffusely in the spleen, and there was no difference in tissue localization for GAB1-knockout versus GAB1-wt cells.

Supplemental Figure 8



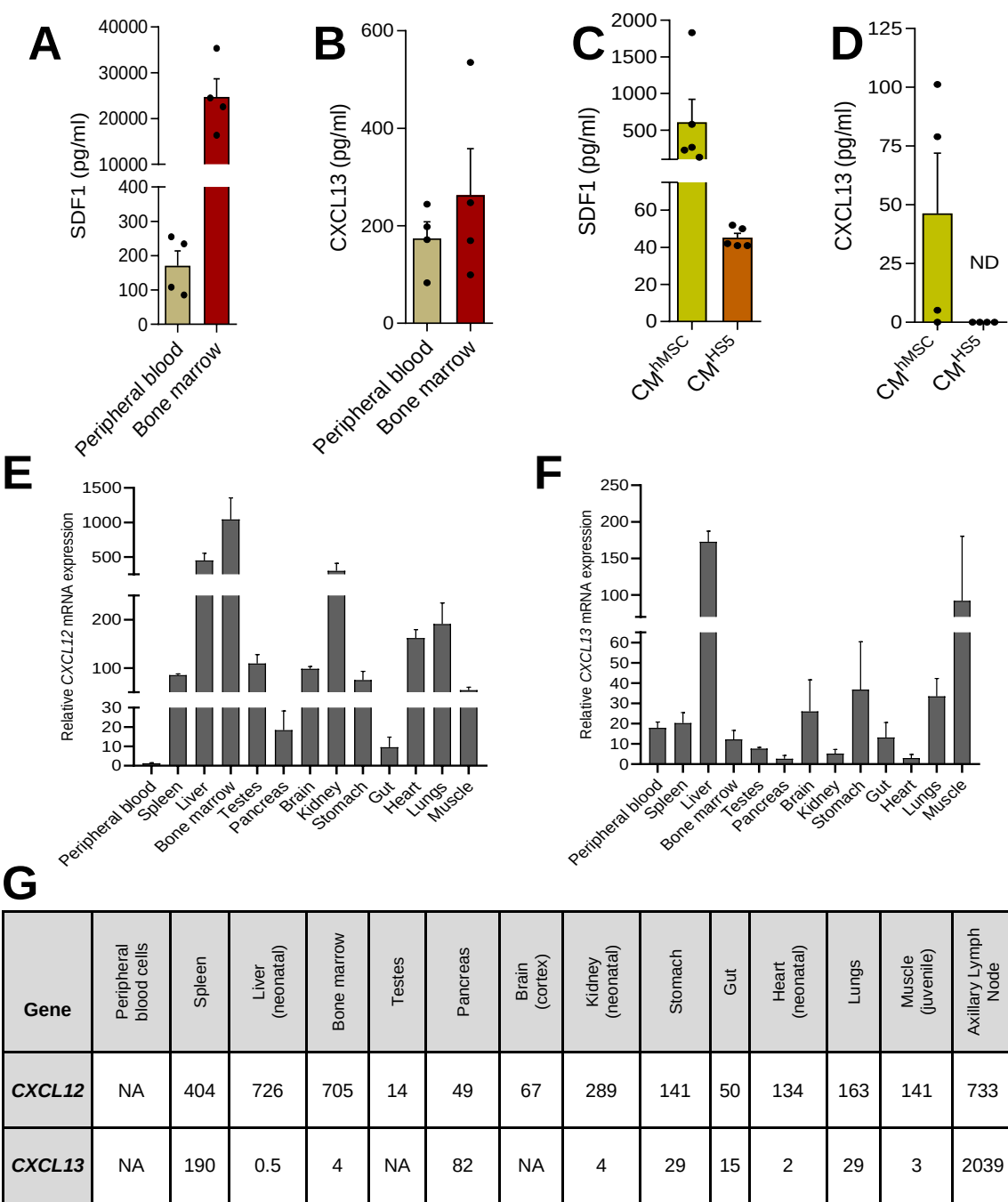
Supplemental Figure 8. Histological evaluation of spleen **(A)**, bone marrow **(B)**, and liver **(C)** of NSG mice transplanted with CLL cells transfected with siRNA against *GAB1* (siRNA-GAB1, *right*) or control siRNA (siRNA-NC, *left*). Cells were incubated for 48 hrs after transfection and then stained with Sytox Blue viability dye and sorted according to viability. Viable CLL cells (10×10^6 of siRNA-GAB1 or siRNA-NC transfected cells) were transplanted to individual NSG mice via a tail vein injection and sacrificed after 6 hrs. Tissue sections were stained with anti-human-CD45 antibody (brown signal) and the post-counterstaining by using Bluing reagent (Ventana). The composition of the spleen structure in NSG mice is different from normal mouse spleen and reflects the absence of mature B/T/NK cells in these animals (see Supplemental Fig. 10D for hematoxylin-eosin staining of the spleen, 20x). Therefore, the white pulp of the spleen is absent, and the organ is mainly formed by red pulp with a high content of overactive erythron and excessive erythropoiesis (including the presence of megakaryocyte). The migrated CLL cells were localized diffusely in the spleen, with a tendency for higher CLL cell infiltration in the trabecular system near the trabecular veins; however, there was no difference in the localization inside the studied organs for siRNA-GAB1 or siRNA-NC CLL cells. For patient characteristics, see Supplemental Tab1, CLL_3.

Supplemental Figure 9



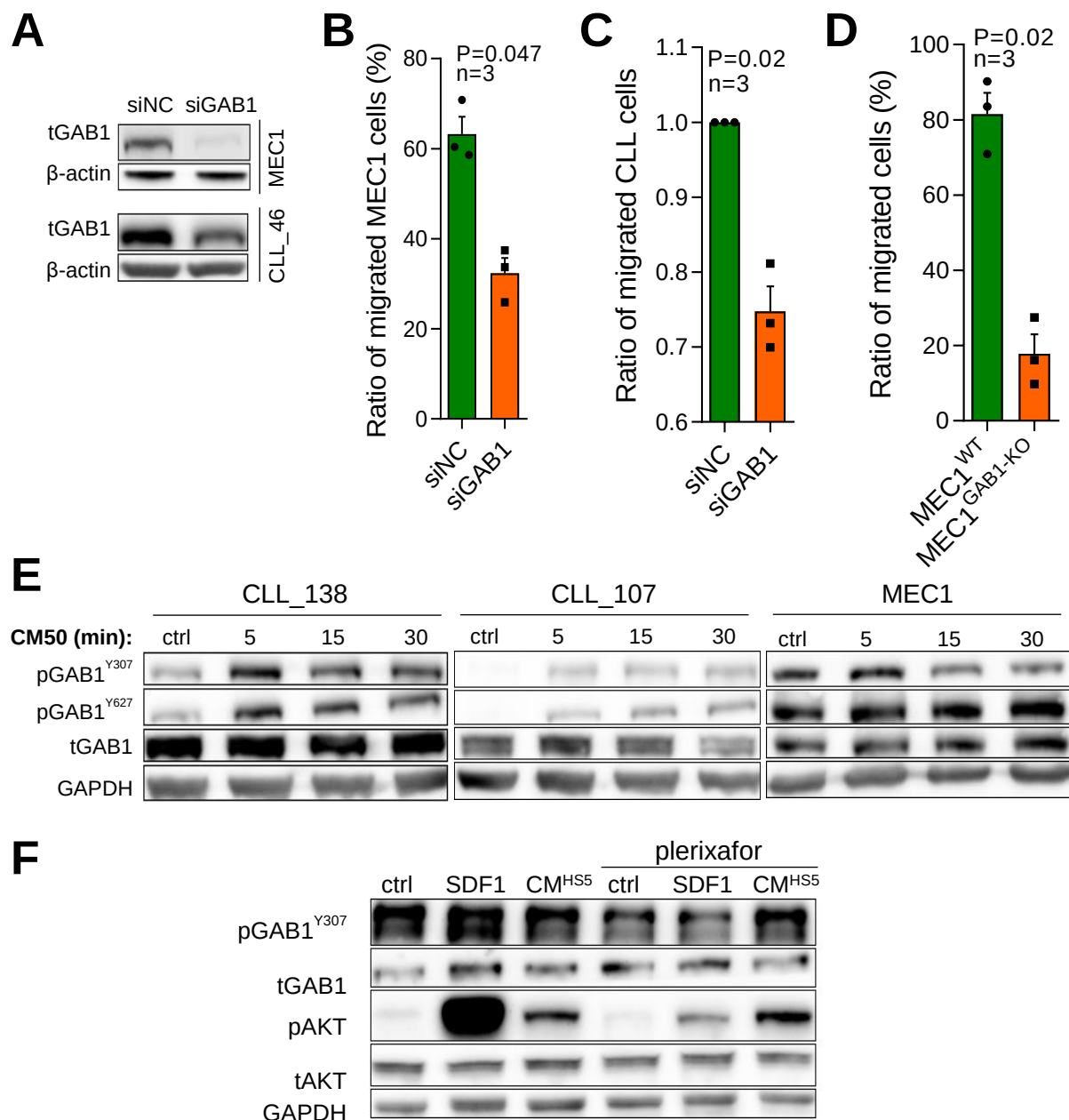
Supplemental Figure 9. Control immunoblots from primary CLL cells transfected with control siRNA (NC) or siRNA against *GAB1* (GAB1). Cells were incubated for 48 hrs and then stained with Sytox Blue viability dye and sorted according to viability. A small aliquot of sorted cells, was used for these control immunoblots, and the remaining cells were used for competitive migration assay in NSG mice.

Supplemental Figure 10



Supplemental Figure 10. (A-B) Serum isolated from peripheral blood or bone marrow of CLL patients (n=4) was tested for SDF1 **(A)** or CXCL13 **(B)** levels by ELISA (R&D Systems). **(C-D)** Conditioned media produced by primary human mesenchymal stromal cells (CM^{hMSC}) or by human stromal cell line HS5 (CM^{HS5}) was analyzed for levels of SDF1 **(C)** or CXCL13 **(D)**. The replicates in C and D represent analyses of conditioned media from various passages of the cells. ND stands for “not-detectable”. **(E-F)** Relative expression of CXCL12 **(E)** and CXCL13 **(F)** mRNAs in different organs of the NSG mice. For this experiment, 3 NSG mice (without transplantation of malignant cells) were sacrificed, organs homogenized, RNA isolated, and subjected to qRT-PCR. **(G)** Publicly available data on CXCL12 and CXCL13 mRNA expression in different compartments of C57BL/6J mice (mice strain not used in this study). NA stands for “data not available”. Data are presented as TPM (Transcripts per Kilobase Million) and were obtained from *Expression Atlas database* (ArrayExpress: experiment E-MTAB-3579).¹⁹

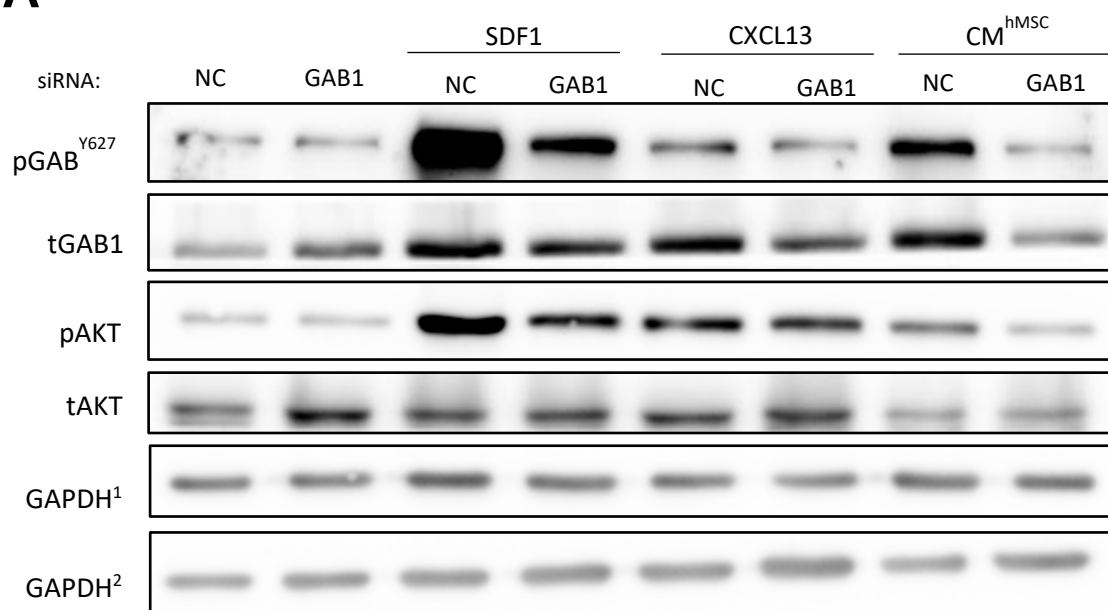
Supplemental Figure 11



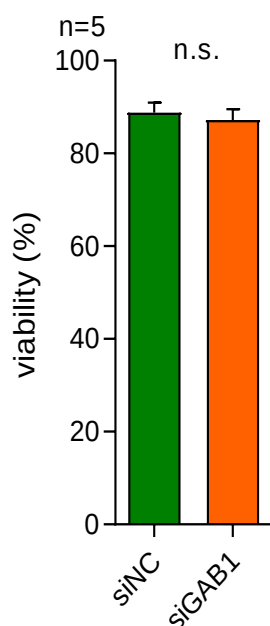
Supplemental Fig. 11 (A) Representative example of GAB1 silencing by siRNA in MEC1 (*top*) and primary CLL cells (*bottom*). **(B-C)** Migration of MEC1 cells (**B**) and CLL cells (**C**) towards 50% conditioned media produced by HS5 cells. CLL or MEC1 cells were transfected with siRNA against *GAB1* (siGAB1) or control siRNA (siNC). Migration across the transwell towards the 50% conditioned media produced by HS5 cells was allowed for 6 hrs (n=3), and the ratio of passed cells was measured by flow cytometry. **(D)** In vitro competitive migration assay of MEC1^{WT} vs. MEC1^{GAB1-KO} cells (cells stained as shown in Fig. 2B). Migration was allowed for 6 hrs towards conditioned media produced by HS5 stromal cells. **(E)** Representative immunoblot for primary CLL cells stimulated for various times (3-60 min) with recombinant SDF1 (100 ng/ml). **(F)** Representative immunoblot from primary CLL cells treated with 50% conditioned media produced by human stromal cell line HS5 (CM^{HS5}), SDF1 (100 ng/ml), or their combination with CXCR4-inhibitor plerixafor. Plerixafor (5 µg/ml) was added 2 hrs prior to the cell treatment with SDF1 or conditioned media to ensure full CXCR4 inhibition. The control stands for cells treated with vehicle and cultured on plastic.

Supplemental Figure 12

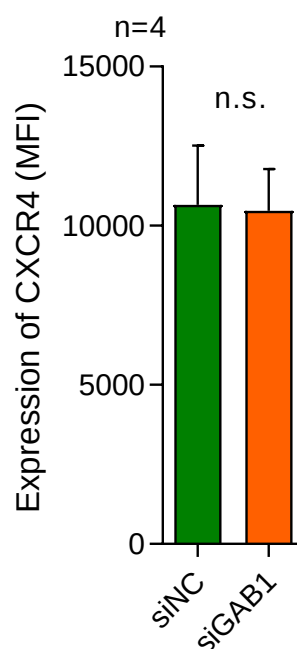
A



B



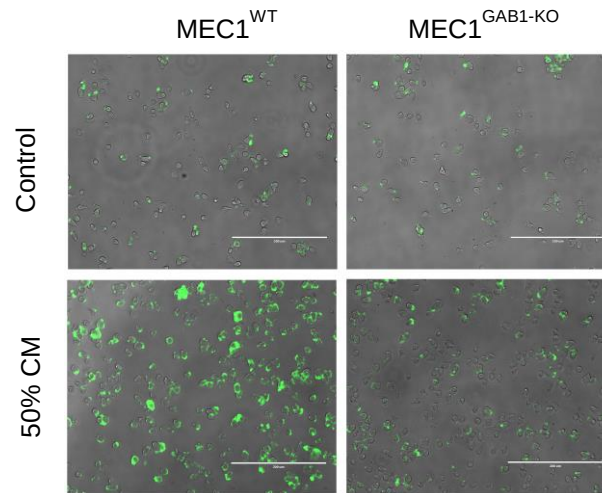
C



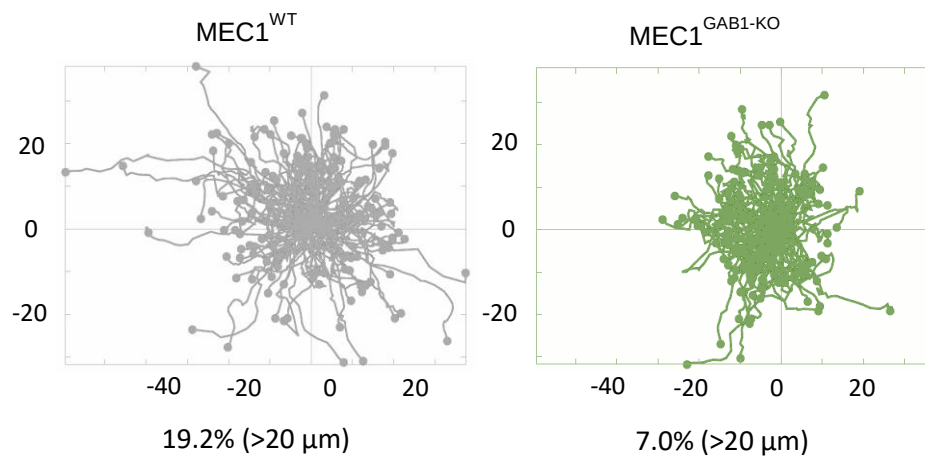
Supplemental Figure 12. (A) Additional immunoblot from primary CLL cells (CLL_133) transfected with siRNA against GAB1 (GAB1) or a negative control (NC). Cells were (48 hrs post-transfection) stimulated for 10 min by SDF1 (100 ng/ml), or CXCL13 (250 ng/ml), or conditioned media produced by primary human mesenchymal stromal cells (CM^{hMSC}). The immunoblot contains 2 endogenous controls (GAPDH) marked by upper index, because for technical reasons pGAB1 and pAKT (loading control GAPDH¹) were analyzed on first gel and remaining proteins (loading control GAPDH²) on the second gel (identical protein loading and conditions). **(B-C)** Analysis of cell viability (A) and CXCR4 cell-surface expression (B) in primary CLL cells transfected with siRNA against GAB1 (siGAB1) or with control scrambled siRNA (siNC). Viability was measured by DiOC6/PI staining.

Supplemental Figure 13

A

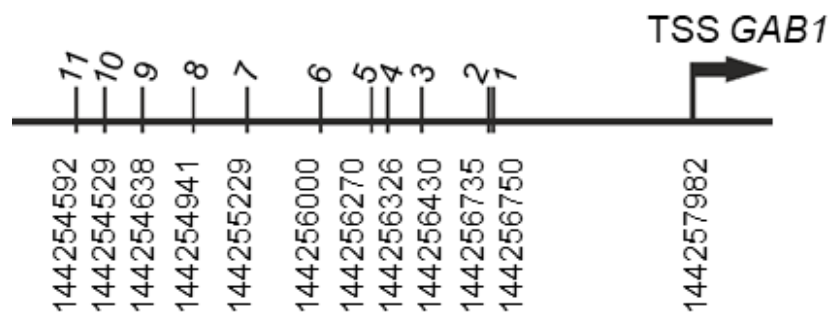


B



Supplemental Figure 13. (A) Representative image of F-actin polymerization in MEC1^{wt} and MEC1^{GAB1-KO} cells after treatment with 50% conditioned media produced by HS5 cells (15 min). MEC1 cells were subsequently fixed, and polymerized F-actin was visualized by fluorescent microscopy after Phalloidin-488 staining. **(B)** Representative rose plots with 5 min migration trajectories of MEC1^{wt} vs. MEC1^{GAB1-KO} cells in the presence of conditioned media produced by HS5 cells (shifted to a common origin, axis in μm). The percentage below the figure indicates the fraction of cells with trajectory >20 μm at 5 min in MEC1^{wt} vs. MEC1^{GAB1-KO} cells (19.2 vs. 7%, respectively; P<0.0001).

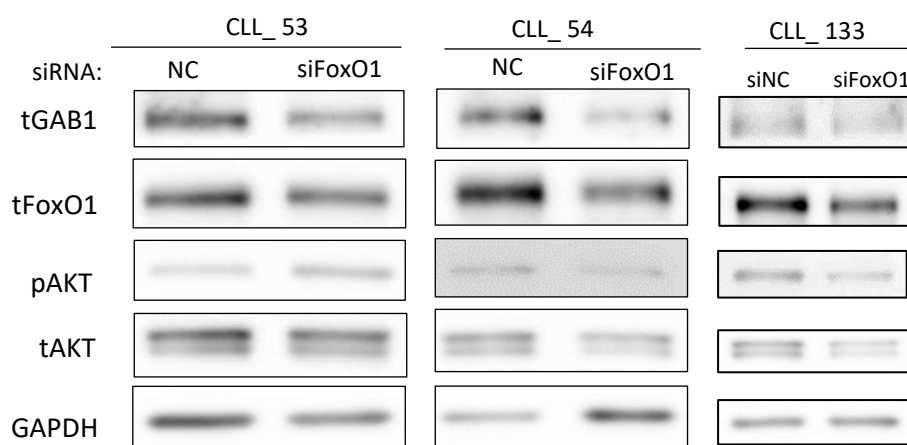
Supplemental Figure 14



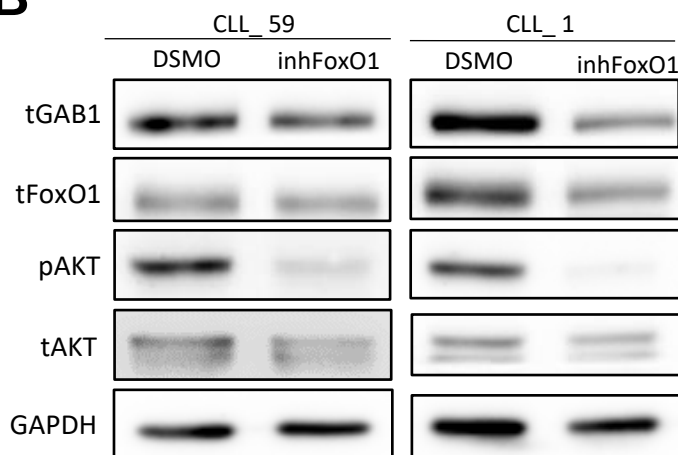
Supplemental Figure 14. Map of predicted transcription binding sites of FoxO1 protein in 3.5 kb promoter region of the *GAB1* gene (ContraV3). Predicted transcription binding sites are number 1-11 (at the top), and their genomic position is indicated.

Supplemental Figure 16

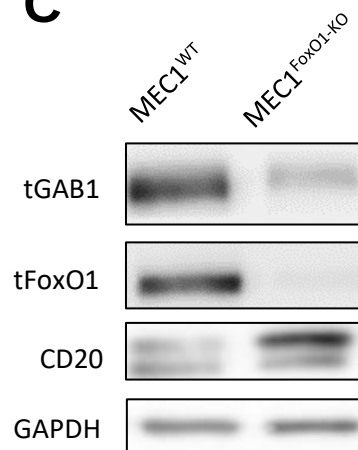
A



B

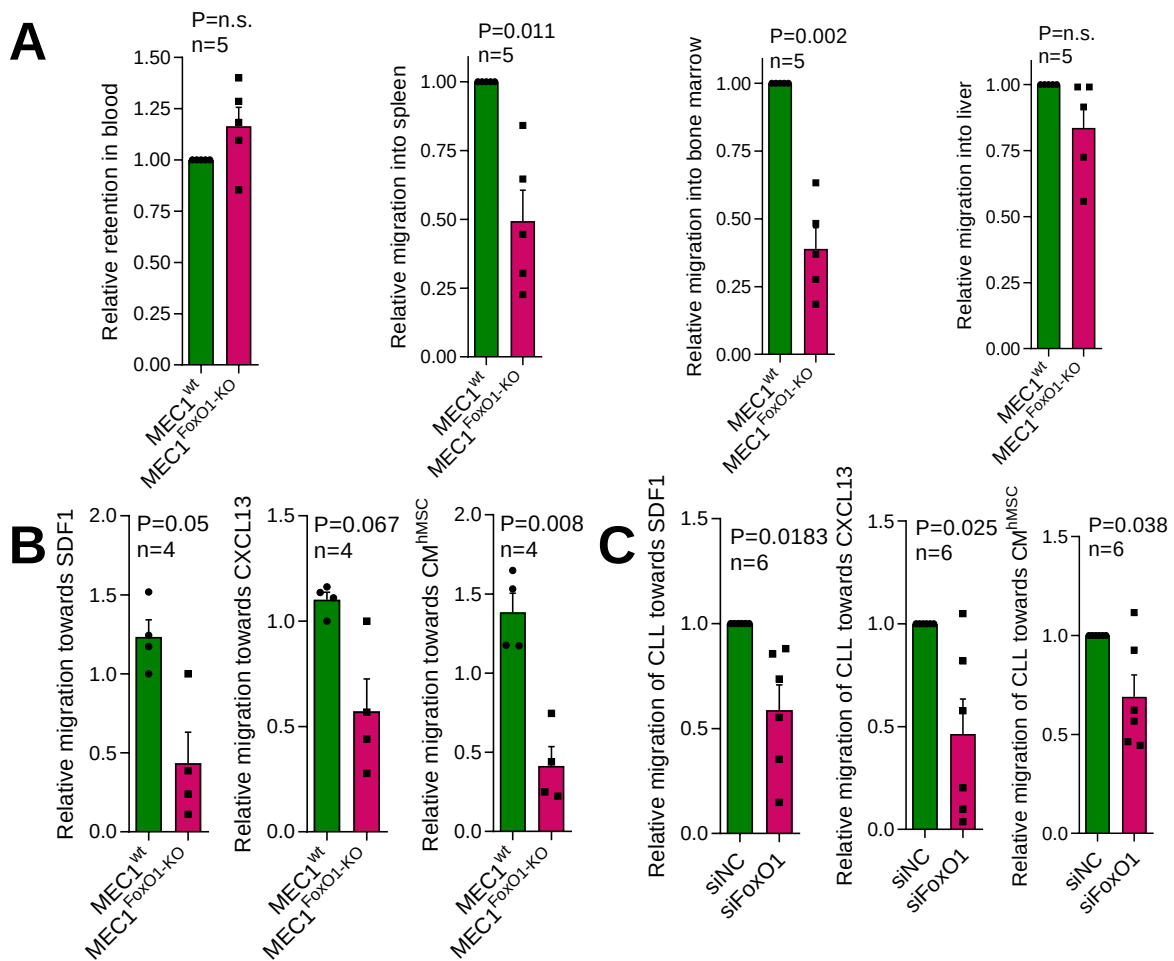


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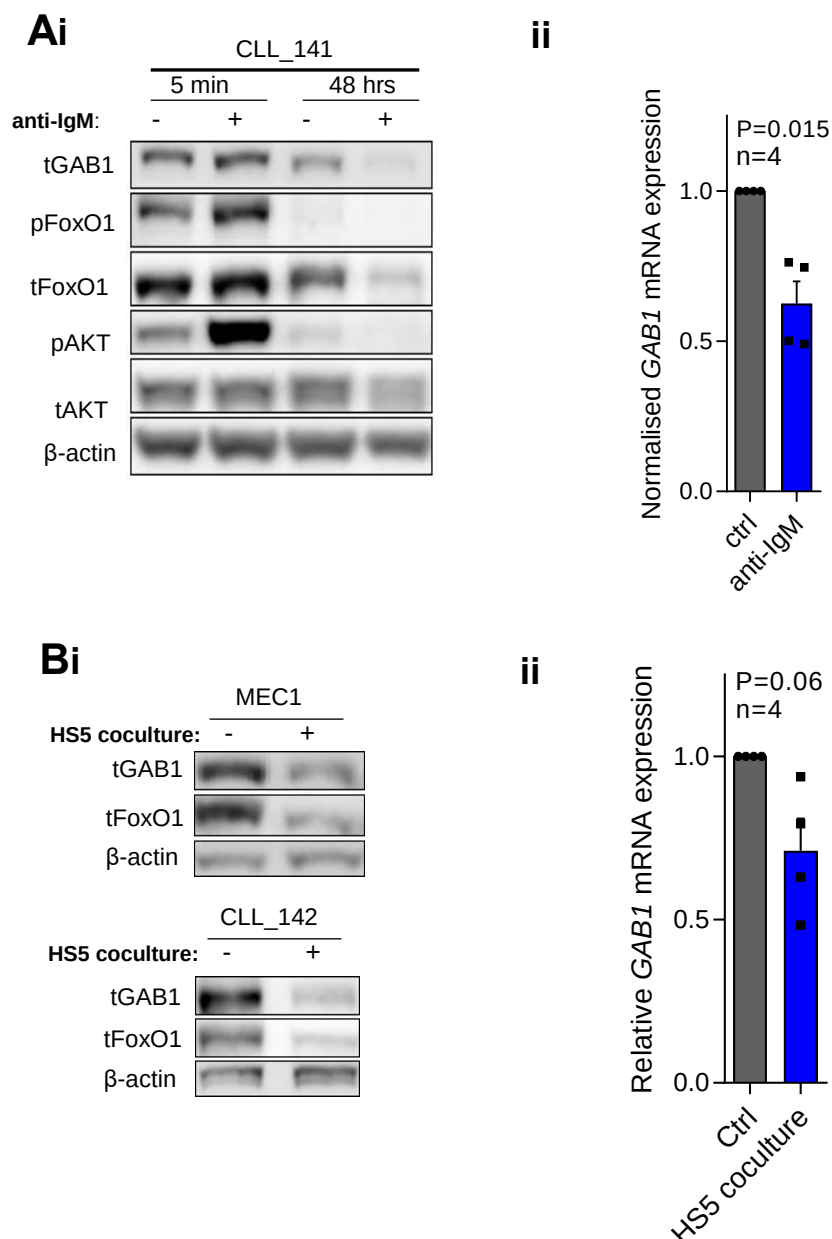
Supplemental Figure 16. (A) Immunoblots from primary CLL cells transfected with non-specific control siRNA (NC) or siRNA against *FoxO1* (siFoxO1). Cells were harvested 48 hrs post transfection. **(B)** Immunoblots from primary CLL cells treated FoxO1 inhibitor (0,5 μ M, 48 hrs). **(C)** Representative immunoblot for MEC1^{wt} and MEC1^{FoxO1-KO} cells. CD20 was used as a positive control of FoxO1 repression since FoxO1 is a negative regulator of CD20 transcription.²¹ The same samples were used in Fig. 4F.

Supplemental Figure 17



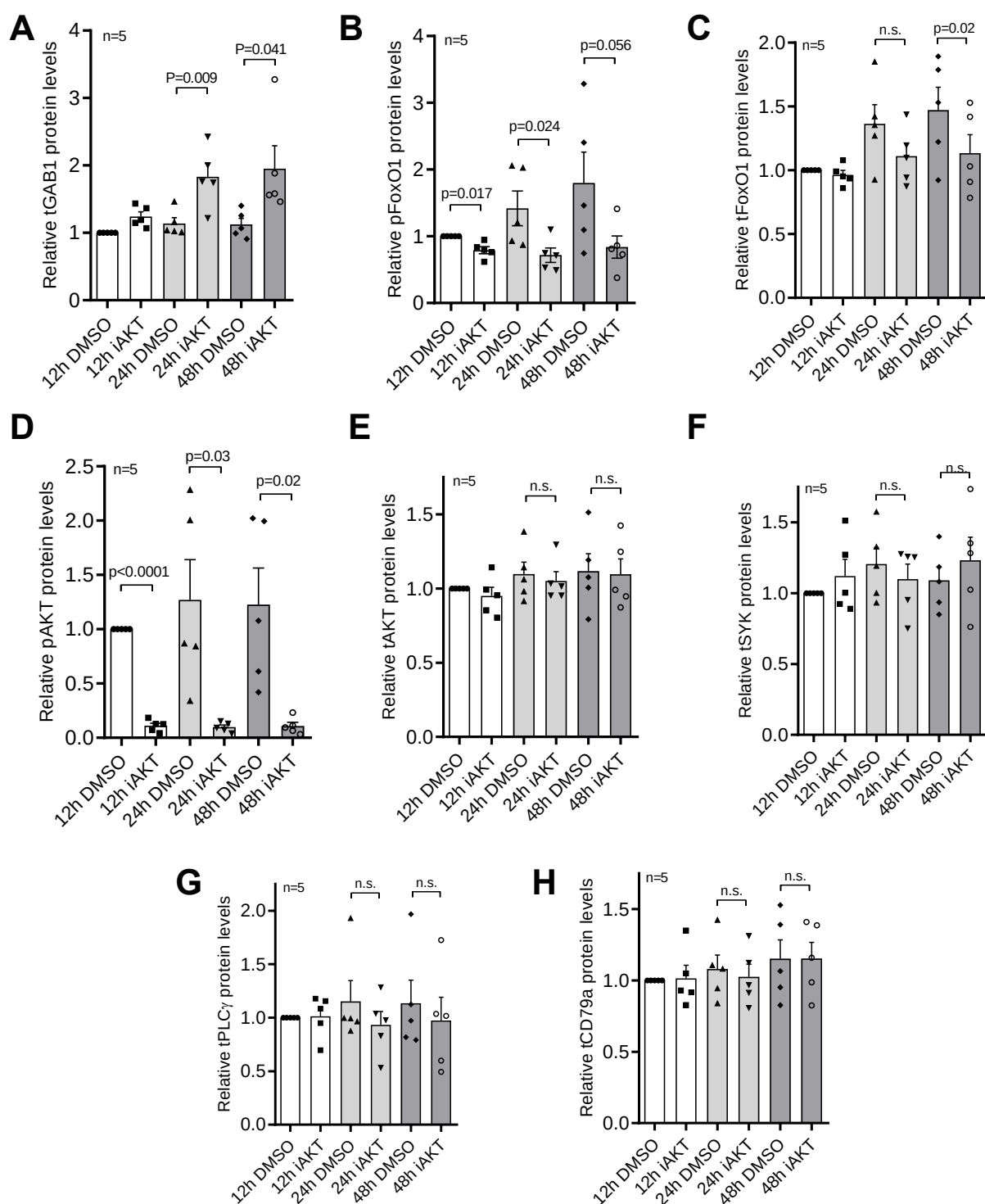
Supplemental Figure 17. (A) *In vivo* competitive migration assay of MEC1^{wt} vs. MEC1^{FoxO1-KO} cells. MEC1^{wt} and MEC1^{FoxO1-KO} were stained with CFSE or FarRed CellTrace dye, respectively, and incubated overnight. The next day equal numbers of CFSE stained and FarRed stained cells (each 50x10⁶) were mixed to obtain a population with their 1:1 ratio (validated by flow cytometry). A mixture of stained cells was injected into NSG mice via the tail vein (total 100x10⁶ cells, n=5). Mice were sacrificed after 6 hrs, and blood, spleen, bone marrow, and liver were analyzed by flow cytometry for the presence of viable CFSE or FarRed positive cells. The amount of MEC1 cells in each examined site is presented as relative migration between CFSE vs. FarRed positive cells. **(B)** *In vitro* competitive migration assay of MEC1^{wt} vs. MEC1^{FoxO1-KO}. MEC1^{wt} and MEC1^{FoxO1-KO} were stained with CFSE or FarRed CellTrace dye, respectively, and incubated overnight. The next day equal numbers of CFSE stained and FarRed stained cells were loaded to transwell. Migration (n=4) towards SDF1 (250 ng/ml), or CXCL13 (500 ng/ml), or conditioned media produced by human bone mesenchymal stromal cells (CM^{hMSC}) was allowed for 6 hrs, and the ratio of passed cells was assessed by flow cytometry. The number of migrated cells was normalized to the migration of cells applied to transwell without chemokines in the bottom chamber. **(C)** Competitive migration assay of CLL cells after *FoxO1* silencing by siRNA (siFoxO1) compared to cells transfected with control siRNA (siNC). Cells were incubated for 48 hrs and then stained with two different CellTrace dyes and loaded in 1:1 ratio on transwell. Migration (n=6) towards SDF1 (100 ng/ml), or CXCL13 (250 ng/ml), or conditioned media produced by human bone mesenchymal stromal cells (CM^{hMSC}) was allowed for 6 hrs, and the ratio of migrated cells was assessed by flow cytometry.

Supplemental Figure 18



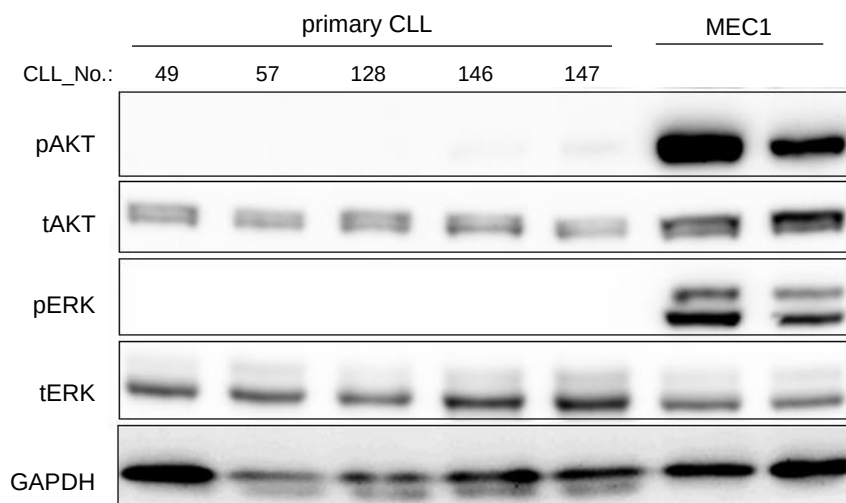
Supplemental Figure 18. (A) (i) Representative immunoblot for FoxO1 and GAB1 levels in primary CLL cells stimulated with anti-IgM (10 µg/ml) for 5 min or 48 hrs. **(ii)** GAB1 mRNA expression from CLL cells (n=4) treated with anti-IgM for 48 hrs. Ctrl stands for cells incubated in culture media only. **(B) (i)** Representative immunoblot of GAB1 and FoxO1 protein levels after coculture of MEC1 cells or primary CLL cells with HS5 stromal cells for 48 hrs. Primary CLL cells were seeded on a confluent layer of HS5 cells in an approx. 5:1 ratio (CLL:HS5). After 48 hrs cell suspension was stained with antibody against CD105 (a specific marker of stromal cells) and sorted by FACS (purity > 99,9% of CD19+CD5+ cells). **(ii)** GAB1 mRNA expression after coculture of primary CLL cells (n=4) with HS5 stromal cells for 48 hrs.

Supplemental Figure 19



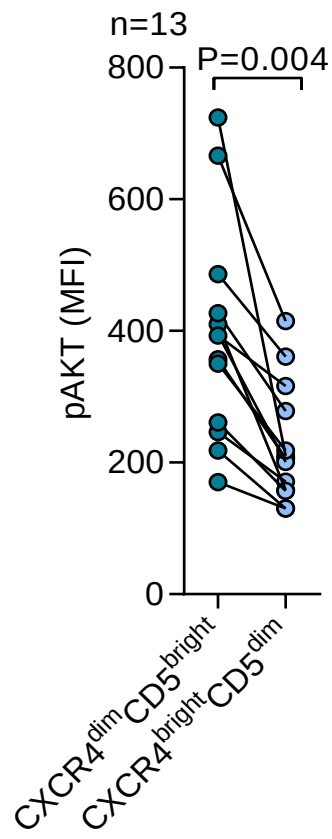
Supplemental Figure 19. Densitometric quantification of tGAB1 (**A**), pFoxO1 (**B**), tFoxO1 (**C**), pAKT (**D**), tAKT (**E**), tSYK (**F**), tPLC γ (**G**), tCD79a (**H**) and protein levels in MEC1 cells treated with 1 μ M inhibitor of AKT (iAKT) or vehicle (DMSO) for 12-48 hrs. MEC1 cells were used in this experiment, since primary CLL cells have relatively very low basal AKT phosphorylation levels to be inhibited (supplemental Figure 20).

Supplemental Figure 20



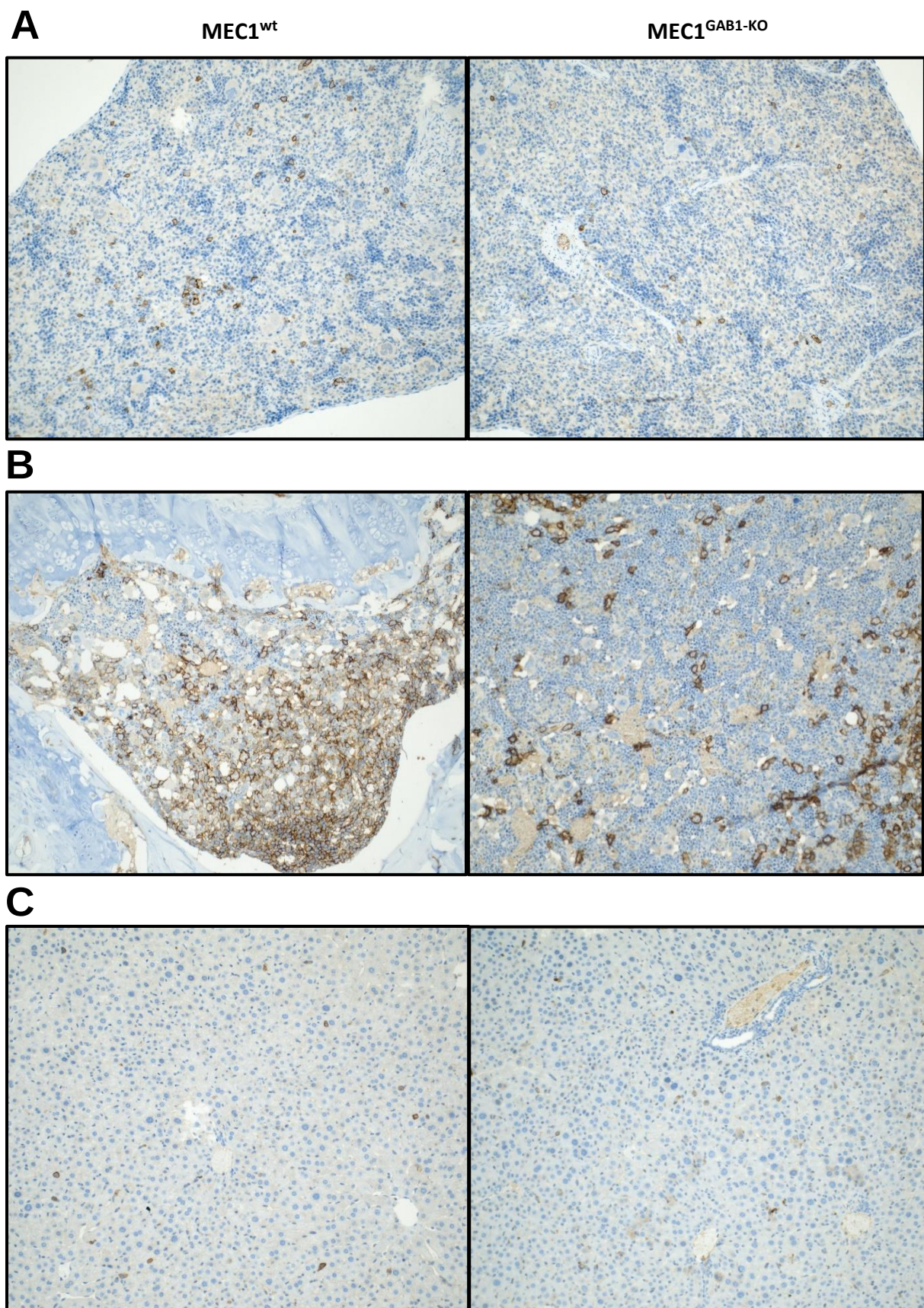
Supplemental Figure 20. Representative immunoblot for freshly isolated primary CLL cells and MEC1 cell line (samples from 2 different cultures of MEC1 cells).

Supplemental Figure 21



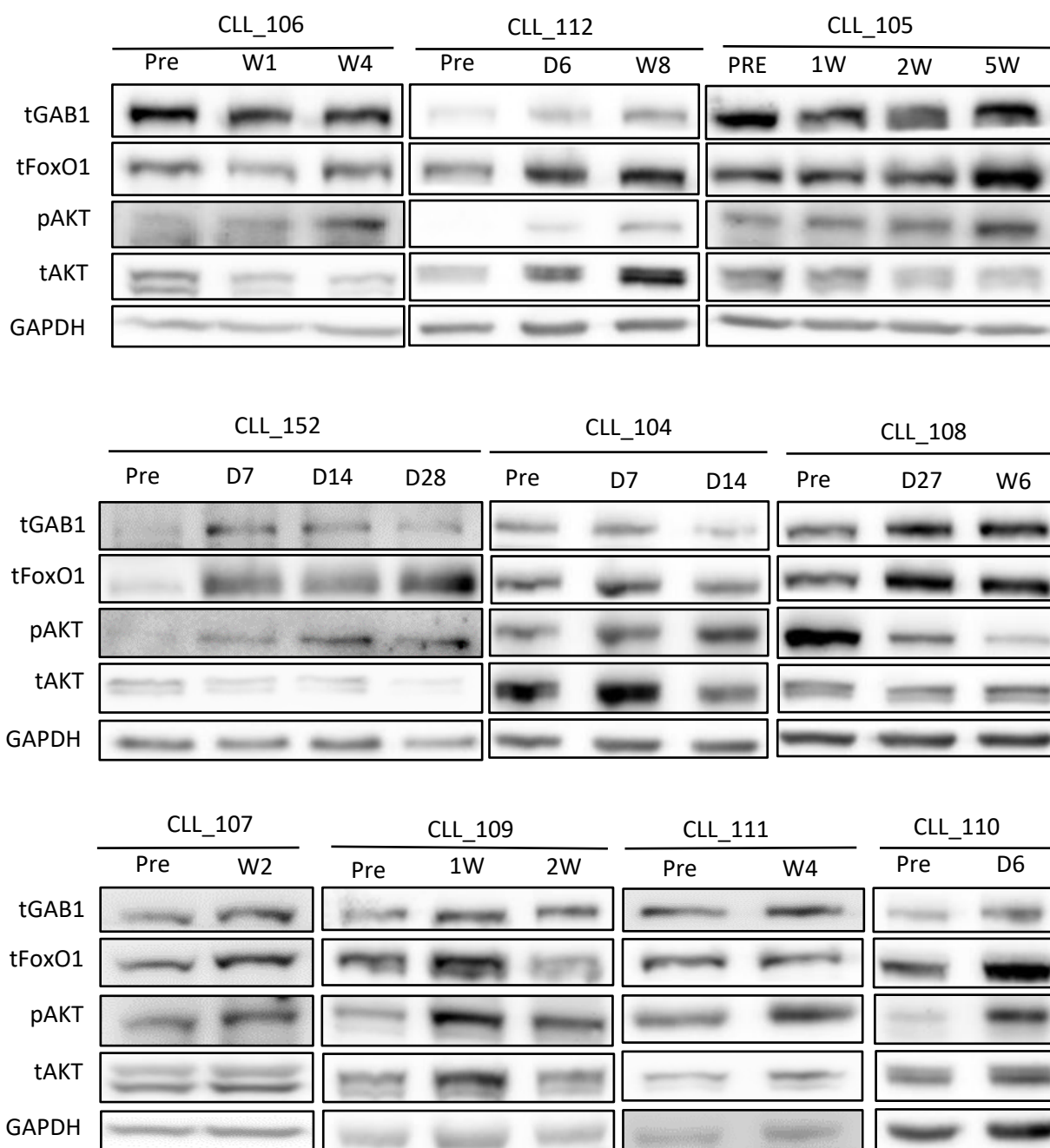
Supplemental figure 21. Intracellular staining of pAKT in CXCR4/CD5 CLL subpopulations.

Supplemental Figure 22



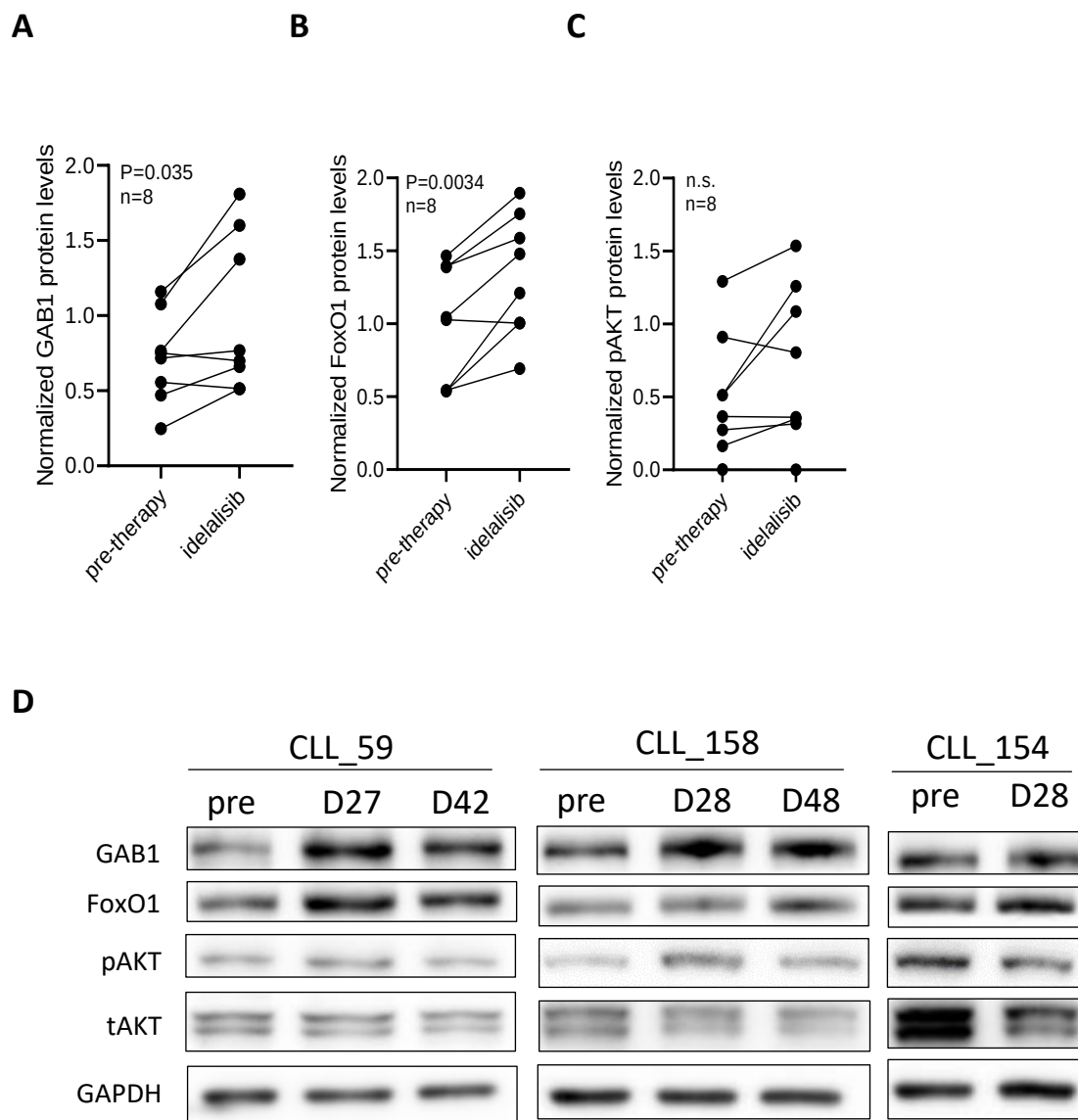
Supplemental Figure 22. Histological evaluation of spleen **(A)**, bone marrow **(B)**, and liver **(C)** of NSG mice transplanted with MEC1^{wt} (left) or MEC1^{GAB1-KO} (right) cells. In this experiment, MEC1^{wt} or MEC1^{GAB1-KO} cells were transplanted separately to individual NSG mice via a tail vein injection and sacrificed after 3 weeks. Tissue sections were stained with anti-human-CD20 antibody (brown signal) and the post-counterstaining by Bluing reagent (Ventana). The composition of the spleen structure in NSG mice is different from normal mouse spleen and reflects the absence of mature B/T/NK cells in these animals (see Supplemental Fig. 10D for hematoxylin-eosin staining of the spleen). Therefore, the white pulp of the spleen is absent, and the organ is mainly formed by red pulp with a high content of overactive erythron and excessive erythropoiesis (including the presence of megakaryocyte). The migrated MEC1 cells were localized diffusely in the spleen, and there was no difference in tissue localization for GAB1-knockout versus GAB1-wt cells (data not shown).

Supplemental Figure 23



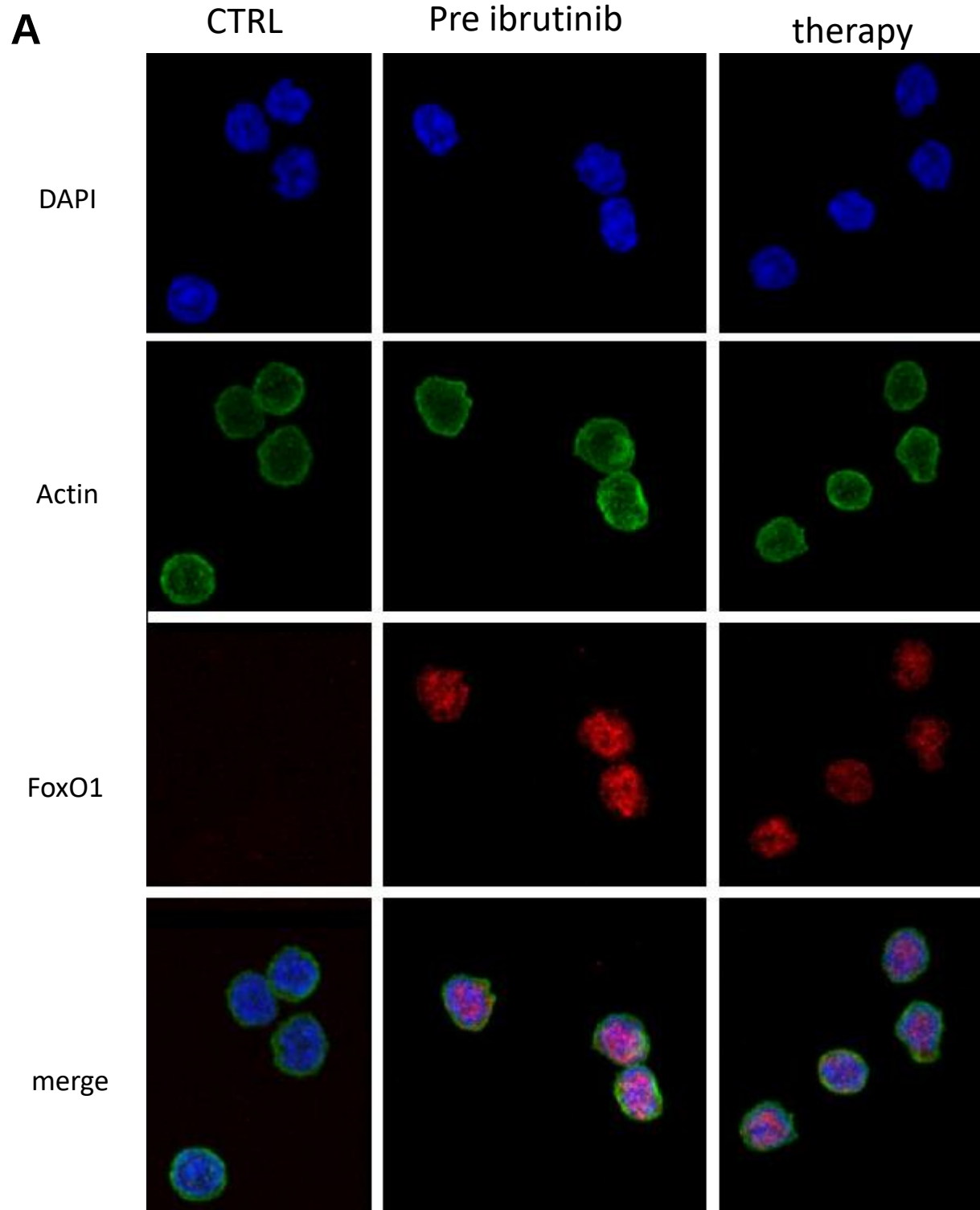
Supplemental Fig. 23 Additional immunoblots from primary CLL cells isolated from peripheral blood of patients before and during ibrutinib therapy. “Pre” stands for samples isolated immediately before the first administration of ibrutinib, and samples obtained during therapy are indicated by the number of days (D) or weeks (W) on therapy.

Supplemental Figure 24

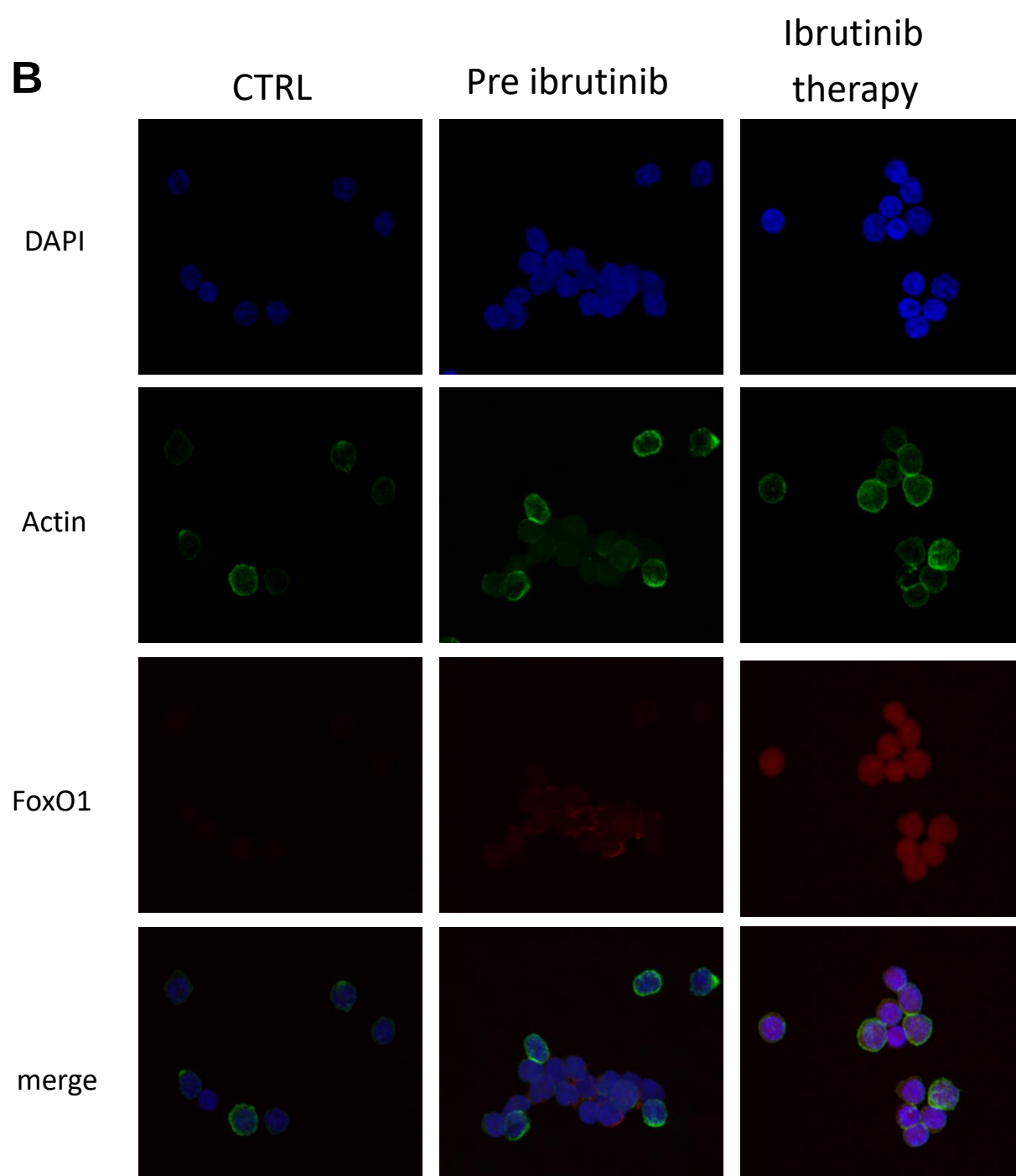


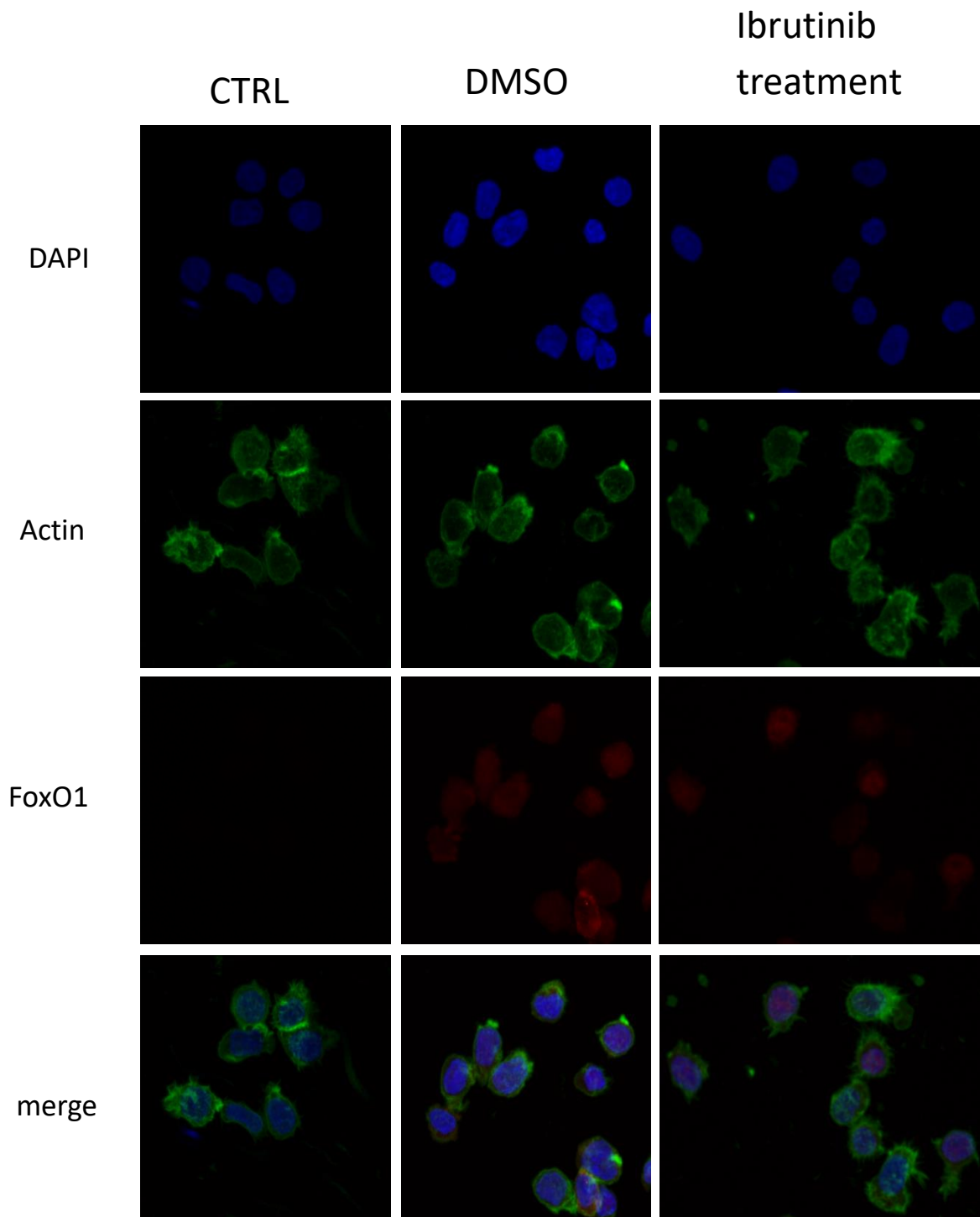
Supplemental Fig. 24 (A-E) Densitometric quantification of tGAB1 (**A**), tFoxO1 (**B**), pAKT (**C**) protein levels in primary CLL cells before (pre-therapy) and during idelalisib therapy as a single agent (week 4). The density of each protein band was normalized to endogenous control. (**D**) Representative immunoblots from primary CLL cells isolated from peripheral blood of patients before (pre) and during idelalisib therapy (D stands for the number of days on therapy).

Supplemental Figure 25



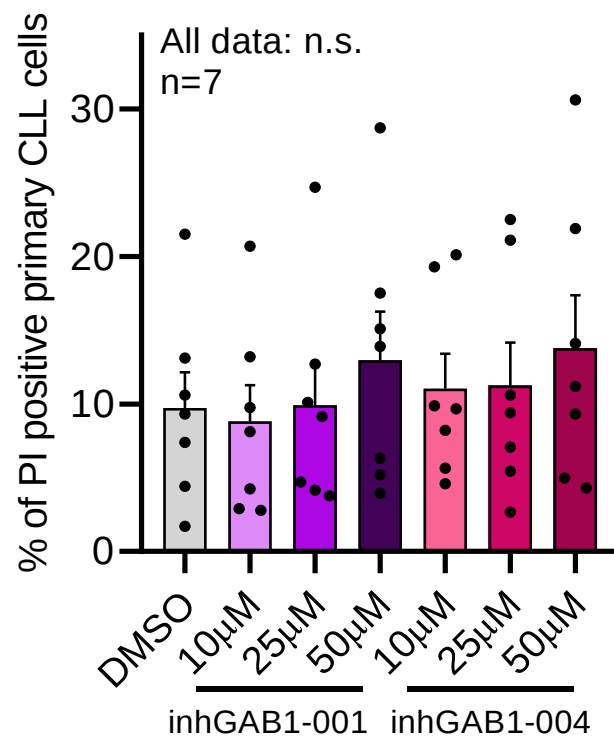
B



C

Supplemental Figure 25. (A-B) Representative confocal microscopy picture of 2 primary CLL samples isolated from the patient before (pre) and during ibrutinib therapy ([**A**] week 2, [**B**] week 8; for patient characteristic, see sample CLL_160 and CLL_112, respectively, Supplemental Tab.1). (**C**) Representative confocal microscopy of MEC1 cells treated with 1 μ M ibrutinib or with an equal volume of control (DMSO) for 5 days. In control sample (CTRL) no anti-FoxO1 primary antibody was used. Blue staining (DAPI) represents the nucleus, green represents the cytoskeleton (actin), and red staining represents FoxO1.

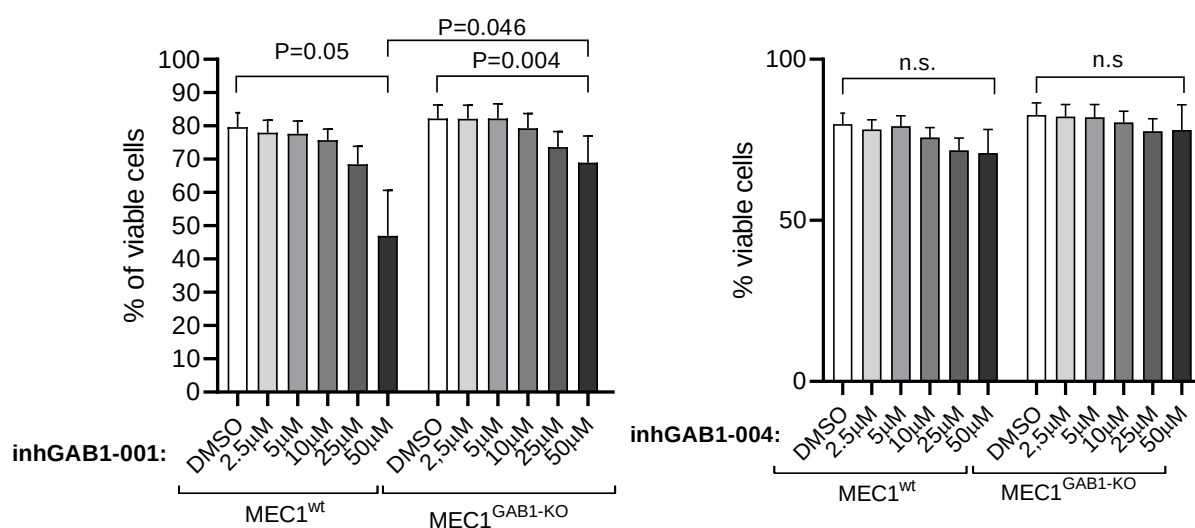
Supplemental Figure 26



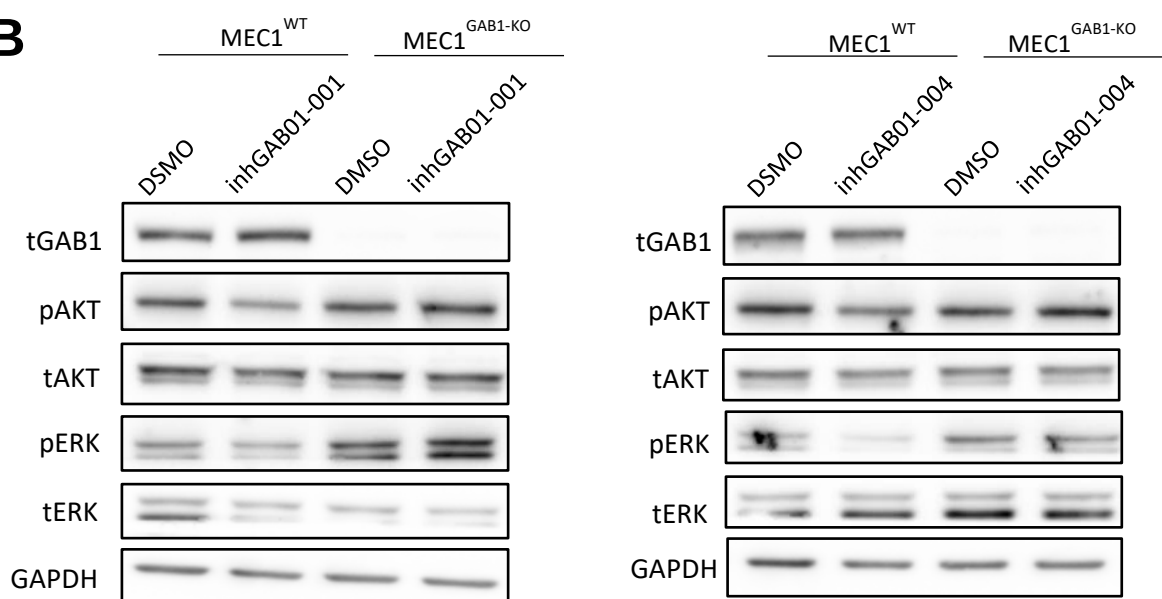
Supplemental Figure 26. Analysis of cell viability in primary CLL cells treated for 6 hrs with different doses of GAB1 inhibitor GAB1-001 or GAB1-004.

Supplemental Figure 27

A



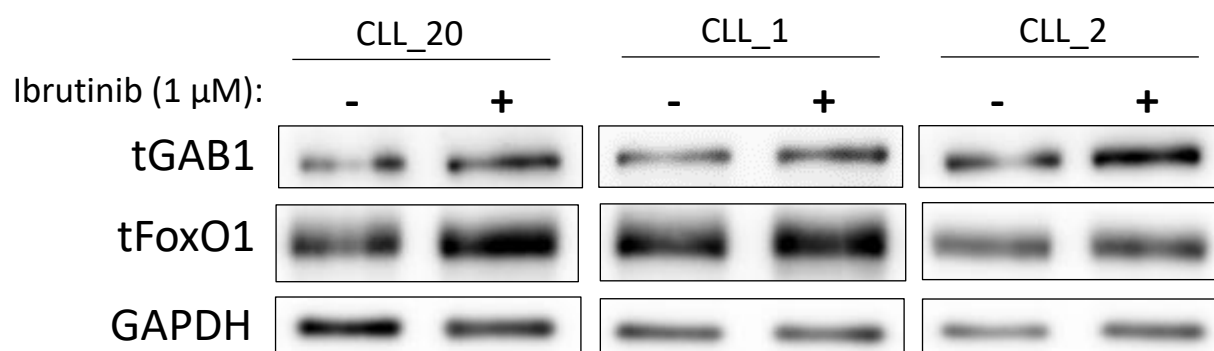
B



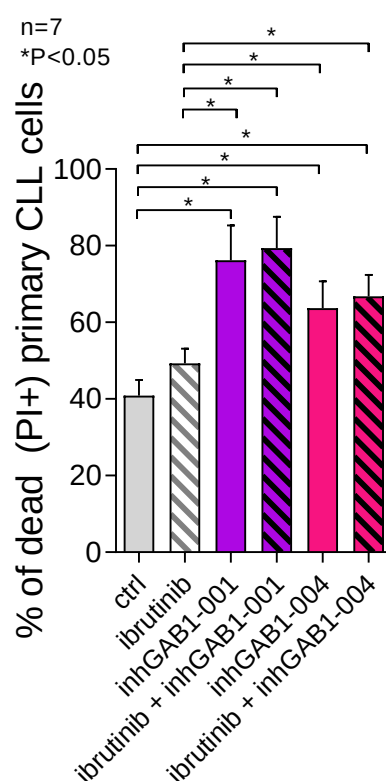
Supplemental Figure 27. (A) The effect of various doses (2.5-50 μM) of inhibitor GAB1-001 (left) or GAB1-004 (right) on the viability of MEC1^{WT} and MEC1^{GAB1-KO} cells (72 hrs, n=7 replicates). **(B)** Immunoblot for MEC1^{WT} and MEC1^{GAB1-KO} cells treated for 6 hrs with 50 μM inhibitor GAB1-001 (left) or GAB1-004 (right).

Supplemental Figure 28

A

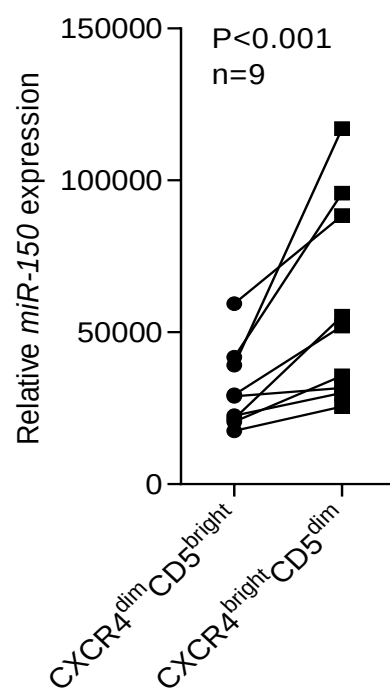


B



Supplemental Figure 28. (A) Representative immunoblot of primary CLL cells treated with 1 μM ibrutinib (+) or equal volume of DMSO (-) for 6 days. Cells were incubated in media containing 5 ng/ml IL4. **(B)** Statistical analysis of primary CLL cells' viability (n=7) after 48 hrs treatment with ibrutinib (1 μM) or two different GAB1 inhibitors (inhGAB1-001 or inhGAB1-004; 25 μM) or their combinations.

Supplemental Figure 29



Supplemental Figure 29. The relative expression of *miR*-150-5p in CXCR4/CD5 CLL subpopulations (n=9).

Supplemental Table 1: Characteristics of CLL patients

Patient ID	Sample used in figure	Gender	Age at time of diagnosis	Rai stage (at sample collection)	FISH results				TP53 mutation status	IGHV status
					del17p13 (%)	del11q23 (%)	trisomy 12 (%)	del13q14 (%)		
CLL 1	1A; 1D; 1G; S1; S2; S5; S6; S6; S16; S17; S28A	F	63	II B	neg	neg	neg	neg	wt	unmut
CLL 2	1A; 1D; 6I; 6J; S1; S2; S5; S26; S28A	M	66	IA	neg	pos (95)	neg	pos (96)	wt	unmut
CLL 3	1A; 2D; S1; S2; S8	M	56	II B	neg	neg	neg	neg	wt	unmut
CLL 4	1A; 2D; S1; S2; S9	F	54	0A	neg	neg	neg	pos (62)	wt	unmut
CLL 5	S9	M	56	II B	neg	neg	neg	neg	wt	unmut
CLL 6	1A; S1; S2	M	64	I A	neg	pos (94)	neg	pos (96)	wt	unmut
CLL 7	1A; S1; S2	F	68	IV	neg	pos (87)	neg	pos (10)	wt	---
CLL 8	1C; 1F; 1G; 3A; 3I; S3; S4; S6; S28	F	59	I A	neg	pos (64)	neg	pos (97)	wt	unmut
CLL 9	1C; 3A; 3I; S3; S4	M	74	IV A	neg	neg	neg	pos (94)	mut100	unmut
CLL 10	1C; 3A; 3I; S3; S4	F	64	III B	neg	pos (9)	neg	pos (47)	wt	---
CLL 11	1C; 3A; 3I; S3; S4; S28	M	71	II A	neg	neg	neg	neg	wt	---
CLL 12	1C; 3A; 3I; S3; S4; S28	F	59	III A-B	neg	neg	neg	pos (72)	wt	unmut
CLL 13	1C; 3A; 3I; S3; S4; S28	F	71	I A	neg	neg	neg	pos (59)	wt	unmut
CLL 14	1C; 3A; 3I; S3; S4; S28	M	79	IIIA	neg	pos (7)	neg	neg	wt	unmut
CLL 15	1C; 3A; 3I; S3; S4; S28	F	62	I A	neg	neg	neg	pos (7)	wt	unmut
CLL 16	1C; 3A; 3I; S3; S4; S28	M	70	I A	neg	neg	neg	pos (99)	wt	unmut
CLL 17	1C; 3A; 3I; S3; S4	F	65	II A	neg	neg	neg	pos (95)	wt	unmut
CLL 18	1D; 1G; 1E; 1F; 3F; 3H; 6C; S2; S5; S6; S28	M	58	I A	neg	neg	neg	43% cells with monoalelic deletion	wt	unmut
CLL 19	1D; 1G; 2H; 6D; 6E; 6I; 6J; S5; S6; S6; S19; S28A	M	62	II A	neg	neg	neg	pos (95)	wt	mut
CLL 20	1D; 1G; 3G; 6I; 6J; S5; S6; S11; S12; S28A	M	64	I A	neg	neg	neg	pos (9)	wt	---

CLL 21	1D; 1G; S2; S5; S6; S6	M	73	III A	neg	pos (74)	neg	neg	wt	unmut
CLL 22	1D; 1G; S5; S6; S6	M	68	IV A	neg	pos (98)	neg	neg	wt	
CLL 23	1D; 6C; S1; S2; S5; S18; S28	F	70	IA	neg	pos (95)	neg	pos (36)	wt	mut
CLL 24	1E; 2H; 3F; 3H; S19; S21	F	77	IVA	neg	neg	neg	81% cells with monoalelic deletion	wt	unmut
CLL 25	1E; 3F; 3H; S21	M	64	ND	tetrasomy (14%)	---	---	---	---	---
CLL 26	1E; 3F; 3H	M	90	IVA/B	neg	pos (34)	neg	pos (42)	wt	---
CLL 27	1E; 3F; 3H	M	58	IIIA	neg	neg	neg	96% cells with monoalelic deletion	mut (ND)	unmut
CLL 28	1E; 3F; 3H	M	68	IIA	neg	pos (97)	neg	neg	wt	unmut
CLL 29	1E; 3F; 3H	M	52		neg	neg	pos (65)	neg	wt	unmut
CLL 30	1E; 3F; 3H; S21	F	71	---	---	---	---	---	---	---
CLL 31	1E; 3F; 3H	M	46	IA	neg	neg	neg	pos (74)	wt	mut
CLL 32	1E; 3F; 3H	F	56	IIIB	neg	neg	neg	27% cells with monoalelic deletion	wt	unmut
CLL 33	1E; 3F; 3H; S21	M	69	OA	neg	neg	neg	neg	wt	unmut
CLL 34	1E; 3F; 3H; S21	F	75	III B	pos (del, 94)	neg	neg	91 % cells with bialelic deletion	mut (77)	mut
CLL 35	1E; 3F; 3H	M	64	OA	neg	neg	neg	pos (10)	wt	mut
CLL 36	1E; 3F; 3H; S21	F	70	A	neg	neg	pos (50)	neg	ND	ND
CLL 37	1E; 3F; 3H; S21	F	75	IIIB	pos (13)	4 copies of gene (63)	tetrasomy (62)	deletion (11); 4 copies (30); 3 copies (32)	wt	---
CLL 38	1E; 3F; 3H; S21	M	55	ND	neg	pos (98)	neg	neg	wt	unmut
CLL 39	1E; 3F; 3H	M	67	OA	neg	neg	neg	pos (41)	wt	mut
CLL 40	1E; 3F; 3H; 6C, S28	M	78	IVA	neg	pos (99)	neg	99% cells with monoalelic deletion	wt	unmut
CLL 41	1E; 3F; 3H; 6C, S28	F	54	IA	neg	neg	neg	25 % cells with monoalelic deletion	wt	mut
CLL 42	1E; 3F; 3H; 6D; 6E	F	66	IA	neg	neg	pos (89)	neg	wt	unmut

CLL 43	1E; 3F; 3H; 6I; 6J; S2	M	30	IIB	neg	neg	neg	neg	wt	unmut
CLL 44	1G; 3D; 6C; 6D; 6E; S6; S28	F	52	IA	neg	neg	neg	97% cells with bialelic deletion	wt	mut
CLL 45	1G; 3G; S6	M	76	IVB	neg	pos (66)	neg	pos (75)	mut	ND
CLL 46	1G; S8; S6; S11; S12	M	73	IIA	neg	neg?	neg	---	wt	unmut
CLL 47	2D; 2G; 4F; 6D; 6E; 6I; 6J; S9; S18; S26	F	54	IIIA	neg	neg	pos (85)	neg	wt	---
CLL 48	2D; S9	F	68	IV B	neg	pos (92)	neg	pos (88)	wt	mut
CLL 49	2D; S9; S20	M	70	IIB	neg	neg	neg	pos (8)	wt	mut
CLL 50	2H	F	76	ND	neg	neg	neg	pos (92)	mut (85)	unmut
CLL 51	2H	M	66	III A	neg	pos (28)	neg	pos (96)	wt	unmut
CLL 52	2H	F	73	II A	neg	neg	neg	neg	wt	unmut
CLL 53	2H; 6I; 6J; S16; S17; S19	M	73	---	neg	neg	neg	pos (95)	wt	---
CLL 54	2H; 6I; 6J; S16; S17; S19; S26	F	73	I A	neg	neg	pos (20)	pos (69)	wt	mut
CLL 55	2H; 6I; 6J; S17; S19; S26	F	75	0A	neg	neg	neg	neg	wt	mut
CLL 56	2H; S19	F	61	I A	neg	neg	neg	pos (46)	wt	mut
CLL 57	2H; S20	M	69	IV	neg	neg	neg	pos (6)	wt	unmut
CLL 58	3D	F	62	1A	neg	neg	neg	pos (81)	wt	mut
CLL 59	3D; 6A; 6B; S16; S17; S24; S26	F	59	---	neg	pos (93)	neg	neg	wt	unmut
CLL 60	3E	F	60	0	neg	neg	neg	pos (85)	wt (10)	mut
CLL 61	3E	F	67	0A	pos (62)	neg	neg	pos (71)	mut (63)	unmut
CLL 62	3E	M	59	IIA	neg	neg	neg	neg	wt	unmut
CLL 63	3E	M	50	IIB	neg	pos (95)	neg	pos (80)	wt	unmut
CLL 64	3E	F	61	IIIA	pos (98)	neg	neg	neg	mut (49)	unmut
CLL 65	3E	M	56	IIIA	pos (84)	neg	neg	pos (86)	mut (75)	unmut
CLL 66	3E	M	67	III	pos (93)	neg	neg	neg	wt	unmut
CLL 67	3E	F	58	IB	pos (47)	neg	neg	pos (95)	mut (95)	mut
CLL 68	3E	M	67	IVA	neg	neg	neg	pos (99)	mut (48)	unmut
CLL 69	3E	M	76	IVB	pos (63)	neg	neg	pos (62)	mut (95)	unmut

CLL 70	3E	F	57	II	neg	neg	neg	neg	mut (44)	unmut
CLL 71	3E	F	63	III	neg	neg	neg	neg	wt	unmut
CLL 72	3E	M	58	IIA	pos (97)	neg	neg	pos (18)	mut (91)	unmut
CLL 73	3E	M	62	IIB	neg	neg	neg	neg	wt	unmut
CLL 74	3E	F	67	I	neg	neg	neg	pos (98)	wt	mut
CLL 75	3E	M	66	O	neg	neg	neg	neg	wt	unmut
CLL 76	3E	M	63	IA-B	neg	neg	pos (81)	neg	wt	unmut
CLL 77	3E	F	56	IVA	neg	neg	neg	pos (15)	wt	unmut
CLL 78	3E	F	56	N/A	pos (66)	neg	neg	pos (94)	mut (47)	mut
CLL 79	3E	M	62	IV	neg	pos (100)	pos (ND)	neg	mut (89)	unmut
CLL 80	3E	F	68	III	neg	pos (96)	neg	pos (49)	wt	unmut
CLL 81	3E	F	57	II	neg	pos (93)	neg	pos (63)	wt	unmut
CLL 82	3E	M	60	II B	neg	pos (98)	neg	pos (99)	wt	unmut
CLL 83	3E	M	54	IVA	neg	neg	neg	neg	wt	unmut
CLL 84	3E	M	67	IIA	pos (93)	neg	pos (76)	pos (97)	mut (46)	mut
CLL 85	3E	M	72	IVA-B	neg	neg	neg	neg	del (82)	---
CLL 86	3E	F	56	III	neg	neg	pos (88)	pos (90)	mut (67)	mut
CLL 87	3E	F	54	IV	neg	neg	neg	pos (62)	wt	unmut
CLL 88	3E	M	49	O	neg	neg	neg	pos (96)	wt	mut
CLL 89	3E	M	65	IV	neg	pos (88)	neg	neg	wt	mut
CLL 90	3E	M	47	IVB	pos (72)	pos (38)	pos (35)	pos (35)	mut (90)	unmut
CLL 91	3E	M	77	III	pos (9)	neg	neg	pos (92)	mut (54)	unmut
CLL 92	3E	M	38	IIA	pos (88)	pos (11)	neg	pos (35)	mut (15)	unmut
CLL 93	3E	M	45	IV	neg	neg	neg	neg	mut (22)	mut
CLL 94	3E	M	55	III	neg	neg	pos (28)	neg	mut (46)	unmut
CLL 95	3E	M	75	III-IV	neg	pos (90)	neg	neg	wt	unmut
CLL 96	3E	M	38	I	neg	pos (99)	neg	pos (50)	wt	unmut
CLL 97	3E	F	59	IIB	neg	neg	neg	pos (21)	mut (99)	unmut
CLL 98	3E	M	43	I	neg	pos (15)	neg	neg	wt	unmut

CLL 99	3E	F	58	IIIA	neg	neg	neg	neg	wt	unmut
CLL 100	3E	M	64	IVA-B	neg	neg	neg	neg	wt	unmut
CLL 101	4A; S12	F	75	---	neg	neg	neg	mut (93)	wt	mut
CLL 102	5Di; 5Dii	M	47	IB	pos	neg	neg	neg	wt	unmut
CLL 103	1E; 3E; 3F; 3H; 5Dii	M	39	IVB	pos (12)	pos (85)	neg	pos (15)	mut (15)	unmut
CLL 104	5Dii; 5E; S23	M	59	IVB	neg	pos (80)	neg	pos (83)	wt	unmut
CLL 105	5Dii; 5E; S23	M	43	ND	neg	pos (96)	pos (80)	neg	wt	unmut
CLL 106	5Dii; 5E; S23	M	48	IVB	neg	neg	neg	pos (85)	wt	unmut
CLL 107	5Dii; 6B; S11; S23	M	65	IA	neg	neg	pos (86)	neg	wt	unmut
CLL 108	5Dii; S23	M	51	IVA	neg	neg	neg	neg	wt	unmut
CLL 109	5Dii; S23	F	64	---	neg	neg	neg	pos (8)	wt	mut
CLL 110	5Dii; S23	M	71	IVB	pos (85)	pos (15)	neg	neg	mut (95)	unmut
CLL 111	5Dii; S23	M	67	IA	neg	pos (86)	neg	pos (11)	wt	unmut
CLL 112	5Dii; S23; S25	M	58	IIB	neg	neg	neg	neg	wt	unmut
CLL 113	5E	M	34	I	pos (16)	neg	neg	neg	ND	mut
CLL 114	5E	F	51	IV	neg	neg	pos (52)	neg	ND	unmut
CLL 115	5E	M	57	IV	pos (60.5)	neg	neg	pos (96)	mut	mut
CLL 116	5E	M	75	I	neg	neg	neg	pos (78.5)	ND	mut
CLL 117	5E	M	56	I	pos (35.5)	neg	neg	pos (4.5)	mut	unmut
CLL 118	5E	F	67	IV	pos (93.5)	pos (93.5)	neg	neg	ND	unmut
CLL 119	5E	M	54	IA	neg	pos (60)	neg	pos (54)	wt	unmut
CLL 120	5E	F	53	IIIA	pos (8)	neg	neg	neg	wt	unmut
CLL 121	6B	M	66	IA	neg	neg	pos (77)	neg	wt	unmut
CLL 122	6B	M	64	IA	neg	neg	pos (81)	neg	wt	unmut
CLL 123	6B	M	64	IV-A	neg	neg	neg	neg	wt	ND
CLL 124	6C; S28	M	72	III A	neg	pos (0.7)	neg	73 % monoalelic deletion	wt	unmut

CLL 125	6C; S28	F	54	IIIB	neg	neg	pos (0.74)	neg	wt	biclonal /unmut
CLL 126	6D; 6E	M	51	0A	neg	neg	pos (82)	neg	wt	unmut
CLL 127	6D; 6E; 6I; 6J; S18	F	46	IVA-B	neg	neg	neg	pos (89)	wt	mut
CLL 128	6G; 6F; S11; S20	F	79	IA-B	neg	neg	neg	pos (8)	wt	mut
CLL 129	6I; 6J; S26	F	60	II B	neg	pos (16)	neg	neg	wt	unmut
CLL 130	6I; 6J	F	72	III A	neg	neg	neg	pos (52)	wt	unmut
CLL 131	6I; 6J	M	87	IV B	---	---	---	---	---	---
CLL 132	6I; 6J; S2	M	67	III B	neg	neg	neg	neg	---	mut
CLL 133	6I; 6J; S2; S12; S16; S26	M	77	I A	neg	neg	neg	pos (9)	wt	---
CLL 134	S10	M	76	0A	neg	neg	neg	pos (20)	wt	---
CLL 135	S10	M	61	IIIB	neg	pos (44)	neg	pos (9)	wt	unmut
CLL 136	S10	M	71	IV B	neg	pos (73)	neg	neg	mut	unmut
CLL 137	S10	F	66	IIIB	neg	pos (70)	neg	neg	wt	unmut
CLL 138	S11	F	80	IIIA	neg	neg	pos (63)	neg	wt	---
CLL 139	S11; S12	F	60	IIB	neg	pos (95)	neg	pos (8)	wt	unmut
CLL 140	S12	F	55	0A	neg	neg	neg	mut (60)	wt	mut
CLL 141	S18	F	73	IVA	neg	neg	pos (70)	neg	wt	---
CLL 142	S18	F	57	0A	neg	neg	neg	pos (45)	mut (40)	mut
CLL 143	S18	M	83	---	neg	neg	pos (78)	neg	wt	---
CLL 144	S18	F	74	IB	neg	neg	pos (80)	neg	wt	---
CLL 145	S18	M	70	IVA	neg	pos (52)	neg	neg	mut (35)	unmut
CLL 146	S20	M	69	IA	neg	neg	pos (81)	neg	wt	unmut
CLL 147	S20	M	68	IIB	neg	neg	pos (67)	pos (66)	wt	mut
CLL 148	S21	M	75	IIB	neg	neg	neg	neg	wt	unmut
CLL 149	S21	F	64	IA	neg	neg	neg	neg	wt	unmut
CLL 150	S21	M	71	IIA	neg	neg	neg	neg	wt	mut
CLL 151	S21	F	69	IIA	neg	neg	pos (72)	pos (61)	wt	mut
CLL 152	S23	M	64	III B	neg	neg	neg	neg	wt	unmut

CLL 153	S24	F	50	IV A	neg	pos (49)	neg	neg	mut	---
CLL 154	S24	F	74	---	neg	neg	pos (78)	neg	wt	---
CLL 155	S24	M	61	IV A	neg	pos (86)	neg	neg	wt	unmut
CLL 156	S24	M	---	---	neg	neg	neg	pos (94)	wt	unmut
CLL 157	S24	M	---	---	neg	pos (96)	neg	pos (94)	wt	mut
CLL 158	S24	F	63	IV A	neg	neg	neg	pos (55)	mut	---
CLL 159	S24	F	82	II A	neg	neg	neg	pos (13)	mut (22)	---
CLL 160	S25	M	34	IA	neg	pos (74)	neg	pos (15)	minor mut (2.5)	unmut
CLL 161	S2	M	80	---	neg	neg	neg	neg	wt	unmut
CLL 162	S29	M	69	IV B	neg	pos (91)	neg	pos (96)	wt	unmut
CLL 163	S29	M	68	III B	neg	neg	neg	pos (98)	wt	unmut

Supplemental Table 2: List of top differentially expressed genes in CXCR4/CD5 subpopulations (top 30 upregulated and top 30 downregulated mRNAs)

Gene ID	Gene name	Base Mean (reads per million)	Fold Change (log2) *	adjusted P-value
ENSG00000118432	CNR1	1722.72	2.39	2.80E-16
ENSG00000171246	NPTX1	1555.61	2.21	1.15E-15
ENSG00000135116	HRK	1111.617	2.05	1.28E-15
ENSG00000112182	BACH2	10947.54	2.05	2.99E-44
ENSG00000111266	DUSP16	219.72	2.01	8.96E-18
ENSG00000069188	SDK2	1503.15	1.91	1.39E-12
ENSG00000065534	MYLK	2140.31	1.91	8.31E-18
ENSG00000179088	C12orf42	256.13	1.87	2.05E-17
ENSG00000166949	SMAD3	289.63	1.81	4.59E-14
ENSG00000131941	RHPN2	276.96	1.81	3.23E-28
ENSG00000152953	STK32B	462.16	1.73	2.35E-06
ENSG00000186350	RXRA	2740.28	1.72	1.55E-11
ENSG00000099958	DERL3	1749.33	1.71	2.61E-14
ENSG00000102053	ZC3H12B	895.52	1.63	1.08E-37
ENSG00000169762	TAPT1	2568.98	1.62	6.45E-12
ENSG00000184897	H1FX	7058.84	1.60	3.21E-37
ENSG00000204569	PPP1R10	2626.45	1.59	2.89E-28
ENSG00000135299	ANKRD6	273.52	1.55	5.14E-33
ENSG00000172794	RAB37	1088.77	1.48	3.46E-17
ENSG00000165029	ABCA1	3209.94	1.47	6.96E-10
ENSG00000254870	ATP6V1G2- DDX39B	227.76	1.46	8.12E-14
ENSG00000141068	KSR1	690.79	1.46	5.01E-29
ENSG00000072134	EPN2	271.56	1.44	1.34E-18
ENSG00000101190	TCFL5	715.48	1.44	1.75E-14
ENSG00000215912	TTC34	440.05	1.44	3.82E-19
ENSG00000118513	MYB	290.40	1.42	5.37E-05
ENSG00000121966	CXCR4	82583.46	1.40	3.26E-06
ENSG00000150672	DLG2	570.30	1.40	3.76E-09
ENSG00000099953	MMP11	421.29	1.40	7.99E-18
ENSG00000166348	USP54	347.07	1.39	4.46E-26
ENSG00000129226	CD68	953.26	-3.76	2.05E-125
ENSG00000197629	MPEG1	2503.31	-3.12	1.13E-35
ENSG00000116701	NCF2	1227.26	-2.90	4.51E-53
ENSG00000124772	CPNE5	2444.89	-2.57	1.17E-21
ENSG00000186818	LILRB4	949.50	-2.56	2.30E-22
ENSG00000277632	CCL3	316.78	-2.54	7.13E-16

ENSG00000140678	ITGAX	7274.70	-2.48	4.60E-28
ENSG00000203896	LIME1	225.21	-2.43	6.65E-47
ENSG00000278195	SSTR3	255.24	-2.29	2.25E-23
ENSG00000173366	AC097637.1	240.20	-2.25	7.01E-15
ENSG00000177685	CRACR2B	1095.87	-2.23	5.75E-37
ENSG00000011028	MRC2	200.59	-2.18	1.07E-18
ENSG00000186810	CXCR3	307.94	-2.18	2.69E-16
ENSG00000126860	EVI2A	579.76	-2.11	2.71E-39
ENSG00000169413	RNASE6	813.61	-2.08	2.40E-19
ENSG00000188389	PDCD1	361.91	-2.02	1.42E-55
ENSG00000167711	SERPINF2	275.58	-2.01	2.32E-28
ENSG00000138623	SEMA7A	724.22	-1.98	2.46E-34
ENSG00000133067	LGR6	228.25	-1.98	7.69E-24
ENSG00000030582	GRN	6864.43	-1.97	6.96E-60
ENSG00000179841	AKAP5	255.83	-1.96	3.41E-17
ENSG00000116771	AGMAT	230.14	-1.96	1.40E-33
ENSG00000261150	EPPK1	281.68	-1.95	8.57E-14
ENSG00000146386	ABRACL	290.48	-1.95	1.78E-24
ENSG00000216490	IFI30	1022.84	-1.92	3.86E-14
ENSG00000115956	PLEK	2534.33	-1.91	1.46E-15
ENSG00000196154	S100A4	3060.76	-1.91	6.17E-42
ENSG00000189159	JPT1	358.79	-1.90	1.57E-22
ENSG00000157388	CACNA1D	574.72	-1.87	4.32E-10
ENSG00000172824	CES4A	426.87	-1.87	1.12E-16
*30 most up-regulated genes (positive fold change) and down-regulated genes (negative fold change) in CXCR4 ^{bright} CD5 ^{dim} subpopulation.				

Supplemental Table 3: List of migration-related genes which were identified as differentially expressed in CXCR4/CD5 subpopulations

Gene ID	Gene name	Base Mean (reads per million)	Fold Change (log2) *	adjusted P-value
ENSG00000114353	GNAI2	8685	-0.95	4.08E-75
ENSG00000100823	APEX1	3708	-1.05	2.8E-59
ENSG00000030582	GRN	6864	-1.97	7.4E-57
ENSG00000128340	RAC2	9335	-1.75	2.46E-53
ENSG00000182054	IDH2	1225	-1.23	1.14E-50
ENSG00000108518	PFN1	8083	-0.96	1.63E-47
ENSG00000100292	HMOX1	1000	-1.76	2.45E-43
ENSG00000159842	ABR	11899	1.05	1.36E-42
ENSG00000205542	TMSB4X	27296	-1.33	3.42E-41
ENSG00000103490	PYCARD	302	-1.53	1.59E-39
ENSG00000197043	ANXA6	6852	-1.34	8.87E-39
ENSG00000172725	CORO1B	2034	-0.75	7.85E-38
ENSG00000149781	FERMT3	2224	-1.24	3.7E-36
ENSG00000176973	FAM89B	519	-1.23	7.76E-36
ENSG00000108561	C1QBP	984	-1.00	2.72E-35
ENSG00000002586	CD99	2088	-1.11	5.98E-35
ENSG00000131375	CAPN7	2115	0.92	5.99E-33
ENSG00000149925	ALDOA	7757	-0.64	1.32E-32
ENSG00000138623	SEMA7A	724	-1.98	2.66E-32
ENSG00000100092	SH3BP1	1891	-0.96	7.89E-32
ENSG00000121879	PIK3CA	3805	0.75	5.77E-31
ENSG00000134242	PTPN22	5538	-0.84	1.36E-30
ENSG00000139626	ITGB7	6295	-1.74	1.79E-30
ENSG00000105429	MEGF8	1215	0.80	1.85E-30
ENSG00000149177	PTPRJ	8064	-0.83	6.66E-30
ENSG00000196712	NF1	2136	1.13	5.83E-28
ENSG00000019995	ZRANB1	2053	0.69	6.03E-28
ENSG00000064666	CNN2	5355	-1.32	2.19E-27
ENSG00000142798	HSPG2	725	-1.51	6.47E-27
ENSG00000140678	ITGAX	7275	-2.48	2.76E-26
ENSG00000023902	PLEKHO1	3666	-0.87	2.88E-26
ENSG00000177169	ULK1	2386	0.62	4.53E-26
ENSG00000136167	LCP1	12293	-1.04	1.06E-25
ENSG00000108854	SMURF2	1459	0.69	2.23E-25
ENSG00000144711	IQSEC1	2265	-0.91	4.25E-25
ENSG00000172757	CFL1	9706	-0.61	9.42E-25
ENSG00000143322	ABL2	2082	0.93	2.11E-24
ENSG00000164190	NIPBL	8141	0.61	4.29E-24
ENSG00000111348	ARHGD1B	9596	-0.75	1.85E-23
ENSG00000163466	ARPC2	10620	-0.81	2.38E-23
ENSG00000132386	SERPINF1	2035	-1.47	3.17E-23
ENSG00000157764	BRAF	3089	0.63	8.28E-23
ENSG00000134318	ROCK2	1826	0.60	1.91E-22
ENSG00000155366	RHOC	1754	-1.33	1.98E-22
ENSG00000133067	LGR6	228	-1.98	2.87E-22
ENSG00000197471	SPN	1125	-1.27	1.59E-21
ENSG00000103769	RAB11A	1740	-0.95	1.78E-21
ENSG00000184481	FOXO4	969	0.70	2.34E-21
ENSG00000139146	SINHCAF	4318	0.92	2.49E-21
ENSG00000139725	RHOF	893	-0.92	4.00E-21

ENSG00000162704	ARPC5	2307	-1.17	7.62E-21
ENSG00000123338	NCKAP1L	12107	-0.74	9.13E-21
ENSG00000160007	ARHGAP35	1749	0.96	1.04E-20
ENSG00000122694	GLIPR2	363	-1.59	1.93E-19
ENSG00000134954	ETS1	21200	1.00	1.97E-19
ENSG00000131504	DIAPH1	5515	-0.60	4.96E-19
ENSG00000100300	TSPO	986	-0.89	4.48E-18
ENSG00000136286	MYO1G	3398	-1.44	7.97E-18
ENSG00000065534	MYLK	2140	.1.91	8.31E-18
ENSG00000110934	BIN2	3042	-1.04	8.99E-18
ENSG00000185950	IRS2	2382	1.12	1.19E-17
ENSG00000102879	CORO1A	13492	-0.65	1.38E-17
ENSG00000083642	PDS5B	2239	0.70	2.36E-17
ENSG00000213654	GPSM3	2977	-0.63	2.51E-17
ENSG00000188906	LRRK2	5584	-0.64	1.35E-16
ENSG00000109919	MTCH2	672	-0.96	1.97E-16
ENSG00000109458	GAB1	6770	0.82	2.44E-16
ENSG00000177697	CD151	339	-1.78	2.55E-16
ENSG00000140443	IGF1R	344	1.42	3.55E-16
ENSG00000079385	CEACAM1	1015	-1.15	5.01E-16
ENSG00000173531	MST1	346	-1.15	7.41E-16
ENSG00000168961	LGALS9	4699	-0.75	1.28E-15
ENSG00000196924	FLNA	29974	-1.15	1.33E-15
ENSG00000109756	RAPGEF2	6076	0.65	1.46E-15
ENSG00000151651	ADAM8	1768	-1.25	1.46E-15
ENSG00000108819	PPP1R9B	5964	-0.89	1.64E-15
ENSG00000160255	ITGB2	480	-1.34	3.23E-15
ENSG00000166974	MAPRE2	1919	-0.66	3.96E-15
ENSG00000186810	CXCR3	308	-2.18	4.32E-15
ENSG00000026025	VIM	17798	-0.88	5.35E-15
ENSG00000277632	CCL3	317	-2.54	1.09E-14
ENSG00000115310	RTN4	1720	-0.63	1.5E-14
ENSG00000087589	CASS4	391	-1.16	1.87E-14
ENSG00000143669	LYST	9027	1.15	2.23E-14
ENSG00000213066	FGFR1OP	873	0.63	4.07E-14
ENSG00000166949	SMAD3	290	1.81	4.59E-14
ENSG00000163832	ELP6	294	-0.65	5.63E-14
ENSG00000146535	GNA12	4735	0.98	8.25E-14
ENSG00000150093	ITGB1	8907	-0.69	8.38E-14
ENSG00000136950	ARPC5L	1205	-0.78	8.98E-14
ENSG00000035664	DAPK2	292	-1.31	9.39E-14
ENSG00000118689	FOXO3	1987	1.06	1.26E-13
ENSG00000039523	RIPOR1	9249	0.84	1.46E-13
ENSG00000137509	PRCP	1533	-0.61	1.56E-13
ENSG00000131236	CAP1	5959	-0.74	5.82E-13
ENSG00000174483	BBS1	532	0.84	9.33E-13
ENSG00000261150	EPPK1	282	-1.95	1.00E-12
ENSG00000108840	HDAC5	2714	0.65	1.29E-12
ENSG00000169508	GPR183	1004	-1.79	2.1E-12
ENSG00000177105	RHOG	886	-0.81	2.31E-12
ENSG00000142347	MYO1F	1331	-0.61	7.07E-12
ENSG00000071051	NCK2	2153	1.09	1.1E-11
ENSG00000079337	RAPGEF3	7587	0.82	2.54E-11
ENSG00000150347	ARID5B	6961	0.60	3.47E-11
ENSG00000100219	XBP1	2708	0.91	4.1E-11
ENSG00000104998	IL27RA	774	-0.99	5.8E-11

ENSG00000174175	SELP	566	1.10	5.86E-11
ENSG00000134352	IL6ST	2266	0.80	1.77E-10
ENSG00000123358	NR4A1	578	1.23	3.3E-10
ENSG00000115109	EPB41L5	254	1.04	6.93E-10
ENSG00000149418	ST14	3406	-0.72	1.02E-09
ENSG00000196220	SRGAP3	520	-0.83	1.66E-09
ENSG00000140564	FURIN	1265	0.66	1.93E-09
ENSG00000127603	MACF1	7205	-0.65	5.98E-09
ENSG00000135622	SEMA4F	544	0.62	6.28E-09
ENSG00000275342	PRAG1	1966	-0.65	9.72E-09
ENSG00000107104	KANK1	730	0.61	9.96E-09
ENSG00000112139	MDGA1	2082	1.06	1.71E-08
ENSG00000152127	MGAT5	6438	0.63	3.27E-08
ENSG00000004399	PLXND1	2216	0.95	3.51E-08
ENSG00000144791	LIMD1	6186	-0.78	4.12E-08
ENSG00000166313	APBB1	1070	0.63	4.25E-08
ENSG00000102760	RGCC	1179	-0.93	7.29E-08
ENSG00000089159	PXN	1592	-0.64	9.01E-08
ENSG00000157193	LRP8	412	-0.67	1.01E-07
ENSG00000163697	APBB2	2695	0.89	1.09E-07
ENSG00000135503	ACVR1B	392	0.89	1.66E-07
ENSG00000163932	PRKCD	1513	-0.87	1.97E-07
ENSG00000079691	CARMIL1	1689	0.60	2.09E-07
ENSG00000068024	HDAC4	237	0.88	2.54E-07
ENSG00000130669	PAK4	208	0.82	1.01E-06
ENSG00000261371	PECAM1	4511	-0.78	1.9E-06
ENSG00000169896	ITGAM	1899	-1.30	1.91E-06
ENSG00000121966	CXCR4	82583	1.40	3.26E-06
ENSG00000107738	VSIR	416	-0.77	6.09E-06
ENSG00000117020	AKT3	1058	0.83	7.23E-06
ENSG00000160683	CXCR5	5662	-0.61	8.47E-06
ENSG00000143537	ADAM15	362	-0.67	1.45E-05
ENSG00000197381	ADARB1	2338	1.10	2.34E-05
ENSG00000158321	AUTS2	492	0.74	3.89E-05
ENSG00000160789	LMNA	334	-1.18	5.73E-05
ENSG00000198648	STK39	201	-0.68	6.67E-05
ENSG00000069702	TGFBR3	1979	-0.61	7.03E-05
ENSG00000182957	SPATA13	874	-0.62	0.000104
ENSG00000171522	PTGER4	847	-1.06	0.000138
ENSG00000067141	NEO1	305	0.62	0.000356
ENSG00000085733	CTTN	295	0.76	0.000739
* positive numbers mean a relative upregulation in CXCR4 ^{bright} CD5 ^{dim} subpopulation				

Supplemental Table 4: GO term analysis of differentially expressed genes in CXCR4/CD5 subpopulations

GO Term*	Description	P-value	FDR q-value	Enrichment	B	b
GO:0110053	regulation of actin filament organization	6.39E-12	7.89E-9	2.72	228	54
GO:0032956	regulation of actin cytoskeleton organization	8.98E-12	1.02E-8	2.48	292	63
GO:0032970	regulation of actin filament-based process	6.71E-11	4.52E-8	2.33	320	65
GO:0030832	regulation of actin filament length	9.03E-11	5.57E-8	3.00	157	41
GO:0008064	regulation of actin polymerization or depolymerization	2.79E-10	1.59E-7	2.94	156	40
GO:0007015	actin filament organization	3.46E-9	1.55E-6	2.46	224	48
GO:0030833	regulation of actin filament polymerization	1.62E-8	5.84E-6	2.79	144	35
GO:0030029	actin filament-based process	1.52E-7	4.68E-5	2.00	345	60
GO:0030036	actin cytoskeleton organization	1.95E-7	5.9E-5	2.10	284	52
GO:0030838	positive regulation of actin filament polymerization	1.63E-6	3.71E-4	2.19	71	20
GO:0032233	positive regulation of actin filament bundle assembly	3.86E-5	3.86E-3	2.91	54	15
GO:0034314	Arp2/3 complex-mediated actin nucleation	7.41E-5	6.42E-3	1.73	14	7
GO:0008154	actin polymerization or depolymerization	1.01E-4	8.13E-3	2.83	52	14
GO:0045010	actin nucleation	3.41E-4	2.02E-2	1.97	22	8
GO:0030834	regulation of actin filament depolymerization	4.3E-4	2.44E-2	2.93	46	12
GO:0032231	regulation of actin filament bundle assembly	7.97E-4	4.13E-2	1.29	84	17
GO:0040012	regulation of locomotion	1.35E-5	1.8E-3	3.44	813	106
GO:0040017	positive regulation of locomotion	1.21E-4	9.35E-3	1.92	461	64
GO:0040011	locomotion	1.65E-4	1.14E-2	1.73	835	103
GO:2000145	regulation of cell motility	3.78E-6	6.75E-4	1.62	753	102
GO:2000147	positive regulation of cell motility	4.5E-5	4.38E-3	2.11	437	63
GO:0048870	cell motility	3.31E-4	1.98E-2	1.25	763	94
GO:0030334	regulation of cell migration	3.58E-6	6.63E-4	2.76	706	97
GO:0030335	positive regulation of cell migration	2.91E-5	3.19E-3	2.11	422	62
GO:1903039	positive regulation of leukocyte cell-cell adhesion	1.77E-6	3.98E-4	1.77	210	40
GO:0030155	regulation of cell adhesion	4.11E-6	7.25E-4	3.42	608	86
GO:0045785	positive regulation of cell adhesion	1.1E-5	1.53E-3	4.51	366	57
GO:0022409	positive regulation of cell-cell adhesion	1.27E-5	1.72E-3	3.04	243	42
GO:1903037	regulation of leukocyte cell-cell adhesion	7.02E-5	6.19E-3	5.74	286	45
GO:0022407	regulation of cell-cell adhesion	2.55E-4	1.63E-2	2.11	373	53

GO:0032956	regulation of actin cytoskeleton organization	8.98E-12	1.02E-8	2.48	292	63
GO:0030036	actin cytoskeleton organization	1.95E-7	5.9E-5	2.10	284	52
GO:0051493	regulation of cytoskeleton organization	5.68E-7	1.5E-4	1.80	467	73
GO:0051495	positive regulation of cytoskeleton organization	1.55E-6	3.63E-4	3.13	179	36
GO:0007010	cytoskeleton organization	1.7E-4	1.17E-2	1.31	739	93
GO:0110053	regulation of actin filament organization	6.39E-12	7.89E-9	2.72	228	54
*GO Term analysis was performed by GOrilla online tool and showed GO Terms are referring to group „Processes“. Enrichment = $(b/n) / (B/N)$ N - is the total number of genes B - is the total number of genes associated with a specific GO term n - is the number of genes in the top of the user's input genes b - is the number of genes in the intersection						

Supplemental Table 5: Statistical analysis of all measurements for cell motility assay

<i>Parameter</i>	MEC1^{wt} cells (mean ± s.e.m.)	MEC1^{GAB1-KO} cells (mean ± s.e.m.)	ANOVA <i>P-value</i>
<i>Circularity*</i>	0.652 ± 0.004	0.653 ± 0.004	n.s.
<i>Elongation*</i>	0.323 ± 0.008	0.332 ± 0.008	n.s.
<i>Mass [pg]*</i>	200.6 ± 5.3	198.7 ± 4.8	n.s.
<i>Spreading [μm²/pg]*</i>	1.75 ± 0.02	1.57 ± 0.02	n.s.
<i>Displacement [μm/60 s]*</i>	3.75 ± 0.14	2.71 ± 0.10	0.035
<i>Persistence*</i>	0.69 ± 0.01	0.60 ± 0.01	0.036
Number of movies	6	6	
Number of tracked cells	195	186	
Number of time point measurements	11 939	15 184	
s.e.m. stands for standard error of the mean * For the description of the categories see <i>Supplemental Methods</i> above			

Supplemental Table 6: Transcription factors differentially expressed in CXCR4/CD5 subpopulations

Gene ID	Gene name	Base Mean (reads per million)	Fold Change (log2) *	adjusted P- value
ENSG00000112182	BACH2	10948	2.05	2.99E-44
ENSG00000166949	SMAD3	290	1.81	4.59E-14
ENSG00000186350	RXRA	2740	1.72	1.55E-11
ENSG00000101190	TCFL5	715	1.44	1.75E-14
ENSG00000118513	MYB	290	1.42	5.37E-05
ENSG00000150907	FOXO1	3615	1.30	7.29E-21
ENSG00000123358	NR4A1	578	1.23	3.30E-10
ENSG00000177683	THAP5	919	1.08	3.70E-22
ENSG00000118689	FOXO3	1987	1.06	1.26E-13
ENSG00000182568	SATB1	1161	1.05	4.81E-20
ENSG00000160961	ZNF333	1259	1.04	5.76E-20
ENSG00000106624	AEBP1	4585	1.03	4.06E-06
ENSG00000197647	ZNF433	497	1.02	1.89E-14
ENSG00000074657	ZNF532	1534	1.00	3.05E-05
ENSG00000102870	ZNF629	365	1.00	9.21E-15
ENSG00000134954	ETS1	21200	1.00	1.97E-19
ENSG00000137309	HMGA1	1173	-1.05	2.13E-26
ENSG00000198715	GLMP	432	-1.20	2.04E-18
ENSG00000198176	TFDP1	1457	-1.26	2.90E-21
ENSG00000011590	ZBTB32	2944	-1.48	3.1715E-24
* positive numbers mean a relative upregulation in CXCR4 ^{bright} CD5 ^{dim} subpopulation				

Supplemental Table 7: Numbers of predicted transcriptional binding sites in promotor region of *GAB1* gene for transcription factors differentially expressed in CXCR4/CD5 subpopulations

Gene ID	Gene name	Number of predicted TBS*
ENSG00000112182	BACH2	1
ENSG00000166949	SMAD3	6
ENSG00000186350	RXRA	15
ENSG00000101190	TCFL5	25
ENSG00000118513	MYB	15
ENSG00000150907	FOXO1	11
ENSG00000123358	NR4A1	5
ENSG00000177683	THAP5	Not in database
ENSG00000118689	FOXO3	7
ENSG00000182568	SATB1	25
ENSG00000160961	ZNF333	4
ENSG00000106624	AEBP1	Not in database
ENSG00000197647	ZNF433	Not in database
ENSG00000074657	ZNF532	Not in database
ENSG00000102870	ZNF629	Not in database
ENSG00000134954	ETS1	58
ENSG00000137309	HMGAI	5
ENSG00000198715	GLMP	Not in database
ENSG00000198176	TFDP1	0
ENSG00000011590	ZBTB32	Not in database
*performed with ContraV3 online tool		

Supplemental Table 8: Table of predicted FoxO1 binding sites in the upstream region of *GAB1* gene

Transcription binding site (TBS) number	Approximate position (hg19 version)	ChIP results for FoxO1-enrichment in the region upstream of <i>GAB1</i>
1	chr4:144256738	No enrichment
2	chr4:144256758	No enrichment
3	chr4:144256434	Not investigated
4	chr4:144256269	Enrichment (Fig. 3B)
5	chr4:144256326	Enrichment (Fig. 3B)
6	chr4:144255992	No enrichment
7	chr4:144255229	Not investigated
8	chr4:144254941	Not investigated
9	chr4:144254638	Not investigated
10	chr4:144254529	Enrichment (Fig. 3B)
11	chr4:144254457	Not investigated
Note: TBS 1 + 2 and 4 + 5 were analyzed with the same primer sets due to close vicinity from each other.		

Supplemental References

1. Mraz M, Malinova K, Kotaskova J, et al. miR-34a, miR-29c and miR-17-5p are downregulated in CLL patients with TP53 abnormalities. *Leukemia*. 2009;23(6):1159-1163.
2. Mraz M, Malinova K, Mayer J, Pospisilova S. MicroRNA isolation and stability in stored RNA samples. *Biochem Biophys Res Commun*. 2009;390(1):1-4.
3. Pavlasova G, Borsky M, Svobodova V, et al. Rituximab primarily targets an intra-clonal BCR signaling proficient CLL subpopulation characterized by high CD20 levels. *Leukemia*. 2018;32(9):2028-2031.
4. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. 2011. 2011;17(1):3 %J EMBnet.journal.
5. Hackenberg M, Sturm M, Langenberger D, Falcon-Perez JM, Aransay AM. miRanalyzer: a microRNA detection and analysis tool for next-generation sequencing experiments. *Nucleic Acids Res*. 2009;37(Web Server issue):W68-76.
6. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014;30(15):2114-2120.
7. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014;42(Database issue):D68-73.
8. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550.
9. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-140.
10. Huber W, Carey VJ, Gentleman R, et al. Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods*. 2015;12(2):115-121.
11. Babicki S, Arndt D, Marcu A, et al. Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res*. 2016;44(W1):W147-153.
12. Yart A, Laffargue M, Mayeux P, et al. A critical role for phosphoinositide 3-kinase upstream of Gab1 and SHP2 in the activation of ras and mitogen-activated protein kinases by epidermal growth factor. *J Biol Chem*. 2001;276(12):8856-8864.
13. Wiederschain D, Wee S, Chen L, et al. Single-vector inducible lentiviral RNAi system for oncology target validation. *Cell Cycle*. 2009;8(3):498-504.
14. Carey MF, Peterson CL, Smale ST. Chromatin immunoprecipitation (ChIP). *Cold Spring Harb Protoc*. 2009;2009(9):pdb prot5279.
15. Strbkova L, Zicha D, Vesely P, Chmelik R. Automated classification of cell morphology by coherence-controlled holographic microscopy. *J Biomed Opt*. 2017;22(8):1-9.
16. Zicha D, Genot E, Dunn GA, Kramer IM. TGFbeta1 induces a cell-cycle-dependent increase in motility of epithelial cells. *J Cell Sci*. 1999;112 (Pt 4):447-454.
17. Dunn GA, Zicha D. Dynamics of fibroblast spreading. *J Cell Sci*. 1995;108 (Pt 3):1239-1249.
18. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*. 2015;43(7):e47.
19. Papatheodorou I, Moreno P, Manning J, et al. Expression Atlas update: from tissues to single cells. *Nucleic Acids Res*. 2020;48(D1):D77-D83.
20. Kreft L, Soete A, Hulpiau P, Botzki A, Saeys Y, De Bleser P. ConTra v3: a tool to identify transcription factor binding sites across species, update 2017. *Nucleic Acids Res*. 2017;45(W1):W490-W494.
21. Pyrzynska B, Dwojak M, Zerrouqi A, et al. FOXO1 promotes resistance of non-Hodgkin lymphomas to anti-CD20-based therapy. *Oncoimmunology*. 2018;7(5):e1423183.

LYMPHOID NEOPLASIA

miR-29 modulates CD40 signaling in chronic lymphocytic leukemia by targeting TRAF4: an axis affected by BCR inhibitors

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KEY POINTS

- *miR-29* down-modulation in an intracлонаl CLL subpopulation from immune niches allows for higher TRAF4 and increased CD40 responsiveness.
- BCR-signaling represses *miR-29* via MYC, allowing for stronger CD40-NFκB signaling, and this regulatory loop is disrupted by BCR inhibitors.

B-cell receptor (BCR) signaling and T-cell interactions play a pivotal role in chronic lymphocytic leukemia (CLL) pathogenesis and disease aggressiveness. CLL cells can use microRNAs (miRNAs) and their targets to modulate microenvironmental interactions in the lymph node niches. To identify miRNA expression changes in the CLL microenvironment, we performed complex profiling of short noncoding RNAs in this context by comparing CXCR4/CD5 intracлонаl cell subpopulations (CXCR4^{dim}CD5^{bright} vs CXCR4^{bright}CD5^{dim} cells). This identified dozens of differentially expressed miRNAs, including several that have previously been shown to modulate BCR signaling (*miR-155*, *miR-150*, and *miR-22*) but also other candidates for a role in microenvironmental interactions. Notably, all 3 *miR-29* family members (*miR-29a*, *miR-29b*, *miR-29c*) were consistently down-modulated in the immune niches, and lower *miR-29(a/b/c)* levels associated with an increased relative responsiveness of CLL cells to BCR ligation and significantly shorter overall survival of CLL patients. We identified tumor necrosis factor receptor-associated factor 4 (TRAF4) as a novel direct target of *miR-29s* and revealed that higher TRAF4 levels increase CLL responsiveness to CD40 activation and downstream nuclear factor-κB (NF-κB) signaling. In CLL, BCR represses *miR-29* expression via MYC, allowing for concurrent TRAF4 upregulation and stronger CD40-NF-κB signaling.

This regulatory loop is disrupted by BCR inhibitors (bruton tyrosine kinase [BTK] inhibitor ibrutinib or phosphatidylinositol 3-kinase [PI3K] inhibitor idelalisib). In summary, we showed for the first time that a miRNA-dependent mechanism acts to activate CD40 signaling/T-cell interactions in a CLL microenvironment and described a novel *miR-29*-TRAF4-CD40 signaling axis modulated by BCR activity. (*Blood*. 2021;137(18):2481-2494)

Introduction

The (de)regulation of microenvironmental interactions, especially B-cell receptor (BCR) signaling and T-cell interactions plays a pivotal role in chronic lymphocytic leukemia (CLL) pathogenesis.^{1,2} It has been postulated that CLL cells depend on constant recirculation between peripheral blood and lymph nodes where they obtain prosurvival/proliferative signals, and inhibition of the recirculation process is an important mechanism of action of BCR inhibitors such as ibrutinib and idelalisib.^{1,3} Microenvironmental interactions and gene expression changes in immune niches can be studied by directly examining lymph node biopsies or more conveniently by analyzing an intracлонаl CLL cell subpopulation that has recently

exited the lymph nodes (characterized as CXCR4^{dim}CD5^{bright} cells).³⁻⁷ These studies revealed that, in the lymph node niches, both BCR activation and T-cell interactions are concurrently available to CLL cells to induce proliferation,^{1,7} and their variable intensity contributes to the heterogeneity in disease aggressiveness.⁸⁻¹¹ T-cell interactions' essential role can be illustrated by the possibility of inducing CLL cell proliferation in vitro by providing T-cell factors such as CD40L,¹²⁻¹⁶ and CLL cotransplantation with activated T cells is required for their significant proliferation in immunodeficient mice.¹⁷ We and others have shown that microRNAs (miRNAs) can regulate microenvironmental interactions such as BCR signaling in CLL.¹⁸⁻²¹ However, it is unknown if miRNAs are

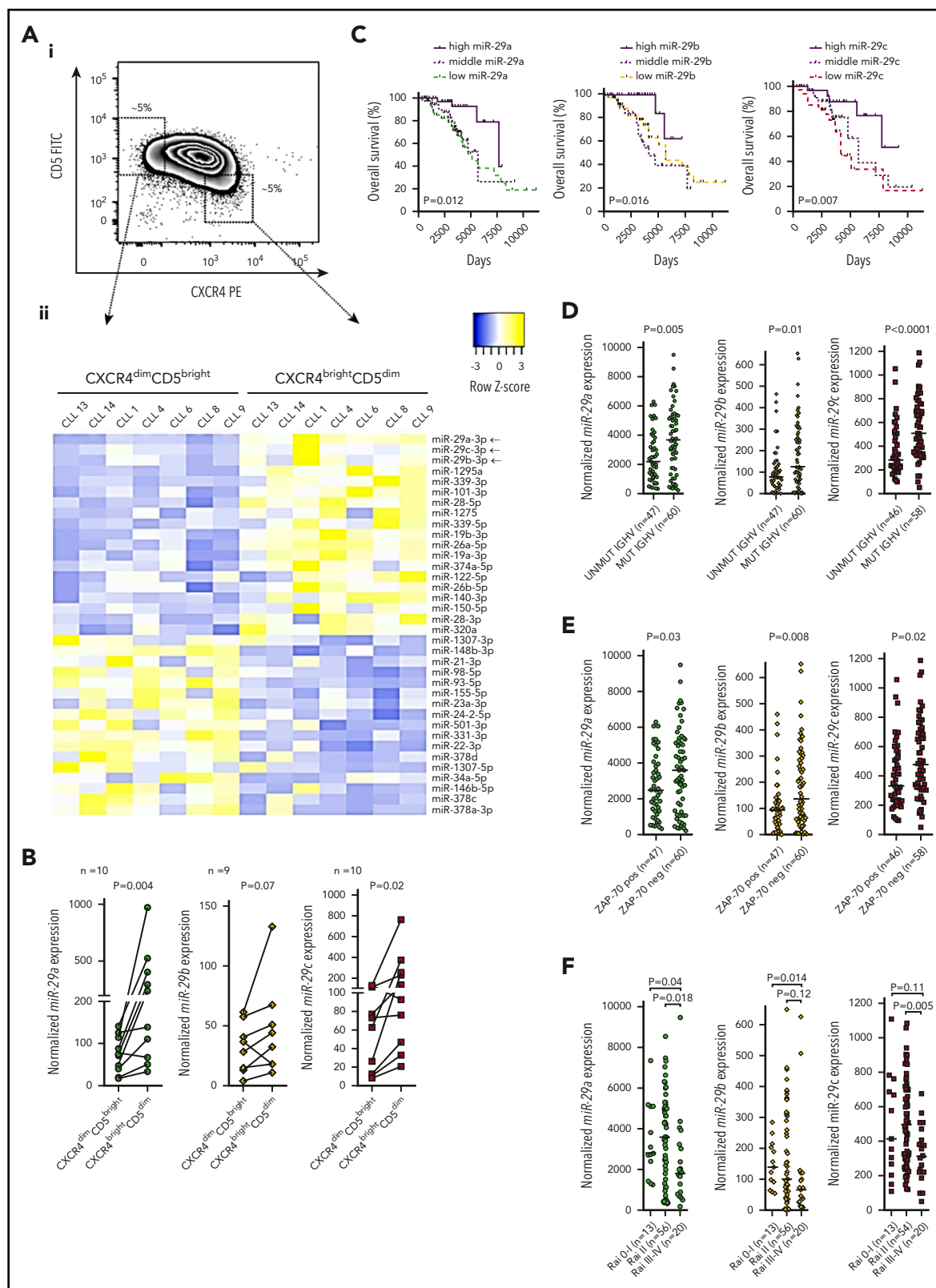


Figure 1. miR-29 levels are downregulated in CXCR4^{dim}CD5^{bright} intraclonal subpopulation and associate with prognosis in CLL. (Ai) Representative example of sorting CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} CLL cell intraclonal subpopulations using flow cytometry. (Aii) Heatmap of differentially expressed miRNAs (fold-change > 1.5, adjusted $P < .0005$) in 7 pairs of CXCR4/CD5 sorted subpopulations (purity > 99%; for sample characteristics, see supplemental Table 1). Heatmap was generated from counts per million reads (rows centered to the median of the row). For details on individual miRNA expression see supplemental Table 3. (B) miR-29a/b/c levels analyzed using qRT-PCR in the CXCR4/CD5 sorted subpopulations from primary CLL samples (miR-29a/c, $n = 10$; miR-29b, $n = 9$; statistical differences were compared by Wilcoxon matched pairs test). (C) Overall survival is depicted using Kaplan-Meier curves in the CLL cohort ($n = 107$) divided by terciles of miR-29a/b/c expression (with log-rank test for comparison of low vs high miR-29s levels; miR-29a: HR, 3.1; 95% CI, 1.3-7.4; miR-29b: HR, 3.6; 95% CI, 1.3-10.20; miR-29c: HR, 3.4; 95% CI, 1.4-8.3). (D-F) Expression of miR-29a/b/c and its relationship

involved in modulating T-cell interactions in the context of CLL (or normal) microenvironment.

MicroRNAs are short noncoding RNAs that can each regulate a variety of different mRNAs by negatively influencing their stability and/or translation.²²⁻²⁵ In lymphoid cells, such regulation of gene dose by miRNAs is needed for proper maturation and survival of B and T cells, BCR signaling, and T-cell receptor signaling proficiency.^{24,26-30} It has been described that several miRNAs are associated with CLL prognosis³¹⁻³⁶ and pathogenesis (*miR-15-16*)^{37,38}; however, the pathways regulated by most miRNAs remain unknown. We and others have shown that miRNAs contribute to the deregulation of apoptosis,^{37,39} BCR signaling,^{18-20,40,41} or metabolism in CLL cells.⁴² Additionally, treatment of CLL patients with chemotherapy or BCR inhibitors affects the expression of miRNAs that are involved in these processes.^{40,43,44}

In this study, we performed the first miRNA expression profiling in sorted CXCR4/CD5 intracolon CLL subpopulations to identify miRNAs potentially relevant in microenvironmental interactions. We identified dozens of differentially expressed miRNAs including the downregulation of *miR-29* in the proliferative CXCR4^{dim}CD5^{bright} cell subpopulation and in CLL lymph nodes. Low levels of *miR-29* were previously consistently reported as associated with unfavorable prognosis in CLL^{31,32,45-47} and other B-cell malignancies,⁴⁸⁻⁵⁰ which underscores the need to understand *miR-29*'s functional relevance. Therefore, we performed an unbiased search for *miR-29* targets and identified tumor necrosis factor receptor-associated factor 4 (TRAF4) as its novel direct target. We further revealed a novel TRAF4 function in controlling T-cell interactions via CD40 pathway and that *miR-29* levels are repressed by BCR-induced MYC. Finally, we observed that therapy with ibrutinib or idelalisib leads to upregulation of *miR-29* expression and subsequent TRAF4 repression, which limits the CLL cell ability to respond to T-cell signals. In summary, the down-modulation of *miR-29* in the context of microenvironmental interactions and BCR activation provides a novel regulatory loop that increases CD40 signaling propensity.

Methods

CLL cohort and cell lines

Blood samples were collected from patients after obtaining written informed consent and approval of the institutional review board. Peripheral CLL cells were isolated from untreated (at diagnosis or least 6 months untreated; Table 1; supplemental Table 1 available on the *Blood* Web site) or treated CLL patients (ibrutinib or idelalisib, both as single agent; supplemental Table 2) by density centrifugation (Ficoll-Paque, HealthCare Life Sciences), followed by magnetic anti-CD3 MicroBeads separation (Miltenyi Biotec), or in some cases, negative selection with RosetteSep Human B Cell Enrichment Cocktail (Stemcell Technologies) was used to obtain purity of $\geq 95\%$ of CD5⁺19⁺ cells (evaluated by flow cytometry). Sorting of CXCR4^{dim}CD5^{bright} vs CXCR4^{bright}CD5^{dim} subpopulations (purity > 99%) was performed as previously described by gating each subpopulation

as ~5% of the clone^{3,5}; flow cytometric analysis is described in the supplemental Methods. The MEC1 and HEK293FT cell lines were obtained from the German Collection of Microorganisms and Cell Cultures or American Type Culture Collection, respectively.

mRNA/miRNA profiling and quantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated by TRI-Reagent (Sigma Aldrich) as previously described (RIN > 8).⁵¹ The NEB Next Small RNA Library Prep kit for miRNA profiling or TruSeq Stranded mRNA LT Sample Prep Kit for mRNA profiling (NextSeq, Illumina) were used as previously described.^{5,40} For details on library preparation and data analyses, see supplemental Methods. The expression of individual miRNAs/mRNAs was assayed with TaqMan MicroRNA or Gene Expression Assays (Thermo Fisher Scientific), respectively, and normalized to endogenous controls (*RNU38B* and *RNU48* for miRNAs, *TBP* and *HPRT1* for mRNA) as previously described.^{35,36}

Cell transfection and CD40/BCR activation

The cells were electroporated using the Neon Transfection System (Thermo Fisher Scientific) with an artificial *miR-29c* (MISSION miRNA Mimic, 1000 nM) or control short RNA (MISSION miRNA Mimic Negative Control, 1000 nM; Sigma Aldrich), siRNA against *TRAF4* (Silencer Select Pre-Designed siRNA, 500 nM) or control siRNA (Silencer Select Negative Control No.1, 500 nM; Thermo Fisher Scientific), LNA *miR-29* family inhibitor (miRCURY LNA miRNA inhibitor, 500 nM) or control miRNA inhibitor (Negative Control A, 500 nM, Qiagen). The cells were harvested for viability analyses, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and immunoblotting (supplemental Methods). Transfected cells were stimulated with recombinant soluble CD40 ligand (CD40L, 1 μ g/mL; Peprotech) in serum-free media (37°C) for the indicated time period and lysed for immunoblotting (see supplemental Methods). For BCR activation by bead-bound anti-immunoglobulin M (IgM), cells were incubated with Dynabeads M-270-Epoxy (Thermo Fisher Scientific) coated with goat F(ab')₂ anti-human IgM or isotype control (see supplemental Methods). BCR crosslinking for the measurement of intracellular calcium flux was performed by soluble goat F(ab')₂ anti-human IgM (Southern Biotechnology; 10 μ g/mL), as described elsewhere^{18,19} (see supplemental Methods).

Luciferase assay

Luciferase reporter assay was carried out using psiCHECK2 reporter plasmid and the Promega Dual-Luciferase Reporter Assay Kit (Promega) as previously described⁴¹ (see supplemental Methods).

Statistical analysis

Apart from next-generation sequencing (NGS) data analysis (for further details, see supplemental Methods), all statistical analyses were performed with GraphPad Prism Software v5.0 (GraphPad Software). $P < .05$ was considered significant.

Figure 1 (continued) with clinico-biological features. *miR-29a*, *b*, and *c* expressions were quantified in a cohort of 107 CLL patients (cohort characteristics in Table 1) and correlated to the clinico-biological characteristics such as (D) IGHV mutation status, (E) ZAP-70 expression, and (F) Rai stage (at sampling). The expression level of *miR-29c* was not available for 3 samples because of technical issues with its quantification. The statistical differences were tested using unpaired *t* test.

Table 1. Cohort characteristics (n = 107)

	N	%
Median age at diagnosis (range), y	56 (34-79)	
Median follow-up, y	9.3	
Median survival, y	15.7	
Median time from diagnosis to blood collection, y	2.7	
Treated during follow-up	76	71.0
Male/female	69/38	64/36
Rai stage at sampling		
0	12	11.2
I	1	0.9
II	56	52.3
III-IV	20	18.7
Not determined	18	
IGHV/ZAP-70/CD38 status		
unmut IGHV/mut IGHV	47/60	(44/56)
ZAP-70 ⁺ /ZAP-70 ⁻	47/60	(44/56)
CD38 ⁺ /CD38 ⁻	30/77	(28/72)
Hierarchical cytogenetics (fluorescence in situ hybridization)		
del 17p13	8	7.5
del 11q23	9	8.4
del 13q14	33	30.8
Trisomy 12	4	3.7
Normal karyotype	16	15.0
Not determined	37	

mut, mutated; unmut, unmutated; y, years.

Results

MicroRNA expression and *miR-29* down-modulation in CXCR4^{dim}CD5^{bright} cell subpopulation

It has been postulated that CLL cells that have recently exited the lymph node microenvironment are characterized by low CXCR4 levels and high CD5 levels (CXCR4^{dim}CD5^{bright}), whereas the CLL cells prone to recirculate back to immune niches are characterized as CXCR4^{bright}CD5^{dim}.³⁻⁵ This model recapitulates the phenotype of CLL cell recirculation, and CXCR4^{dim}CD5^{bright} cells retain a "fingerprint" of gene expression reflecting their activation in the lymph node niches.³⁻⁵ Here, we performed the first complex miRNA expression profiling in sorted CXCR4/CD5 intraclonal subpopulations (n = 14, 7 pairs; Illumina miRNA-seq). This identified 36 differentially expressed miRNAs in CXCR4/CD5 subpopulations (Figure 1A; supplemental Table 3) including lower *miR-150* levels and higher *miR-155*, *miR-22* levels in CXCR4^{dim}CD5^{bright} cells, which is in line with their known role in regulating BCR signaling in the CLL microenvironment¹⁸⁻²⁰ (Figure 1A). In this analysis, we noticed that all 3 members of the *miR-29* family (*miR-29a/b/c*) were consistently down-modulated (~2-fold; $P < 10^{-6}$) in the CXCR4^{dim}CD5^{bright} cells (Figure 1A); this was validated by qRT-PCR (Figure 1B). We also observed lower *miR-29* levels in paired peripheral blood and lymph node

CLL samples (supplemental Figure 1A). This is in concordance with a similar transcriptional regulation of all *miR-29* family⁵² and suggests that these miRNAs might have a role in regulating microenvironmental signaling. *miR-29* levels assessed from the total RNA isolated from the bulk of CLL cells in peripheral blood were intermediate between its expression in CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} subpopulations for the individual patient (supplemental Figure 1B; data not shown), which is similar to what we have previously observed for other genes such as *miR-155* and *CD20*.^{3,5,18} It has previously been shown that the expression of *miR-29* family from peripheral blood CLL cells is independently associated with prognosis in CLL.^{31,32} Indeed, in our CLL cohort, lower levels of all 3 *miR-29*s were associated with shorter overall survival (Figure 1C; supplemental Figure 2; *miR-29a*: hazard ratio [HR], 3.1; *miR-29b*: HR, 3.6; *miR-29c*: HR, 3.4; all $P < .05$) and unfavorable clinico-biological characteristics, namely unmutated immunoglobulin heavy chain variable region (IGHV), expression of ZAP-70, or higher Rai stage (Figure 1D-F; RNA isolated from the bulk of purified peripheral blood CLL cells; cohort characteristics in Table 1). The levels of *miR-29a/b/c* had no discernible relationship with the CD38 expression or with specific chromosomal abnormalities (supplemental Figure 1C-D). Altogether, *miR-29* levels are downregulated in the proliferative CXCR4^{dim}CD5^{bright} cell subpopulation, and lower *miR-29* levels associate with disease aggressiveness; however, the *miR-29* target(s) and its biological relevance in immune niches remain largely unknown.

Identification of *miR-29* target in CLL cells

Antipoptotic myeloid cell leukemia 1 (MCL1) and T-cell leukemia/lymphoma (TCL1) have been identified as potentially regulated by *miR-29* in CLL^{46,47}; however, an unbiased search for *miR-29* targets in CLL has not been performed. As such, we performed expression analyses of all predicted evolutionarily conserved mRNA targets of *miR-29* in 10 pairs of samples sorted according to CXCR4/CD5 cell-surface levels (Illumina RNA-seq), searching for mRNAs with expression anticorrelated to *miR-29*. We and others have shown that such a strategy is useful because miRNAs influence their target mRNA(s) stability.^{19,22} All *miR-29* family members have an identical "seed sequence," determining the binding to the 3' untranslated region (3'UTR) of mRNAs, and thus they share all the predicted targets (TargetScan tool v7.2). This analysis identified 26 genes that differed in their relative expression between intraclonal CXCR4/CD5 subpopulations and have predicted evolutionary conserved binding sites (TargetScan) for *miR-29* (Figure 2A; for details, see supplemental Table 4). Fourteen of these genes had an anticorrelated expression to *miR-29*, with higher levels in CXCR4^{dim}CD5^{bright} cells, and thus represent potential target mRNAs repressed by *miR-29*. We further focused on *TRAF4* (~2.4-fold upregulated in CXCR4^{dim}CD5^{bright} cells) because it has 2 evolutionary conserved predicted binding sites for *miR-29*, and *miR-29* is predicted as the most likely *TRAF4* miR-regulator (TargetScan tool v7.2). Moreover, high *TRAF4* levels associated with significantly shorter overall survival in CLL patients ($P = .02$; HR, 2.4; 95% confidence interval [CI], 1.2-4.9; see supplemental Figure 3 also for other genes), which is in line with low *miR-29* levels being associated with a more aggressive disease (see above). Additionally, we also observed a general anticorrelation of *TRAF4* mRNA and *miR-29* levels in a large CLL cohort (n = 97; supplemental Figure 4A-C). Next, we validated the *TRAF4*/*miR-29* levels in CXCR4/CD5 sorted CLL samples (n = 10) by individual qRT-PCR. This confirmed higher *TRAF4* levels in the CXCR4^{dim}CD5^{bright} cells compared with the CXCR4^{bright}CD5^{dim}

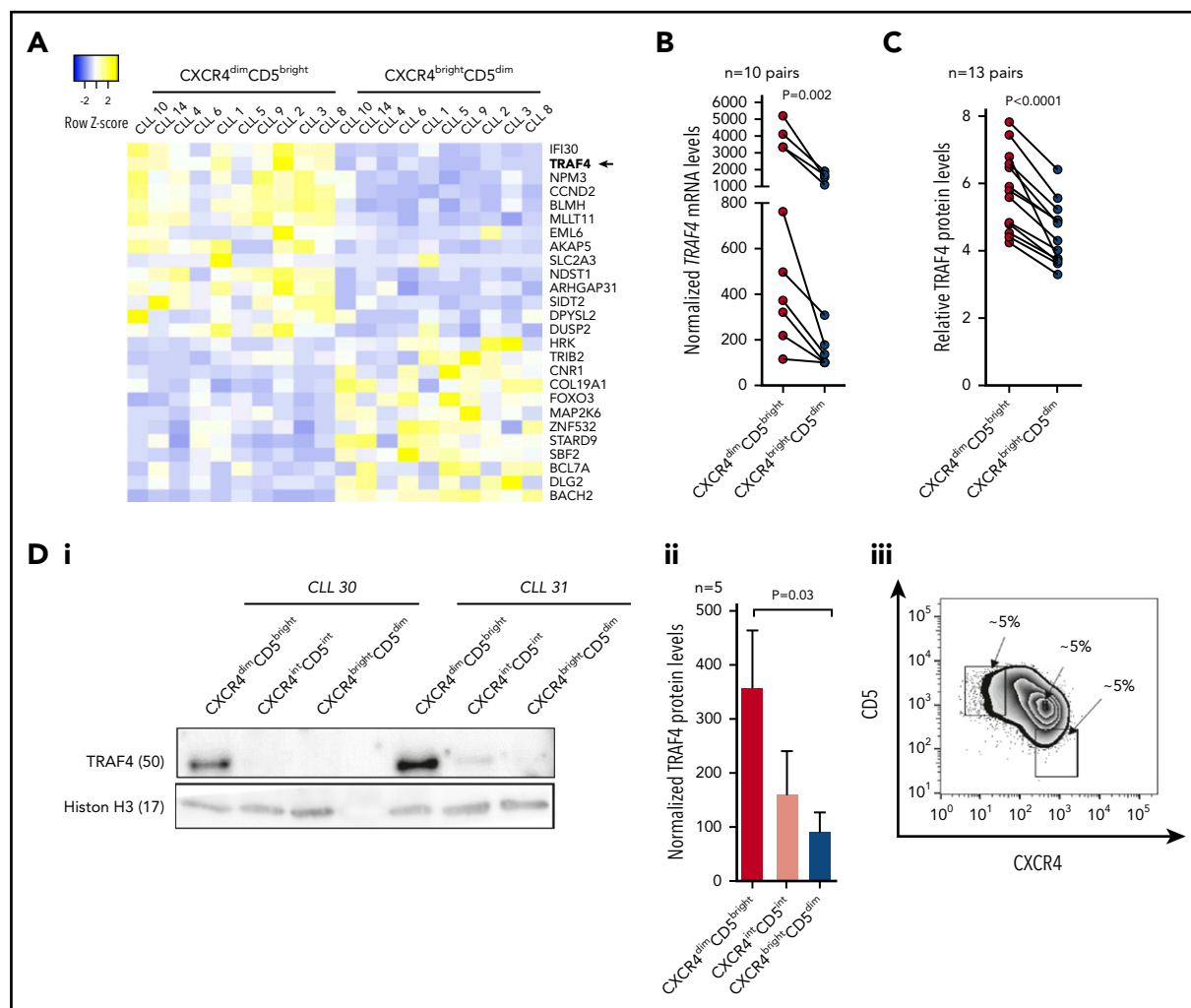


Figure 2. TRAF4 is upregulated in CXCR4^{dim}CD5^{bright} intraclonal subpopulation. (A) Heatmap of differentially expressed mRNAs (fold-change > 2; adjusted $P < .0005$) in 10 pairs of CXCR4/CD5 sorted subpopulations (purity > 99%; for sample characteristics, see supplemental Table 1). Plotted mRNAs represent an overlap of differentially expressed mRNAs and predicted evolutionary conserved *miR-29* targets (TargetScan tool). Samples CLL1, CLL4, CLL6, CLL8, CLL9, and CLL14 are identical to Figure 1A (6 of 7 pairs for *miR*-29 target prediction). Heatmap was generated from counts per million reads (rows centered to the median of the row). For details on mRNA expression and *miR*-29 target prediction see supplemental Table 4. (B) TRAF4 mRNA levels analyzed using qRT-PCR in the CXCR4/CD5 sorted subpopulations from 10 primary CLL samples. Differences were compared by Wilcoxon matched pairs test. (C) Intracellular staining for TRAF4 protein levels in the CXCR4/CD5 subpopulations from 13 CLL samples. Results are presented as the ratio of TRAF4 expression to the isotype control, and the statistical differences were compared by paired t test. (D) The expression of TRAF4 protein in CXCR4^{dim}CD5^{bright}, CXCR4^{intermediate(int)}CD5^{intermediate(int)}, and CXCR4^{bright}CD5^{dim} intraclonal cell populations. (i) Representative immunoblot blot analysis of TRAF4 in sorted CXCR4/CD5 subpopulations from 2 patient samples. Histone H3 was used as a loading control. (ii) Statistical analysis of TRAF4 protein levels in the CXCR4/CD5 sorted subpopulations (n = 5). The statistical differences were tested by paired t test, and the error bars indicate the standard error of the mean (SEM). (iii) Representative example of a gating strategy for CXCR4^{dim}CD5^{bright}, CXCR4^{intermediate(int)}CD5^{intermediate(int)}, and CXCR4^{bright}CD5^{dim} intraclonal cell populations. The CXCR4^{int}CD5^{int} represents a transitional subpopulation between CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} cells.

subpopulation (Figure 2B). Furthermore, intracellular protein staining (independent cohort of 13 CLL samples) and immunoblot analysis of sorted intraclonal CXCR4/CD5 subpopulations confirmed higher TRAF4 levels in CXCR4^{dim}CD5^{bright} cells (Figure 2C-D). The TRAF4 level was also higher in CLL lymph nodes samples compared with paired peripheral blood CLL cells (supplemental Figure 4D-E). Moreover, high TRAF4 levels were detected by immunohistochemistry in the proliferative pseudo-follicles of CLL lymph nodes (supplemental Figure 4F). Altogether, this shows that CLL cells have anticorrelated TRAF4/*miR*-29 levels, and cells interacting in the microenvironment have lower *miR*-29 and higher TRAF4 levels.

Validation of TRAF4 as a target of *miR*-29

To validate TRAF4 regulation by *miR*-29, the MEC1 CLL cell line was transfected with a synthetic *miR*-29c (shares identical

binding site in TRAF4 with *miR*-29a/b). This revealed a reduction in the TRAF4 protein levels by ~30% in cells transfected with synthetic *miR*-29c (Figure 3A) and a similar reduction at the mRNA level (Figure 3B). The TRAF4 protein and mRNA levels were also down-modulated in primary CLL cells transfected with a synthetic *miR*-29c, and this was comparable to the effect of siRNA against TRAF4 (Figure 3C-D). The increase in the expression of *miR*-29c achieved by transfecting the synthetic *miR*-29c was approximately 2-fold for both MEC1 and primary CLL cells (data not shown). All the *miR*-29 family members have an identical seed sequence, and transfecting B cells with synthetic *miR*-29c or synthetic *miR*-29a led to a comparable reduction in TRAF4 protein levels (supplemental Figure 5A). The *miR*-29 family inhibitor increased TRAF4 protein levels in B cells (supplemental Figure 5B). Our data also confirmed some *miR*-29's effect on

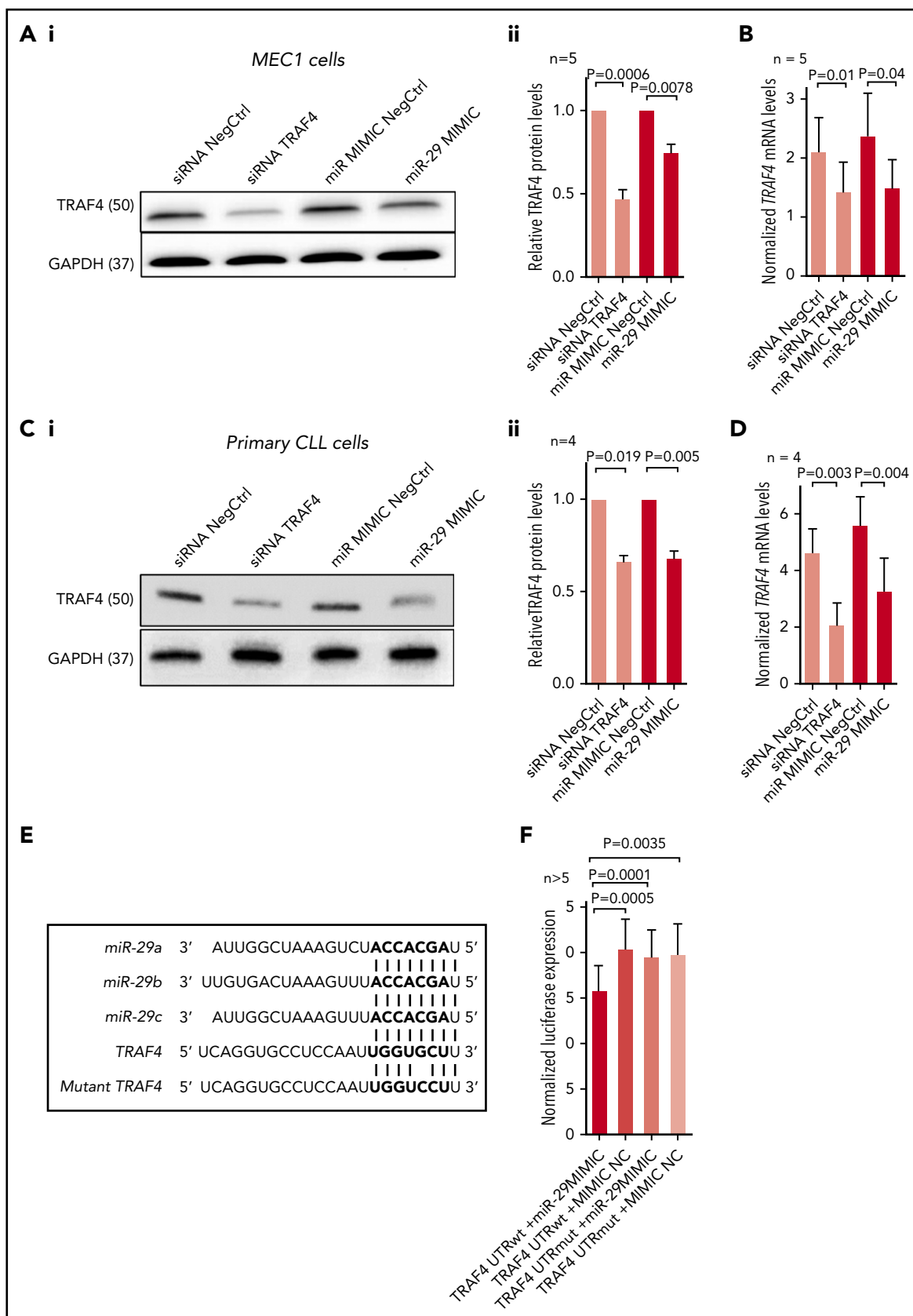


Figure 3. Validation of TRAF4 as a target of miR-29. (Ai) Representative example of immunoblot for TRAF4 levels in MEC1 cells transfected (72 hours) with siRNA against TRAF4 (siRNA TRAF4), control siRNA (siRNA NegCtrl), artificial miR-29c (miR-29 MIMIC), or control miRNA (miR MIMIC NegCtrl). (Aii) Densitometric quantification of TRAF4 levels for independent replicates of the experiment (n = 5) described in panel Ai. (B) Analysis of TRAF4 mRNA levels in MEC1 cells transfected by synthetic miR-29 or siRNA against TRAF4 (n = 5) as described in panel A. (Ci) Representative example of immunoblot analysis for TRAF4 levels in primary CLL cells transfected (72 hours) with siRNA against TRAF4

MCL1 and TCL1 down-modulation described previously^{46,47}; however, this seemed less prominent than the effect on TRAF4 levels (supplemental Figure 5C).

To test for direct interaction of *miR-29* with the 3'UTR of *TRAF4*, we cotransfected HEK293FT cells with the synthetic *miR-29c* or a negative control and a luciferase reporter containing the putative *miR-29* binding site from 3'UTR of *TRAF4* gene or a mutated site (containing a point mutation in the *miR* binding seed region). To validate *miR-29* binding, we selected the predicted binding site at position 161-167 of 3'UTR, which has a higher overall binding score compared with the second *miR-29* binding site (for *miR-29* alignments with the 3'UTR region, see Figure 3E and supplemental Figure 6). This confirmed *miR-29*'s direct physical binding at positions 161 to 167 of the *TRAF4* 3'UTR (Figure 3F); however, it does not exclude the possibility that other regions of *TRAF4* 3'UTR might bind *miR-29*. On the other hand, the *miR-29* mimic did not affect the luciferase activity in cells cotransfected with a luciferase-reporter construct containing the mutated *TRAF4* binding site (Figure 3F). We conclude that the seed sequence of *miR-29* family members inhibits *TRAF4* expression by directly binding to its 3'UTR region.

***miR-29* is repressed by BCR-induced MYC**

The transcriptional regulation of all three *miR-29* family members is known to be similar,⁵² and it has previously been demonstrated in aggressive B-cell lymphomas that *miR-29* is directly transcriptionally repressed by MYC binding to its promoter.^{50,53} CXCR4^{dim}CD5^{bright} cells have higher MYC protein levels than CXCR4^{bright}CD5^{dim} cells, suggesting that MYC might regulate *miR-29* levels in this context (Figure 4A). Indeed, the activation of BCR signaling by a bead-bound anti-IgM induces MYC levels and leads to *miR-29* down-modulation and upregulation of *TRAF4* mRNA and protein levels in CLL cells (Figure 4B-D). In line with this, CLL samples that were more responsive to BCR ligation with soluble anti-IgM had significantly higher MYC, lower *miR-29a* and *miR-29c* levels, and higher *TRAF4* levels than samples that were relatively less responsive to BCR crosslinking (Figure 4E-F). Additionally, samples expressing higher MYC levels had significantly lower *miR-29a* and *miR-29c* levels and higher levels of *TRAF4* (Figure 4G-H; supplemental Figure 7B). Moreover, B cells from transgenic iMyc^{Cx} mice (MYC transgene controlled by the Ig heavy-chain enhancer) have lower *miR-29a* and *miR-29c* levels than wild-type mice (Figure 4I), and CLL samples with a MYC aberration ($n = 23$, all with unmutated IGHV) also showed lower *miR-29a* and *miR-29c* levels than unmutated IGHV CLL cells that did not have a gain in MYC (supplemental Figure 8). Altogether, these data demonstrate that BCR-induced MYC represses *miR-29a* and *miR-29c*, but *miR-29b* is not directly dependent on MYC activity.

TRAF4 is a novel regulator of CD40 signaling

Members of the TRAF family (TRAF2/3/6) play a role in B-cell-T-cell interactions by regulating CD40 signaling.^{54,55} We

hypothesized that TRAF4 might act as a novel CD40 signaling regulator. Indeed, MEC1 cells transfected with siRNA against *TRAF4* had impaired CD40 ligation responsiveness as evidenced by reduced phosphorylation of immediate downstream signaling molecules in the nuclear factor κ B (NF- κ B) pathway, namely IKK α / β (Figure 5A). Identical data were obtained when primary CLL cells were transfected with siRNA against *TRAF4* and treated by CD40L (Figure 5B). Similarly, transfection of MEC1 cells or primary CLL cells with synthetic *miR-29c* reduced TRAF4 levels and impaired IKK α / β phosphorylation after stimulation with CD40 ligand (Figure 5C-D). TRAF4 silencing or cell transfection by the synthetic *miR-29* did not have any significant effect on MEC1 or CLL cell viability (supplemental Figure 9). TRAF4 overexpression led to an increased IKK α / β phosphorylation after CD40L (supplemental Figure 10A), and transfecting MEC1 cells with the TRAF4 construct (not containing the *miRNA* binding site) rescued the effect of *miR-29* on CD40 signaling (supplemental Figure 10B). We also validated the role of the *miR-29*/TRAF4 axis by coculturing primary CLL cells transfected with synthetic *miR-29* (or negative control) with autologous T cells. In concordance with data above, CLL cells transfected by *miR-29* had reduced pIKK α / β levels after the CLL-T-cell interaction (supplemental Figure 11A). Similar data were obtained when coculturing MEC1 cells transfected by synthetic *miR-29* (vs negative control) with CD40L⁺CD4⁺ Jurkat T cells (supplemental Figure 11B). Interestingly, we noticed that TRAF4 silencing also repressed the basal extracellular signal-regulated kinase (ERK) phosphorylation level (supplemental Figure 12), suggesting a CD40-independent function of TRAF4 in CLL cells. Overall, the results demonstrate the existence of a novel *miR-29*-TRAF4 regulatory axis in CD40 signaling in CLL.

***miR-29* is induced and TRAF4 repressed by BCR inhibitors**

We observed that BCR activity in CLL represses *miR-29*, and we further examined the in vivo effects of BCR inhibitors on MYC (*miR-29* repressor), *miR-29s*, and TRAF4 (*miR-29* target) levels. In line with the data above, MYC expression was repressed, and all members of the *miR-29* family were induced in CLL patients after several weeks on single-agent therapy with ibrutinib (Figure 6A-B). All patients in this analysis (supplemental Table 2) were responsive to therapy at the time of sampling and for a period of >1 year (with the exception of 1 patient with a relapse after 7 months). The TRAF4 mRNA and protein levels were clearly down-modulated during ibrutinib therapy (Figure 6C-D). Similar data were also obtained for patients treated with idelalisib as a single agent (Figure 6E-F; for patient's characteristics, see supplemental Table 2) with the exception of 1 sample with *TRAF4* upregulation during therapy. However, we were not able to identify any specific characteristics for this patient that would explain it (CLL 69 in supplemental Table 2; NOTCH1 mutated, TP53 wt, trisomy 12 [69%], ZAP70 [13%], IGHV V3-64); the patient responded well to idelalisib (achieved partial remission), and therapy was discontinued for reasons unrelated to CLL. CLL cells obtained directly from ibrutinib

Figure 3 (continued) (siRNA TRAF4), control siRNA (siRNA NegCtrl), artificial *miR-29c* (*miR-29* MIMIC), or control *miRNA* (*miR* MIMIC NegCtrl). (Cii) Densitometric quantification of TRAF4 protein levels for independent replicates of the experiment ($n = 4$) described in panel Ci. (D) Analysis of *TRAF4* mRNA expression in primary CLL cells transfected by synthetic *miR-29* or siRNA against *TRAF4* ($n = 4$) as described in panel Ci. (E) Alignment of *miR-29a/b/c* with 3'UTR of *TRAF4* mRNA (at position 161-167). (F) Luciferase activity in HEK293FT cells cotransfected with psiCHECK2 vector containing the cloned 3'UTR region of *TRAF4* encoding the putative *miR-29* binding site at position 161-167 (*TRAF4* UTRwt) and either artificial *miR-29c* (*miR-29* MIMIC) or control *miRNA* (mimic NC), or containing a cloned mutated 3'UTR of *TRAF4* (*TRAF4* UTRmut; G>C at position 165) and either artificial *miR-29c* (*miR-29* MIMIC) or control *miRNA* (mimic NC). Renilla activity was measured 24 hours after transfection, and activity was normalized to the endogenous firefly control of the psiCHECK2 vector ($n > 5$). In all experiments, the differences were compared by paired *t* test. The error bars indicate SEM.

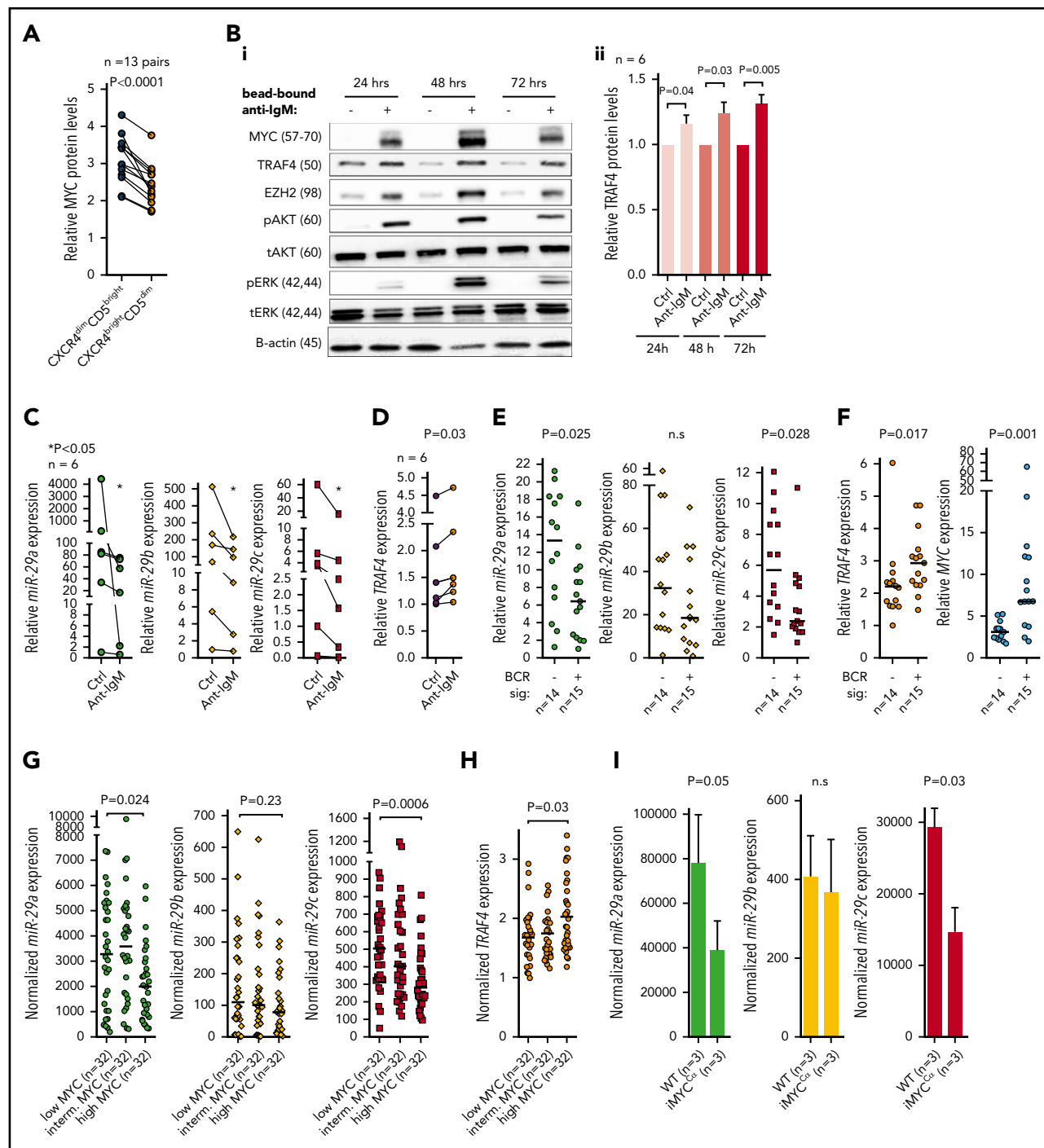


Figure 4. Negative regulation of miR-29a/b/c by BCR-induced MYC. (A) Intracellular staining for MYC in CXCR4/CD5 sorted subpopulations from 13 CLL samples. Results are represented as the ratio of MYC protein expression to the isotype control. The statistical differences were tested by paired t test. (Bi) Representative immunoblot of TRAF4 and MYC protein levels in primary CLL cells with BCR signaling activated by bead bound anti-IgM for indicated period of time. pAKT (phosphorylated AKT) and pERK (phosphorylated ERK) serve as positive controls for BCR pathway activation. (Bii) Densitometric quantification of TRAF4 levels for replicates of the experiment (n = 6) described in panel Bi. The statistical differences were tested by paired t test. (C-D) Analyses of miR-29a/b/c (C) and TRAF4 mRNA levels (D) in primary CLL cells (n = 6) with BCR signaling activated by bead-bound anti-IgM for 72 hours as described in panel B. For MYC induction by bead-bound anti-IgM, see panel Bi. The bead-bound anti-IgM did not affect CLL cell viability (supplemental Figure 7A). The statistical differences were tested by Wilcoxon matched pairs test. (E-F) Relationship between expression of miR-29a/b/c, MYC, or TRAF4 and the relative sensitivity to BCR ligation. CLL samples (n = 29) were treated with soluble anti-IgM and monitored for BCR-induced calcium flux by flow cytometry, which allowed us to stratify cases as relatively responsive to BCR ligation (n = 15) or unresponsive to treatment with soluble anti-IgM (n = 14; see supplemental Methods). The associations between BCR responsiveness and miR-29a/b/c (E) and TRAF4 or MYC (F) expression were plotted. Below each panel is a - or + in the row labeled BCR sig: on the left to indicate samples that were low responders or high responders, respectively. P value was tested by Mann-Whitney test. (G-H) Expression of miR-29a/b/c (G) or TRAF4 (H) in CLL patients (n = 96) divided according to MYC expression. All samples with available data on MYC mRNA levels were used in the analysis, and samples were stratified according to the terciles of MYC levels (low vs intermediate [interm] vs high; for distribution of MYC expression, see supplemental Figure 7B). The difference was tested by Mann-Whitney test. (I) Differences in miR-29 levels in B cells from transgenic iMYC^{cc} mice (MYC transgene controlled by the Ig heavy-chain enhancer, n = 3) compared with wildtype (WT) mice (n = 3). The differences were tested by unpaired t test. The error bars indicate SEM.

treated patients ($n = 5$) were clearly less responsive to CD40 ligation (lower IKK α/β phosphorylation) than paired samples obtained before therapy (Figure 6G). This suggests that by inducing *miR-29s*, BCR inhibitors suppress CD40 signaling/T-cell interactions, and this might at least partially explain the previously noted decreased CD40 signaling during ibrutinib/idelalisib treatment *in vitro*.^{56,57}

Discussion

In this study, we identified *miR-29* as being consistently down-modulated in the proliferative CXCR4^{dim}CD5^{bright} CLL cell subpopulation by BCR-induced MYC and identified *miR-29s* novel target, TRAF4, as being upregulated in this context. We revealed that the higher TRAF4 levels determine an increased responsiveness to CD40 ligand. This demonstrates for the first time that miRNAs can act to coordinate malignant B-cell responsiveness to T-cell signals and facilitate the co-occurrence of BCR and CD40 signaling activation. Moreover, we showed that during therapy with BCR inhibitors, the *miR-29* levels are upregulated, and this at least partially explains the impaired CD40 signaling in CLL cells during such therapy.

We performed the first miRNA expression profiling in the CXCR4/CD5 intraclonal CLL cell subpopulation to identify changes in miRNA expression in the immune microenvironment. We and others have previously suggested that the CXCR4^{dim}CD5^{bright} CLL cells correspond to the cells that have recently exited the lymph node niches,^{3,6} which can be used to study gene expression's "fingerprint" in the microenvironment.^{3,5} In this analysis, we identified 36 differentially expressed miRNAs, including several that have previously been shown by us and others to modulate BCR signaling (such as *miR-155*, *miR-150*, and *miR-22*),^{18-20,58} but also other miRNAs with a potentially interesting role in microenvironmental interactions. We noticed that all 3 members of the *miR-29* family (*miR-29a/b/c*) are consistently down-modulated (~2-fold) in the CXCR4^{dim}CD5^{bright} CLL cells and in CLL lymph node samples. Additionally, the lower *miR-29a/b/c* levels associated with significantly shorter overall survival in CLL and unfavorable prognostic markers linked to BCR responsiveness such as unmutated IGHV and ZAP-70 expression. The expression of *miR-29s* was shown not to be related to p53 activity.^{40,59} This is in line with observations of others in CLL, mantle cell lymphoma, follicular lymphoma, and diffuse large B-cell lymphoma, where low levels associate with a more aggressive disease,^{31,32,48-50} and underscores the need to identify *miR-29* targets. Moreover, *miR-29a/b/c* are the most frequently mutated miRNA genes in CLL, and such mutations lead to inefficient *miR-29* biogenesis and its low levels.^{31,59}

It has been shown that all *miR-29* family members have an identical seed sequence determining binding to their target mRNAs. It has been suggested that low-level *miR-29* expression supports CLL cell aggressiveness by upregulating the antiapoptotic proteins MCL1 and TCL1.^{32,46,47} However, as with many other miRNAs, these targets were identified based on a relatively biased preselection of potential *miR-29* targets, which most likely leads to omission of some crucial miR targets in a given (patho)physiologic context.^{21,60} To address this, we performed whole transcriptome profiling in CXCR4/CD5 subpopulations searching for mRNAs that have anti-correlated expression levels to *miR-29*. This is a useful strategy for miRNA target identification because miRNAs typically destabilize their target mRNAs.^{19,21,22} Additionally, this approach obviated

experiments using forced overexpression of the studied miRNA that can lead to shifts in target mRNAs and off-target effects.^{21,60,61} This approach allowed us to identify a novel *miR-29* target in CLL, namely TRAF4, which was further validated by transfecting malignant B cells with synthetic *miR-29*, *miR-29* inhibitor, and a luciferase assay proving a direct *miR-29* binding to 3'UTR TRAF4 mRNA (at positions 161-167). Altogether, the low *miR-29* levels in CLL immune niches allow for higher TRAF4 protein levels. TRAF4 regulation by *miR-29* has also recently been reported in gliomas.⁶² We also confirmed that *miR-29* affects MCL1 and TCL1 levels in CLL^{46,47} but to a lesser extent than its effects on TRAF4 levels.

We further studied TRAF4's molecular function in CLL because this is unknown, and TRAF4 represents a relatively understudied member of the TRAF protein family. Several studies have suggested that other TRAF proteins (TRAF 1, 2, and 3) can contribute to CLL aggressiveness, including the development of a CLL-like disease in double TRAF1/BCL2 transgenic mice.⁶³⁻⁶⁵ In germinal center B cells, TRAF family members (TRAF1/2/3/5/6) act as major signal transducers for lymphocyte activation by Toll-like receptor, B-cell activating factor, and especially CD40.^{54,55,66} Notably, the Epstein-Barr virus recruits TRAFs to the cell membrane via Epstein-Barr virus-encoded LMP1 protein, and this provides a key proliferative signal mimicking CD40 ligation in B cells.⁶⁷⁻⁶⁹ However, TRAF4's function in CD40 signaling is debated because TRAF4 does not directly interact with CD40 but binds other TRAFs interacting with CD40 (such as TRAF2 and 6)⁷⁰ and also binds directly to the cell membrane via phosphoinositide phosphates.⁷¹ Here, we showed for the first time that higher TRAF4 levels determine a higher sensitivity to CD40 ligation in CLL cells and subsequent phosphorylation of immediate downstream effectors in the NF- κ B pathway, namely IKK α/β . Notably, TRAF4 overexpression enhanced B-cell responsiveness to CD40 ligation and rescued their transfection by synthetic *miR-29*. We also verified the role of the *miR-29*-TRAF4 axis on CD40 signaling during CLL-T-cell interactions by coculturing purified CLL cells transfected by synthetic *miR-29* (vs negative control) with autologous activated T cells. We did not observe a role for TRAF4 levels in CLL cell responsiveness to Toll-like receptor activation by cytosine guanine dinucleotides (data not shown). However, we noticed that modulating TRAF4 levels affects basal ERK phosphorylation levels independently of receptor-ligand binding (the basal level of IKK α/β phosphorylation was not affected). This is in line with studies in non-lymphoid cells, suggesting that TRAF4 forms homodimers and contributes to AKT (also known as protein kinase B) and ERK activity by a yet unclear mechanism.⁷¹⁻⁷⁴ Notably, higher TRAF4 levels associate with a shorter survival in CLL, and this was independent of 7 other routinely used prognostic markers in a multivariate analysis ($P = .001$; HR, 6.0; 95% CI, 2-17.9; supplemental Table 5). Altogether, these data indicate that the regulatory connection between *miR-29* and TRAF4 is an important one for CLL-T-cell interactions and malignant B-cell behavior. The importance of T-cell interactions in CLL biology can be illustrated by the possibility of inducing CLL cell proliferation *in vitro* by providing T-cell factors such as CD40L,^{12-16,75} and T-cell interaction intensity contributes to the heterogeneity in disease aggressiveness.⁸⁻¹⁰ However, miRNA targets are known to be context dependent, and it is likely that *miR-29* regulates other gene(s) that also contribute to B-cell biology and/or might be involved in the CD40 pathway. For example, it has been shown that in malignancies, such as mantle cell

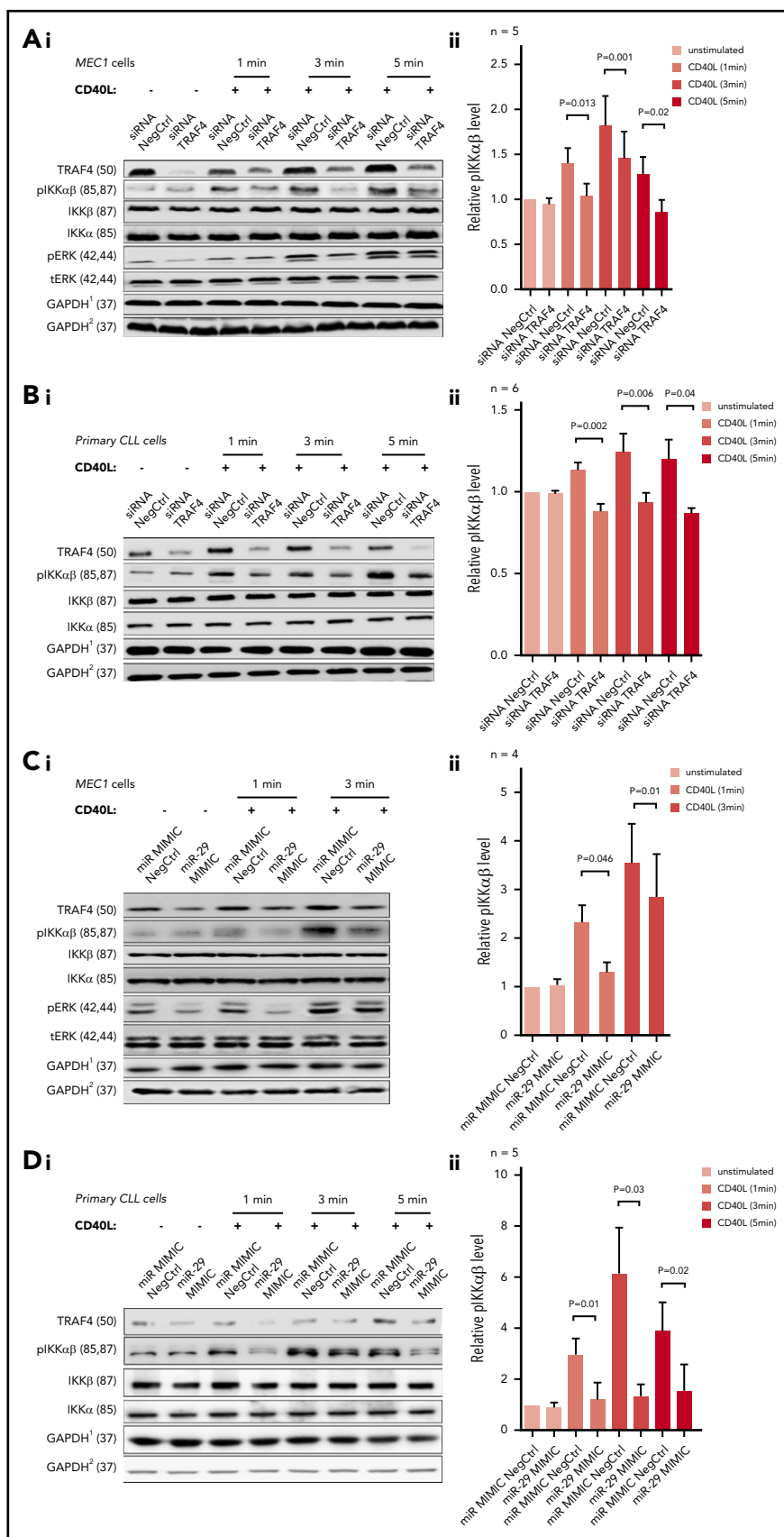
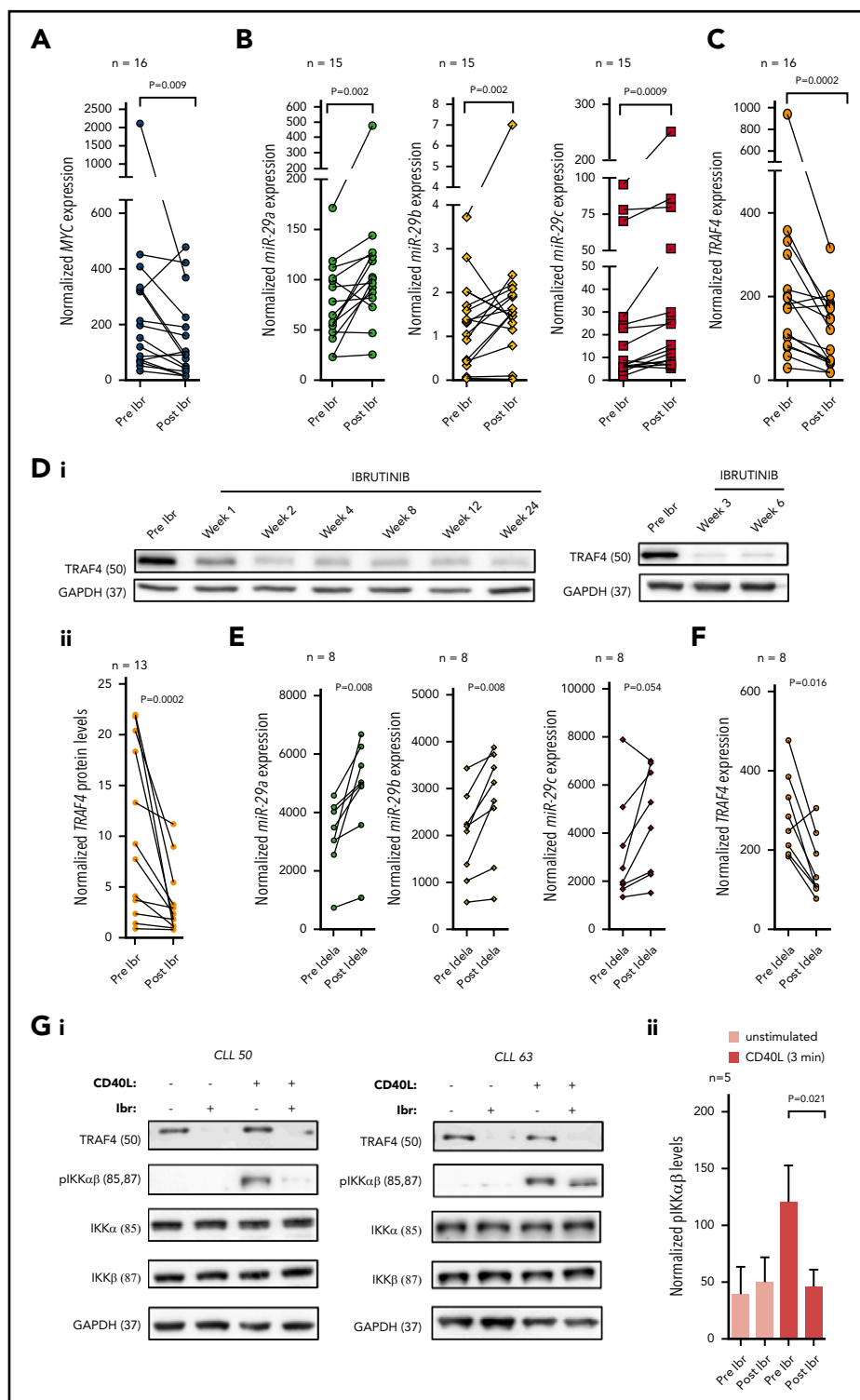


Figure 5. miR-29 targets TRAF4 involved in CD40-mediated NF- κ B signaling. (Ai) Representative example of an immunoblot for MEC1 cells transfected with siRNA against TRAF4 (siRNA TRAF4) or negative control (siRNA NegCtrl). Seventy-two hours after transfection, the cells were stimulated with CD40L (1 μ g/mL) for indicated period of time and harvested for analysis by immunoblotting. (Aii) Densitometric quantification of pIKK α / β levels for replicates of the experiment (n = 5) described in panel Ai. (Bi) Representative example of immunoblot for primary CLL cells transfected with siRNA against TRAF4 (siRNA TRAF4) or negative control (siRNA NegCtrl). Seventy-two hours after transfection, the cells were stimulated with CD40L (1 μ g/mL) for indicated period of time and harvested for analysis by immunoblotting. (Bii) Densitometric quantification of pIKK α / β levels for replicates of the experiment (n = 6) described in panel Bi. (Ci) Representative example of immunoblot for MEC1 cells transfected with artificial miR-29c (miR-29 MIMIC) or negative control (miR MIMIC NegCtrl). Seventy-two hours after transfection, the cells were stimulated with CD40L (1 μ g/mL) and harvested for analysis by immunoblotting. (Cii) Densitometric quantification of pIKK α / β levels for independent replicates of the experiment (n = 4) described in panel Ci. The error bars indicate the standard error of the mean. (Di) Representative example of immunoblot for primary CLL cells transfected with artificial miR-29c (miR-29 MIMIC) or negative control (miR MIMIC NegCtrl). Seventy-two hours after transfection, the cells were stimulated with CD40L (1 μ g/mL) and harvested for analysis by immunoblotting at the indicated time points. (Dii) Densitometric quantification of pIKK α / β levels for replicates of the experiment (n = 5) described in panel Di. (A-D) Each of the immunoblots contains 2 endogenous controls (GAPDH) marked by upper index, because for technical reasons, pIKK, TRAF4, pERK, and total ERK (+ loading control GAPDH¹) were analyzed on first gel and tIKK α and tIKK β (+ loading control GAPDH²) on the second gel (identical protein loading and conditions). In all experiments, the statistical difference was tested using a paired t test, and the error bars indicate SEM.

Figure 6. *miR-29* is upregulated and TRAF4 repressed during therapy with BCR inhibitors.

(A) Normalized MYC expression in 16 CLL patients before (Pre lbr) and during ibrutinib (Post lbr) therapy (weeks 2-12 on therapy depending on the sample availability; for patient characteristics, see supplemental Table 2; CLL 44-46, 48-51, 54, 56-63). (B-C) Normalized expression of *miR-29a/b/c* in 15 CLL patients (B) or TRAF4 mRNA (C) in 16 CLL patients before (Pre lbr) and during ibrutinib (Post lbr) therapy (weeks 2-12 on therapy depending on sample availability; for patient characteristics, see supplemental Table 2; CLL 44-46, 48-51, 54, 56-63 for panel B; CLL 44-46, 48-51, 54, 56-63 for panel C). (Di) Representative immunoblot of TRAF4 protein levels in 2 CLL patients before (Pre lbr) and during ibrutinib therapy (CLL 48 and 50). (Dii) Densitometric quantification of TRAF4 protein levels analyzed by immunoblotting in all available CLL patients before (Pre lbr) and during ibrutinib (Post lbr) therapy ($n = 13$; weeks 2-12, $n = 12$; week 1, $n = 1$; for patient characteristics, see supplemental Table 2; CLL 44-53, 61-63). (E-F) Normalized expression of *miR-29a/b/c* (E) and TRAF4 (F) in 8 CLL patients before (Pre Idela) and during single agent idelalisib (Post Idela) therapy (weeks 5-6 of therapy depending on sample availability; for patient characteristics, see supplemental Table 2; CLL 64-71). The statistical differences for A to F were tested by Wilcoxon matched pairs test. (Gi) Representative immunoblot of pIKK α / β expression after stimulation with CD40L (1 μ g/mL; 3 minutes) in 2 CLL patients before (lbr -) and during ibrutinib (lbr +) therapy in vivo (CLL 50 and 63). (Gii) Densitometric quantification of pIKK α / β levels for replicates of the experiment described in panel Gi ($n = 5$; week 4, $n = 2$; week 6, $n = 1$; week 12, $n = 1$; week 15, $n = 1$). CLL patients before (Pre) and during ibrutinib (Post lbr) were analyzed (for patient characteristics, see supplemental Table 2; CLL 47, 50, 61-63). P value was tested by paired t test, and the error bars indicate SEM.



lymphoma or AML, the *miR-29* targets CDK6⁴⁹ and DNA methyltransferases,⁷⁶ respectively.

We further showed that BCR activation leads to repression of *miR-29s* and a subsequent increase in TRAF4 levels. This is directly dependent on BCR-induced MYC activation in case of *miR-29a* and *miR-29c*, but not *miR-29b*, suggesting that other factors affect the processing and/or stability of *miR-29b*. The data suggest that BCR signaling acts to coordinate CLL cell

responsiveness to the CD40 ligand provided by T cells by modulating *miR-29*-TRAF4 levels. This is in line with a recent observation in normal germinal center B cells, where concurrent synchronous activation of both BCR and CD40 is required to properly induce NF- κ B activity, MYC levels, and entry of B cells into the cell cycle.⁷⁷ This is also partially analogous to a reversed situation with *miR-155*, *miR-150*, and *miR-22*, which are regulated by CD40 or stromal interactions, and this subsequently increases BCR signaling propensity of CLL cells in immune niches.^{18-20,26,58}

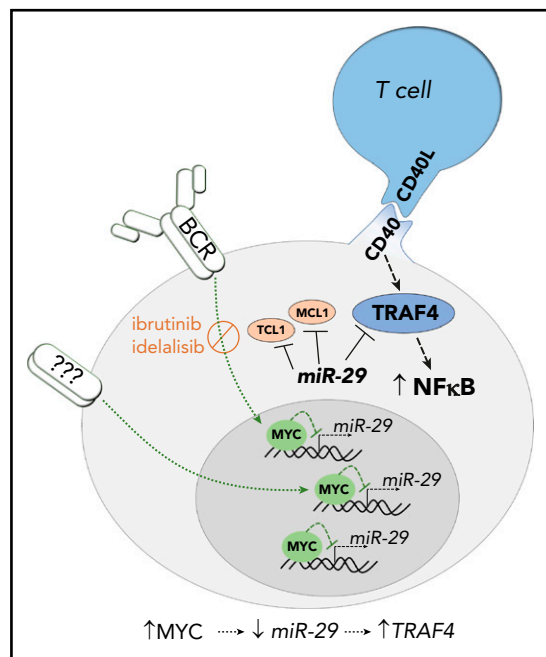


Figure 7. Schematic overview of the role of miR-29-TRAF4 axis in regulation of CD40-induced NF-κB signaling. MYC upregulation by BCR signaling activation downregulates *miR-29* expression in the immune niches. This allows for higher levels of its direct target TRAF4, which acts as a positive regulator of CD40 signaling. BCR signaling inhibitors (ibrutinib/idelalisib) inhibit the BCR-induced MYC levels leading to higher *miR-29* levels and repression of TRAF4. *miR-29* also targets antiapoptotic proteins MCL1 and TCL1.

We also showed that the BCR inhibitors ibrutinib or idelalisib (as single agents) lead in vivo to *miR-29* upregulation, whereas the target, TRAF4, is repressed. We also observed that CLL cells obtained from patients on ibrutinib therapy were clearly less responsive to CD40 ligation than paired samples obtained from the same patient before therapy. The impaired CD40 signaling has been previously noted in vitro ibrutinib treated cells,^{56,57} and CD40-regulated genes are known to be repressed during ibrutinib therapy in vivo.⁷⁸ The effect of *miR-29* on limiting CD40 signaling might have some interesting therapeutic implications. Recently, it has been proposed that synthetic *miR-29* might be used as a therapeutic agent in CLL,⁷⁹ and our data provide clues toward the effects of such a therapy. For example, an *miR-29* mimic might potentially be efficiently combined with venetoclax, because CD40 signaling represents a major mechanism for resistance to BCL2 inhibitors,⁸⁰ and *miR-29* also targets the key antiapoptotic proteins MCL1 and TCL1.

In summary, we used integrated miRNA and mRNA profiling analysis in intracлонаl CLL subpopulations to identify a novel target

of *miR-29* involved in T-cell interactions. Regulation of TRAF4 by *miR-29* contributes to CD40 ligation sensitivity in CLL cells after BCR activation and associates with differences in disease prognosis. This represents an example of how miRNAs can modulate the competency of CLL-T-cell interactions (summarized in Figure 7).

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Authorship

Contribution: S.S. performed experiments, analyzed data, and wrote the paper; G.M.P. and K.A.C. performed NGS experiments; V. Seda, E.V., L.O., V. Sandova, P.F.Z., M.B., D.F., and L. Kostalova performed experiments; K.L. and L. Kren performed IHC; J.O. analyzed NGS data; M.M. designed the study, interpreted data, and wrote the paper; and all authors provided samples and clinical data, interpreted data, and edited and approved the paper for submission.

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Footnotes

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The results of the miRNA/RNAseq analysis can be found in the data supplement. For the original data and detailed protocols, please contact the corresponding author at marek.mraz@email.cz.

The online version of this article contains a data supplement.

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REFERENCES

- Kipps TJ, Stevenson FK, Wu CJ, et al. Chronic lymphocytic leukaemia [published correction in *Nat Rev Dis Primers*. 2017;3:17008]. *Nat Rev Dis Primers*. 2017;3(1):16096.
- Ondrisova L, Mraz M. Genetic and non-genetic mechanisms of resistance to BCR signaling inhibitors in B cell malignancies. *Front Oncol*. 2020;10:591577.
- Pavlasova G, Borsky M, Seda V, et al. Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis. *Blood*. 2016;128(12):1609-1613.
- Calissano C, Damle RN, Marsilio S, et al. Intracлонаl complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. *Mol Med*. 2011;17(11-12):1374-1382.
- Pavlasova G, Borsky M, Svobodova V, et al. Rituximab primarily targets an intra-clonal BCR signaling proficient CLL subpopulation characterized by high CD20 levels. *Leukemia*. 2018;32(9):2028-2031.
- Hemdon TM, Chen SS, Saba NS, et al. Direct in vivo evidence for increased proliferation of CLL cells in lymph nodes compared to bone marrow and peripheral blood. *Leukemia*. 2017;31(6):1340-1347.

7. Herishanu Y, Pérez-Galán P, Liu D, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood*. 2011;117(2):563-574.
8. D'Avola A, Drennan S, Tracy I, et al. Surface IgM expression and function are associated with clinical behavior, genetic abnormalities, and DNA methylation in CLL. *Blood*. 2016;128(6):816-826.
9. Rossmann ED, Jeddi-Tehrani M, Osterborg A, Mellstedt H. T-cell signaling and costimulatory molecules in B-chronic lymphocytic leukemia (B-CLL): an increased abnormal expression by advancing stage. *Leukemia*. 2003;17(11):2252-2254.
10. Palmer S, Hanson CA, Zent CS, et al. Prognostic importance of T and NK-cells in a consecutive series of newly diagnosed patients with chronic lymphocytic leukaemia. *Br J Haematol*. 2008;141(5):607-614.
11. Scielzo C, Apollonio B, Scarfò L, et al. The functional in vitro response to CD40 ligation reflects a different clinical outcome in patients with chronic lymphocytic leukemia [published correction in *Leukemia*. 2011;25:1794]. *Leukemia*. 2011;25(11):1760-1767.
12. Schleiss C, Ilias W, Tahar O, et al. BCR-associated factors driving chronic lymphocytic leukemia cells proliferation ex vivo. *Sci Rep*. 2019;9(1):701.
13. Lezina L, Spriggs RV, Beck D, et al. CD40L/IL-4-stimulated CLL demonstrates variation in translational regulation of DNA damage response genes including ATM. *Blood Adv*. 2018;2(15):1869-1881.
14. Fluckiger AC, Rossi JF, Bussel A, Bryon P, Banchereau J, Defrance T. Responsiveness of chronic lymphocytic leukemia B cells activated via surface Igs or CD40 to B-cell tropic factors. *Blood*. 1992;80(12):3173-3181.
15. Ahearn MJ, Willmott S, Piñon L, et al. Enhancement of CD154/IL4 proliferation by the T follicular helper (Tfh) cytokine, IL21 and increased numbers of circulating cells resembling Tfh cells in chronic lymphocytic leukaemia. *Br J Haematol*. 2013;162(3):360-370.
16. Pascutti MF, Jak M, Tromp JM, et al. IL-21 and CD40L signals from autologous T cells can induce antigen-independent proliferation of CLL cells. *Blood*. 2013;122(17):3010-3019.
17. Bagnara D, Kaufman MS, Calissano C, et al. A novel adoptive transfer model of chronic lymphocytic leukemia suggests a key role for T lymphocytes in the disease. *Blood*. 2011;117(20):5463-5472.
18. Cui B, Chen L, Zhang S, et al. MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia. *Blood*. 2014;124(4):546-554.
19. Mraz M, Chen L, Rassenti LZ, et al. miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1. *Blood*. 2014;124(1):84-95.
20. Palacios F, Abreu C, Prieto D, et al. Activation of the PI3K/AKT pathway by microRNA-22 results in CLL B-cell proliferation. *Leukemia*. 2015;29(1):115-125.
21. Musilova K, Mraz M. MicroRNAs in B-cell lymphomas: how a complex biology gets more complex. *Leukemia*. 2015;29(5):1004-1017.
22. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*. 2010;466(7308):835-840.
23. Dorsett Y, McBride KM, Jankovic M, et al. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity*. 2008;28(5):630-638.
24. O'Connell RM, Baltimore D. MicroRNAs and hematopoietic cell development. *Curr Top Dev Biol*. 2012;99:145-174.
25. Henao-Mejia J, Williams A, Goff LA, et al. The microRNA miR-181 is a critical cellular metabolic rheostat essential for NKT cell ontogenesis and lymphocyte development and homeostasis. *Immunity*. 2013;38(5):984-997.
26. Mraz M, Kipps TJ. MicroRNAs and B cell receptor signaling in chronic lymphocytic leukemia [published correction in *Leuk Lymphoma*. 2014;55(4):969]. *Leuk Lymphoma*. 2013;54(8):1836-1839.
27. Vigorito E, Perks KL, Abreu-Goodger C, et al. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity*. 2007;27(6):847-859.
28. Koralov SB, Muljo SA, Galler GR, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell*. 2008;132(5):860-874.
29. Ebert PJ, Jiang S, Xie J, Li QJ, Davis MM. An endogenous positively selecting peptide enhances mature T cell responses and becomes an autoantigen in the absence of microRNA miR-181a. *Nat Immunol*. 2009;10(11):1162-1169.
30. Li G, Yu M, Lee WW, et al. Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. *Nat Med*. 2012;18(10):1518-1524.
31. Calin GA, Ferracin M, Cimmino A, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med*. 2005;353(17):1793-1801.
32. Stamatopoulos B, Meuleman N, Haibe-Kains B, et al. microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification. *Blood*. 2009;113(21):5237-5245.
33. Rossi S, Shimizu M, Barbarotto E, et al. microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. *Blood*. 2010;116(6):945-952.
34. Fulci V, Chiaretti S, Goldoni M, et al. Quantitative technologies establish a novel microRNA profile of chronic lymphocytic leukemia. *Blood*. 2007;109(11):4944-4951.
35. Mraz M, Malinova K, Kotaskova J, et al. miR-34a, miR-29c and miR-17-5p are down-regulated in CLL patients with TP53 abnormalities. *Leukemia*. 2009;23(6):1159-1163.
36. Mraz M, Dolezalova D, Plevova K, et al. MicroRNA-650 expression is influenced by immunoglobulin gene rearrangement and affects the biology of chronic lymphocytic leukemia. *Blood*. 2012;119(9):2110-2113.
37. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*. 2002;99(24):15524-15529.
38. Klein U, Lia M, Crespo M, et al. The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. *Cancer Cell*. 2010;17(1):28-40.
39. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2 [published correction in *Proc Natl Acad Sci USA*. 2006;103(7):2464]. *Proc Natl Acad Sci USA*. 2005;102(39):13944-13949.
40. Cerna K, Oppelt J, Chochola V, et al. MicroRNA miR-34a downregulates FOXP1 during DNA damage response to limit BCR signalling in chronic lymphocytic leukaemia B cells. *Leukemia*. 2019;33(2):403-414.
41. Musilova K, Devan J, Cerna K, et al. miR-150 downregulation contributes to the high-grade transformation of follicular lymphoma by upregulating FOXP1 levels. *Blood*. 2018;132(22):2389-2400.
42. Tili E, Michaille JJ, Luo Z, et al. The down-regulation of miR-125b in chronic lymphocytic leukemias leads to metabolic adaptation of cells to a transformed state. *Blood*. 2012;120(13):2631-2638.
43. Guinn D, Ruppert AS, Maddocks K, et al. miR-155 expression is associated with chemotherapeutic outcome and is modulated by Bruton's tyrosine kinase inhibition with Ibrutinib. *Leukemia*. 2015;29(5):1210-1213.
44. Guinn D, Lehman A, Fabian C, et al. The regulation of tumor-suppressive microRNA, miR-126, in chronic lymphocytic leukemia. *Cancer Med*. 2017;6(4):778-787.
45. Mraz M, Pospisilova S, Malinova K, Slapak I, Mayer J. MicroRNAs in chronic lymphocytic leukemia pathogenesis and disease subtypes. *Leuk Lymphoma*. 2009;50(3):506-509.
46. Pekarsky Y, Santanam U, Cimmino A, et al. Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. *Cancer Res*. 2006;66(24):11590-11593.
47. Mott JL, Kobayashi S, Bronk SF, Gores GJ. miR-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene*. 2007;26(42):6133-6140.
48. Leich E, Zamo A, Horn H, et al. MicroRNA profiles of t(14;18)-negative follicular lymphoma support a late germinal center B-cell phenotype. *Blood*. 2011;118(20):5550-5558.
49. Zhao JJ, Lin J, Lwin T, et al. microRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. *Blood*. 2010;115(13):2630-2639.
50. Zhang X, Zhao X, Fiskus W, et al. Coordinated silencing of MYC-mediated miR-29 by HDAC3 and EZH2 as a therapeutic target of histone modification in aggressive B-cell lymphomas. *Cancer Cell*. 2012;22(4):506-523.

51. Mraz M, Malinova K, Mayer J, Pospisilova S. MicroRNA isolation and stability in stored RNA samples. *Biochem Biophys Res Commun*. 2009;390(1):1-4.
52. Kollinerova S, Vassanelli S, Modriansky M. The role of miR-29 family members in malignant hematopoiesis. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2014;158(4):489-501.
53. Chang TC, Yu D, Lee YS, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet*. 2008;40(1):43-50.
54. Rickert RC, Jellusova J, Miletic AV. Signaling by the tumor necrosis factor receptor superfamily in B-cell biology and disease. *Immunol Rev*. 2011;244(1):115-133.
55. Bishop GA. The multifaceted roles of TRAFs in the regulation of B-cell function. *Nat Rev Immunol*. 2004;4(10):775-786.
56. Herman SE, Gordon AL, Hertlein E, et al. Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood*. 2011;117(23):6287-6296.
57. Slinger E, Thijssen R, Kater AP, Eldering E. Targeting antigen-independent proliferation in chronic lymphocytic leukemia through differential kinase inhibition. *Leukemia*. 2017;31(12):2601-2607.
58. Cerna K, Mraz M. p53 limits B cell receptor (BCR) signalling: a new role for *miR-34a* and *FOX P1*. *Oncotarget*. 2018;9(92):36409-36410.
59. Kminkova J, Mraz M, Zaprazna K, et al. Identification of novel sequence variations in microRNAs in chronic lymphocytic leukemia. *Carcinogenesis*. 2014;35(5):992-1002.
60. Akbari Moqadam F, Pieters R, den Boer ML. The hunting of targets: challenge in miRNA research. *Leukemia*. 2013;27(1):16-23.
61. Arvey A, Larsson E, Sander C, Leslie CS, Marks DS. Target mRNA abundance dilutes microRNA and siRNA activity. *Mol Syst Biol*. 2010;6(1):363.
62. Shi C, Rao C, Sun C, et al. miR-29s function as tumor suppressors in gliomas by targeting TRAF4 and predict patient prognosis. *Cell Death Dis*. 2018;9(11):1078.
63. Paiva C, Rowland TA, Sreekantham B, et al. SYK inhibition thwarts the BAFF - B-cell receptor crosstalk and thereby antagonizes Mcl-1 in chronic lymphocytic leukemia. *Haematologica*. 2017;102(11):1890-1900.
64. Munzert G, Kirchner D, Stobbe H, et al. Tumor necrosis factor receptor-associated factor 1 gene overexpression in B-cell chronic lymphocytic leukemia: analysis of NF-kappa B/Rel-regulated inhibitors of apoptosis. *Blood*. 2002;100(10):3749-3756.
65. Zapata JM, Krajewska M, Morse HC III, Choi Y, Reed JC. TNF receptor-associated factor (TRAF) domain and Bcl-2 cooperate to induce small B cell lymphoma/chronic lymphocytic leukemia in transgenic mice. *Proc Natl Acad Sci USA*. 2004;101(47):16600-16605.
66. Xie P, Kraus ZJ, Stunz LL, Bishop GA. Roles of TRAF molecules in B lymphocyte function. *Cytokine Growth Factor Rev*. 2008;19(3-4):199-207.
67. Xie P, Hostager BS, Bishop GA. Requirement for TRAF3 in signaling by LMP1 but not CD40 in B lymphocytes. *J Exp Med*. 2004;199(5):661-671.
68. Bishop GA, Hostager BS, Brown KD. Mechanisms of TNF receptor-associated factor (TRAF) regulation in B lymphocytes. *J Leukoc Biol*. 2002;72(1):19-23.
69. Arcipowski KM, Stunz LL, Graham JP, Kraus ZJ, Vanden Bush TJ, Bishop GA. Molecular mechanisms of TNFR-associated factor 6 (TRAF6) utilization by the oncogenic viral mimic of CD40, latent membrane protein 1 (LMP1). *J Biol Chem*. 2011;286(12):9948-9955.
70. Zhang X, Wen Z, Sun L, et al. TRAF2 regulates the cytoplasmic/nuclear distribution of TRAF4 and its biological function in breast cancer cells. *Biochem Biophys Res Commun*. 2013;436(2):344-348.
71. Rousseau A, McEwen AG, Poussin-Courmontagne P, et al. TRAF4 is a novel phosphoinositide-binding protein modulating tight junctions and favoring cell migration. *PLoS Biol*. 2013;11(12):e1001726.
72. Li JM, Fan LM, Christie MR, Shah AM. Acute tumor necrosis factor alpha signaling via NADPH oxidase in microvascular endothelial cells: role of p47phox phosphorylation and binding to TRAF4. *Mol Cell Biol*. 2005;25(6):2320-2330.
73. Li W, Peng C, Lee MH, et al. TRAF4 is a critical molecule for Akt activation in lung cancer. *Cancer Res*. 2013;73(23):6938-6950.
74. Kédinger V, Alpy F, Baguet A, et al. Tumor necrosis factor receptor-associated factor 4 is a dynamic tight junction-related shuttle protein involved in epithelium homeostasis [published correction appears in *PLoS One*. 2008;3(11):e882]. *PLoS One*. 2008;3(10):e3518.
75. Panayiotidis P, Ganeshaguru K, Jabbar SA, Hoffbrand AV. Interleukin-4 inhibits apoptotic cell death and loss of the bcl-2 protein in B-chronic lymphocytic leukaemia cells in vitro. *Br J Haematol*. 1993;85(3):439-445.
76. Garzon R, Liu S, Fabbri M, et al. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood*. 2009;113(25):6411-6418.
77. Luo W, Weisel F, Shlomchik MJ. B cell receptor and CD40 signaling are rewired for synergistic induction of the c-Myc transcription factor in germinal center B cells. *Immunity*. 2018;48(2):313-326.
78. Landau DA, Sun C, Rosebrock D, et al. The evolutionary landscape of chronic lymphocytic leukemia treated with ibrutinib targeted therapy. *Nat Commun*. 2017;8(1):2185.
79. Chiang CL, Goswami S, Frissora FW, et al. ROR1-targeted delivery of miR-29b induces cell cycle arrest and therapeutic benefit in vivo in a CLL mouse model. *Blood*. 2019;134(5):432-444.
80. Thijssen R, Slinger E, Weller K, et al. Resistance to ABT-199 induced by microenvironmental signals in chronic lymphocytic leukemia can be counteracted by CD20 antibodies or kinase inhibitors. *Haematologica*. 2015;100(8):e302-e306.



Chronic lymphocytic leukemia

MicroRNA *miR-34a* downregulates FOXP1 during DNA damage response to limit BCR signalling in chronic lymphocytic leukaemia B cells

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Abstract

The variable clinical course in chronic lymphocytic leukaemia (CLL) largely depends on p53 functionality and B-cell receptor (BCR) signalling propensity; however, it is unclear if there is any crosstalk between these pathways. We show that DNA damage response (DDR) activation leads to down-modulating the transcriptional factor FOXP1, which functions as a positive BCR signalling regulator and its high levels are associated with worse CLL prognosis. We identified microRNA (miRNA) *miR-34a* as the most prominently upregulated miRNA during DDR in CLL cells in vitro and in vivo during FCR therapy (fludarabine, cyclophosphamide, rituximab). *MIR-34a* induced by DDR activation and p53 stabilization potently represses FOXP1 expression by binding in its 3'-UTR. The low FOXP1 levels limit BCR signalling partially via derepressing BCR-inhibitory molecule CD22. We also show that low *miR-34a* levels can be used as a biomarker for worse response or shorter progression free survival in CLL patients treated with FCR chemoimmunotherapy, and shorter overall survival, irrespective of *TP53* status. Additionally, we have developed a method for the absolute quantification of *miR-34a* copies and defined precise prognostic/predictive cutoffs. Overall, herein, we reveal for the first time that B cells limit their BCR signalling during DDR by down-modulating FOXP1 via DDR-p53/*miR-34a* axis.

Introduction

Chronic lymphocytic leukaemia is the most common leukaemia among adults in the western world and its

conventional therapy mainly constitutes of anti-CD20 antibodies, DNA damaging drugs and inhibitors of BCR-associated kinases [1–3]. The clinical course and therapy response of CLL patients is, among other factors, largely dependent on p53 protein functionality [4–6], and the CLL cells' BCR signalling propensity [7, 8]. The differences in the expression and activity of BCR-associated kinases, phosphatases, and their adaptor molecules modulate BCR signalling, and contribute to disease progression and therapy response [8–11]. However, it is unclear if and how B cells limit signalling from their cell-surface BCR under the conditions that involve stabilizing p53 and activating the DNA damage pathway, and if there is any crosstalk between these pathways. It has been described that p53 activation/stabilization leads to signalling inhibition from surface receptors and/or associated kinases in other cell types [12–14].

We have recently shown that the transcription factor FOXP1 functions as a positive BCR signalling propensity regulator in CLL, and its high expression associates with shorter overall survival and treatment free survival [15].

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FOXP1 has also been previously described as upregulated in more aggressive cases of other B-cell malignancies, such as DLBCL, FL, and MALT lymphoma [16–19]. FOXP1 is over-expressed in the ABC subtype of DLBCL [20], and acts as a key regulator for a large number of genes, including those involved in BCR signalling, lymphocyte activation, NF- κ B pathway, Wnt signalling, and chemotaxis [19–25]. We have previously demonstrated that the basal FOXP1 levels in CLL cells are regulated by the miRNA *miR-150*, which was one of the first described roles of miRNAs in BCR signalling [11, 15, 26, 27]. MiRNAs regulate stability and translation of messenger RNAs and thus influence the essential B-cell functions, such as survival and maturation, immunoglobulin production, and their relative BCR signalling proficiency [11, 15, 27–29].

Here we show that FOXP1 expression is down-modulated during the CLL cells' response to DNA damage induced by chemotherapy, and this is mediated by *miR-34a*. The *miR-34a* is, unlike *miR-150*, massively induced during DNA damage response (DDR), and this leads to FOXP1 repression through evolutionary conserved binding sites for *miR-34a*. We and others have previously shown that low *miR-34a* levels are associated with *TP53* aberrations in CLL [30–33]. Here we demonstrate that down-modulation of FOXP1 mediated by the *p53/miR-34a* axis acts to limit the signalling from BCR, which provides a negative regulation between the DDR pathway and BCR signalling. Additionally, we show that low *miR-34a* levels can be used as a biomarker for shorter overall survival (OS) and worse response or shorter progression free survival (PFS) in CLL patients treated with FCR chemoimmunotherapy, irrespective of *TP53* status.

Material and methods

CLL samples and cell lines

Peripheral blood samples were collected with written informed consent and the study was approved by the Institutional Review Boards. Freshly isolated CLL cells were purified (purity $\geq 95\%$ of CD5+19+ cells) with RosetteSep Human B Cell Enrichment Cocktail (Stemcell Technologies; Vancouver, Canada). The clinical and biological characteristics of CLL patients are summarized in Supplementary Tables S2–7. The cell lines (WSU-NHL, RAMOS, RAJI, MEC-1, 293FT, SW-48^{TP53-/-}, SW-48^{TP53+/+}) were obtained from the German Collection of Micro-organisms and Cell Cultures (DSMZ) and American Type Culture Collection (ATCC), and cultured according to the recommendations (see Supplementary Methods).

Small RNA sequencing and real-time PCR

Total RNA was isolated with TRIzol Reagent (Thermo Fisher Scientific; Waltham, MA, USA) as described previously [30] and RNA integrity was analysed (RIN >8). Two different NGS platforms and library preparation protocols were used, namely SOLiD Total RNA-Seq Kit-Small RNA Library (SOLiD5500, Applied Biosystem; Foster City, CA, USA) and TruSeq Small RNA sample preparation kit (HiSeq 2500, Illumina; San Diego, CA, USA). For further details on library preparation and data analyses, see Supplementary Methods.

The expression of individual miRNAs/mRNAs was performed with TaqMan MicroRNA or Gene Expression Assays (Thermo Fisher Scientific), and normalized to endogenous controls (RNU38B or GAPDH, respectively), as described previously [29, 30]. For the absolute quantification of *miR-34a* copies, see Supplementary Methods.

Transfections

The B-cell lymphoma cell line WSU-NHL or freshly isolated CLL cells were transfected by Neon Transfection System (Thermo Fisher Scientific) with miRNA mimics (Sigma Aldrich; St Louis, MO, USA), miRNA inhibitors (Dharmacon; Lafayette, CO, USA), siRNAs (Dharmacon) or respective controls, and harvested for further analyses by immunoblotting and flow cytometry (see Supplementary Methods).

BCR stimulation and calcium flux

BCR signalling was evaluated by examining the intracellular calcium flux induced by ligating the cell-surface IgM using goat F(ab')₂ anti-human IgM as described previously [15, 34], for details see Supplementary Methods.

Luciferase assay

Luciferase reporter assay was performed in HEK293FT cells using the LightSwitch Luciferase Assay System (SwitchGear Genomics; San Francisco, CA, USA). According to the manufacturer's protocol as described previously [15] (see Supplementary Methods).

Statistical analysis

Survival analysis was performed with the use of the Kaplan–Meier survival estimator, univariate and multivariate analyses employed Cox regression models and were performed on Statistica 10 (StatSoft Inc.). Other statistical analyses were performed with GraphPad Prism Software v5.0 (GraphPad Software) or using freeware R. P-values

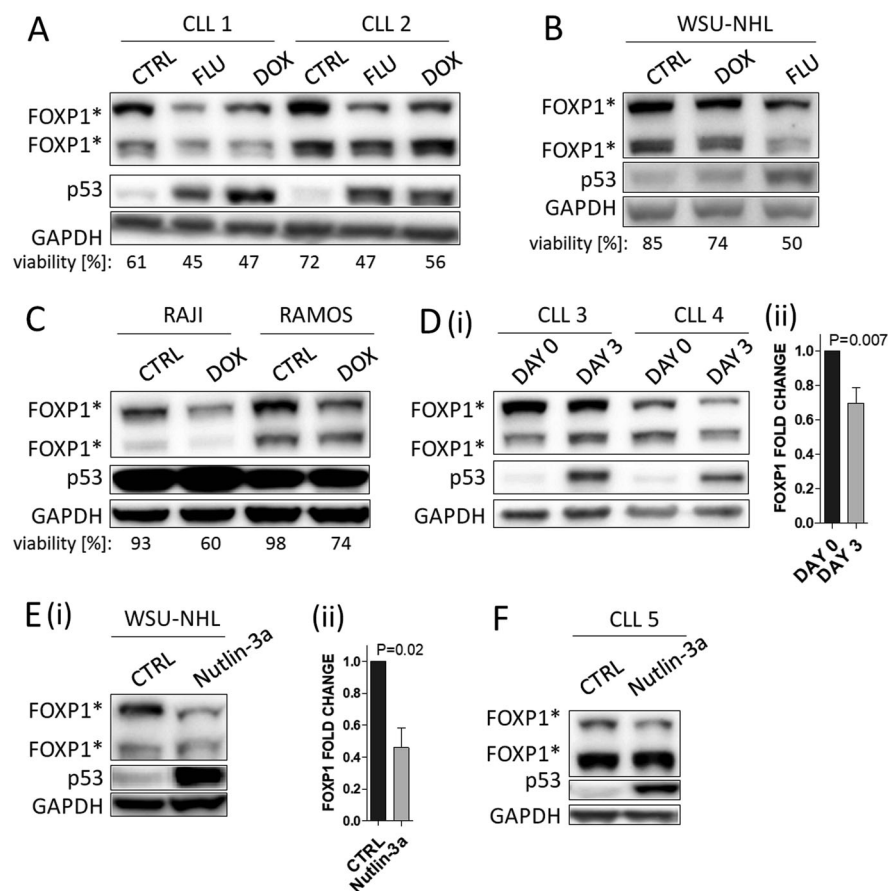


Fig. 1 FOXP1 is downregulated during DDR. **a** Representative immunoblot from CLL cells (purity >95% CD19+CD5+) treated in vitro (48 h) with fludarabine (FLU; 1.75 μ M) or doxorubicin (DOX; 400 ng/mL). **b** Representative immunoblot from WSU-NHL cells treated in vitro (48 h) with doxorubicin (25 ng/ μ L) or fludarabine (0.5 μ M). **c** A representative immunoblot from RAJI and RAMOS cell lines treated in vitro (48 h) with doxorubicin (100 ng/mL). **d** (i) Representative immunoblot from freshly isolated CLL cells (purity >95% CD19+CD5+) obtained from patients before the

therapy administration (day 0) and on day 3 (48 h post FC administration). (ii) Expression of FOXP1 protein from in vivo treated patients ($n = 3$) normalized to GAPDH. **e** (i) Representative immunoblot from WSU-NHL cells treated with Nutlin-3a (10 μ M; 24 h). (ii) FOXP1 fold change expression normalized to GAPDH ($n = 3$). **f** Representative immunoblot from freshly isolated CLL cells treated with Nutlin-3a (10 μ M; 24 h). **a–f** The double bands identified by the anti-FOXP1 antibody represent 2 splicing variants of FOXP1 as described previously [15, 20, 25, 46]

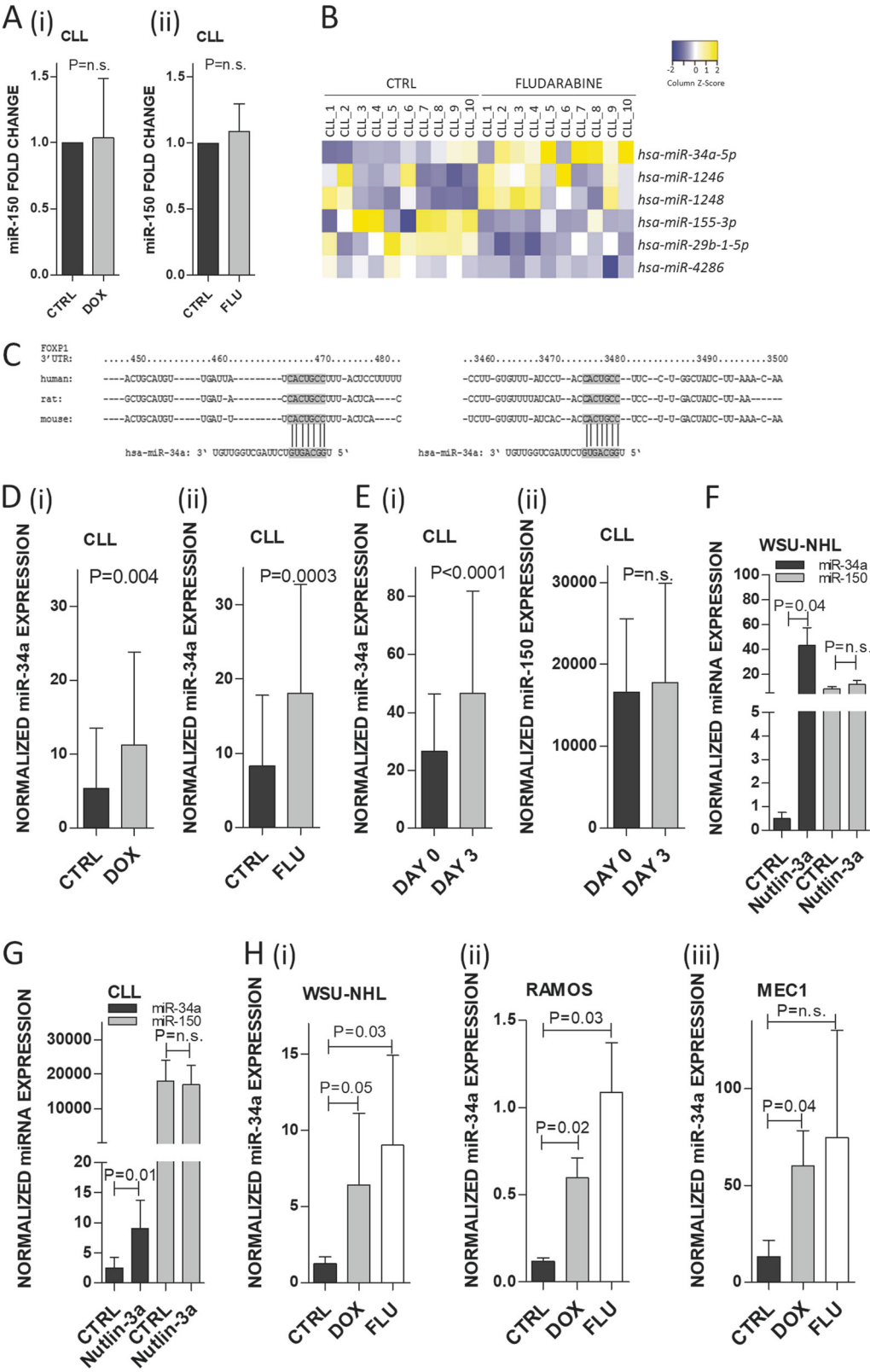
<0.05 were considered significant. For other data processing and NGS data analysis, see Supplementary Methods.

Results

FOXP1 is downregulated during DNA damage response

The transcription factor FOXP1 positively regulates BCR signalling, and its high levels associate with worse prognosis in CLL [15] and B-cell lymphomas [16–18, 35, 36]; however, the mechanisms affecting the FOXP1 levels in malignant B cells are largely unknown. We noticed that FOXP1 expression is down-modulated with the DNA-damage response (DDR) in CLL B cells. This was observed with classical DNA damage inducing drugs (fludarabine,

doxorubicin) in primary CLL cells and B-cell lines under conditions inducing relatively low-level apoptosis (Fig. 1a–c), and also following administration of FCR chemoimmunotherapy in CLL in vivo (Fig. 1d). The DDR led to down-modulation of both major splicing variants of FOXP1 that were described previously [15, 20, 25, 37]. This suggests that the p53 pathway might be directly responsible for FOXP1 down-modulation. Indeed, applying the specific p53-stabilizer Nutlin-3a (inhibitor of MDM2 ubiquitin-ligase) down-modulated FOXP1 levels in WSU-NHL and CLL cells (Fig. 1e, f). However, we also noted FOXP1 down-modulation in *TP53*-deficient cell lines RAMOS and RAJI (Fig. 1c), suggesting that this phenotype can also be present in cells with impaired p53 functionality (also see below). Altogether, this data reveal that FOXP1 is negatively regulated within the context of DDR in B cells.



◀ **Fig. 2** *MiR-34a* represents the most prominently upregulated miRNA during DDR and therapy response. **a** *MiR-150* fold change expression in CLL cells (purity >95% CD19+CD5+; Supplementary Table 4) treated for 48 h with (i) doxorubicin (DOX; 100 ng/mL; CLL1-10) or (ii) fludarabine (FLU; 3.5 µg/mL; CLL11-25). For a time course and dose dependent analyses see Supplementary Figure 1. **b** Heatmap of significantly changed miRNAs in 10 paired CLL samples treated with fludarabine (3.5 µg/mL; Supplementary Table 2). Heatmap was generated from the sequencing data mapped with miRanalyzer and normalized to counts per million reads (rows centred to the median of the row). **c** The evolutionary conserved binding sites for *miR-34a* in the FOXP1 3'-UTR (TargetScanHuman, Release 7.1). **d** *MiR-34a* expression in CLL cells (purity >95% CD19+CD5+; $n = 10$; CLL1-25, Supplementary Table 4) treated for 48 h with (i) doxorubicin (100 ng/mL; CLL1-10) or (ii) fludarabine (3.5 µg/mL; CLL11-25). **e** The expression of (i) *miR-34a* or (ii) *miR-150* in freshly isolated CLL cells (purity >95% CD19+CD5+) obtained from patients ($n = 50$; Supplementary Table 5) undergoing FCR therapy on day 0 (before the therapy) and at day 3 (48 h post FC administration). **f** The expression of *miR-34a* and *miR-150* in WSU-NHL cells treated with Nutlin-3a (10 µM, 24 h). **g** The expression of *miR-34a* and *miR-150* in freshly isolated CLL cells (purity >95% CD19+CD5+) treated with Nutlin-3a (10 µM, 24 h; $n = 5$). **h** The expression of *miR-34a* in B-cell lines WSU-NHL (i) treated with doxorubicin (25 ng/mL) or fludarabine (0.5 µg/mL) and cultured for 48 h and in RAMOS (ii) and MEC-1 cells (iii) treated with doxorubicin (100 ng/mL) or fludarabine (10 µg/mL) for 48 h

***MiR-34a* is the most robustly upregulated miRNA during DNA damage response in CLL**

It has been previously shown that the FOXP1 levels in CLL cells are regulated by *miR-150*, however, we did not observe any *miR-150* induction that would explain FOXP1 repression during DDR in vitro or in vivo (Fig. 2a, Supplementary Figure 1, and see below). Additionally, the *miR-150* promoter or the 10 kb upstream region do not contain any experimentally obtained (ChIP-Seq) or predicted p53 binding sites (Supplementary Tables 1A, B). To investigate whether other miRNAs could regulate FOXP1 during DDR, we performed miRNA profiling (NGS miRNA-Seq) in CLL samples treated in vitro with fludarabine (component of the FCR therapy), which induces FOXP1 down-modulation (Fig. 1a–d; Supplementary Table 2). Paired control and fludarabine treated samples were analysed; fludarabine concentration was selected based on a sufficient cell viability decrease (Supplementary Figure 2A) and an achievable level in patient's plasma in vivo [38]. The profiling with two different NGS platforms and in a validation cohort identified six miRNAs as differentially expressed after fludarabine (fold change >1.5; FDR <0.05; Fig. 2b, Supplementary Table 3). Three upregulated miRNAs were all major miRNA sequences, but two of the downregulated miRNAs (*miR-155-3p*, *miR-29b-1-5p*) were the minor sequences that are generally thought not to have a biological function (Supplementary Figures 2B, C). From the six differentially expressed miRNAs, only *miR-34a* contains

putative evolutionary conserved binding sites in the 3'-UTR region of FOXP1 (Fig. 2c) and therefore, we selected this miRNA as a candidate for FOXP1 regulation during DDR. We confirmed the induction of *miR-34a* expression in CLL cells treated in vitro with doxorubicin or fludarabine (Fig. 2d, for patient characterization see Supplementary Table 4). To evaluate these observations in vivo, we obtained samples from patients undergoing FCR therapy ($n = 50$; for cohort characterization see Supplementary Table 5). In this analysis, *miR-34a* was not induced after rituximab alone (day 2; Supplementary Figure 3), but was clearly induced after the FC administration (day 3), while *miR-150* levels were unchanged (Fig. 2e). Additionally, *miR-34a* (but not *miR-150*) was massively induced after p53 stabilization with Nutlin-3a in WSU-NHL cells or primary CLL cells (Fig. 2f, g). The induction of *miR-34a*, which is a known p53-downstream target [39], was also observed in p53-aberrant RAMOS and MEC-1 cells (Fig. 2h). To test this in a system with no residual p53 activity, we analysed syngeneic SW-48^{TP53+/+} and SW-48^{TP53-/-} (*TP53*-deficient) cells, and these also induced some levels of *miR-34a* during DDR (Supplementary Figure 4A). The incomplete dependency of *miR-34a* induction on p53 has also been reported by others [40, 41], and resembles the situation with other DDR-induced genes such as p21 (refs. [42, 43]). In line with this observation, FOXP1 down-modulation was also observed in RAJI and RAMOS cells (Fig. 1c), and in SW-48^{TP53-/-} to a lesser extent than in *TP53* wild-type SW-48 cells (Supplementary Figure 4B). Altogether, this suggests that *miR-34a* is induced during DDR and represents a primary candidate for FOXP1 regulation under DDR conditions.

***MiR-34a* is a direct and dominant regulator of FOXP1 during DNA damage response**

We hypothesized that *miR-34a* induction is responsible for down-modulating FOXP1 during DDR, while *miR-150* levels are of main relevance for regulation of FOXP1 basal levels. This is supported by very low basal *miR-34a* levels in CLL cells (median number of reads in miRNA NGS profiling experiment is 78 counts per million [CPM] compared to *miR-150* with 94 630 CPM), and lack of correlation (in contrary to *miR-150*) with the basal levels of FOXP1 (Supplementary Figure 5). To study the FOXP1-miRNA interactions, we performed a luciferase assay in HEK293FT cells and compared the *miR-34a*'s ability against *miR-150* to inhibit the luciferase activity of a vector containing the 3'-UTR of FOXP1. The down-modulation of the luciferase activity was approximately twofold more prominent for *miR-34a* than *miR-150* ($P < 0.001$, Fig. 3a). This is likely due to the presence of two binding sites for *miR-34a* in the 3'-UTR of FOXP1 compared to one for

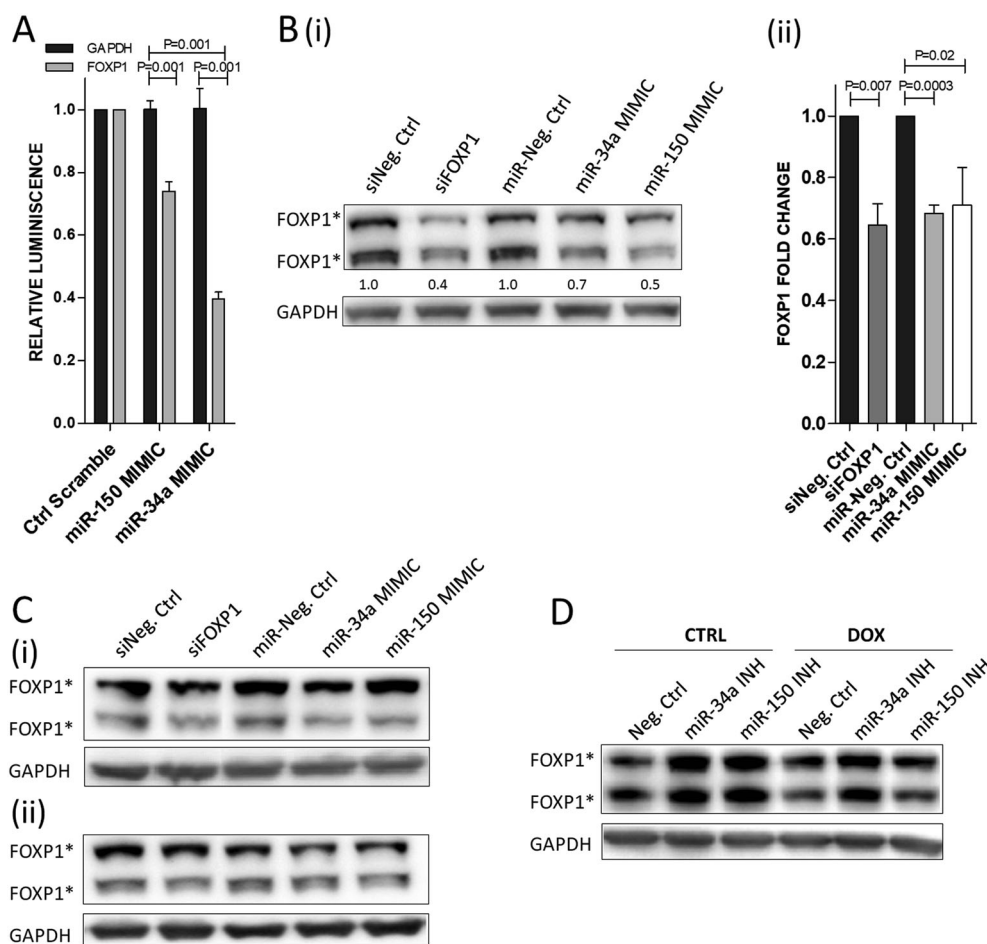


Fig. 3 *MiR-34a* is a direct and dominant FOXP1 regulator during DDR. **a** Luciferase activity in HEK293FT cells co-transfected with either *miR-34a* mimic (*miR-34a* MIMIC, 100 nM) or *miR-150* mimic (*miR-150* MIMIC; positive control) or the negative control (Ctrl Scramble, 100 nM) and luciferase reporter construct (100 ng), containing the 3'-UTR region of FOXP1 or GAPDH (as a control). The HEK293FT cells express very low and comparable endogenous levels of *miR-34a* and *miR-150*. **b** (i) Representative immunoblot of FOXP1 levels in WSU-NHL cells transfected with siRNA against FOXP1 (siFOXPI) or siRNA negative control (siNeg. Ctrl) or *miR-34a* mimic (*miR-34a* MIMIC), *miR-150* mimic (*miR-150* MIMIC), or miRNA negative control (*miR-Neg. Ctrl*), and collected 48 h after transfection. SiFOXPI and *miR-150* MIMIC serve as positive controls.

miR-150 (Supplementary Figure 6). Importantly, the transfection of synthetic *miR-34a* or *miR-150* induced significant down-modulation of FOXP1 levels in WSU-NHL cells compared to the negative control (Fig. 3b). WSU-NHL cells were selected because of the *TP53* wild-type status (unlike the CLL derived *TP53*-aberrant MEC-1 cells), relatively high-FOXP1 levels, and good viability after transfection (Supplementary Figure 7). The effect of *miR-34a* mimic on FOXP1 could be also replicated with primary CLL samples (Fig. 3c). Next, we inhibited *miR-34a* or *miR-150* with miRNA inhibitors, and subsequently induced DDR with doxorubicin (Fig. 3d). In this experiment the *miR-34a*

(ii) Statistical analyses of the synthetic *miR-34a*'s effect on FOXP1 levels (48 h; $n = 3$). The FOXPI/GAPDH ratios for the siNeg. Ctrl and *miR-Neg. Ctrl* were arbitrarily set as 1.0. **c** (i, ii) Representative immunoblots from two freshly isolated CLL cells (purity >95% CD19 +CD5+) transfected in the same manner as described above in **b**. **d** Representative immunoblot of WSU-NHL cells transfected with inhibitors of *miR-34a*, *miR-150* or negative control (Neg. Ctrl), and subsequently treated with doxorubicin (25 ng/mL). The FOXPI/GAPDH ratios for the siNeg. Ctrl with or without doxorubicin were arbitrarily set as 1.0. **b–d** The double bands identified by the anti-FOXP1 antibody represent 2 splicing variants of FOXP1 as described previously [15, 20, 25, 46]

inhibitor (but not *miR-150* inhibitor) prevented FOXP1 down-modulation under DDR conditions. This suggests that *miR-34a* is a dominant direct FOXP1 regulator under DDR conditions.

***MiR-34a* induction by DNA damage response inhibits BCR signalling**

We and others have previously suggested that FOXP1 is a positive BCR signalling pathway regulator [15, 20, 23]. We observed that under DDR conditions, the B-cell response to BCR activation is mitigated (Supplementary Figure 8).

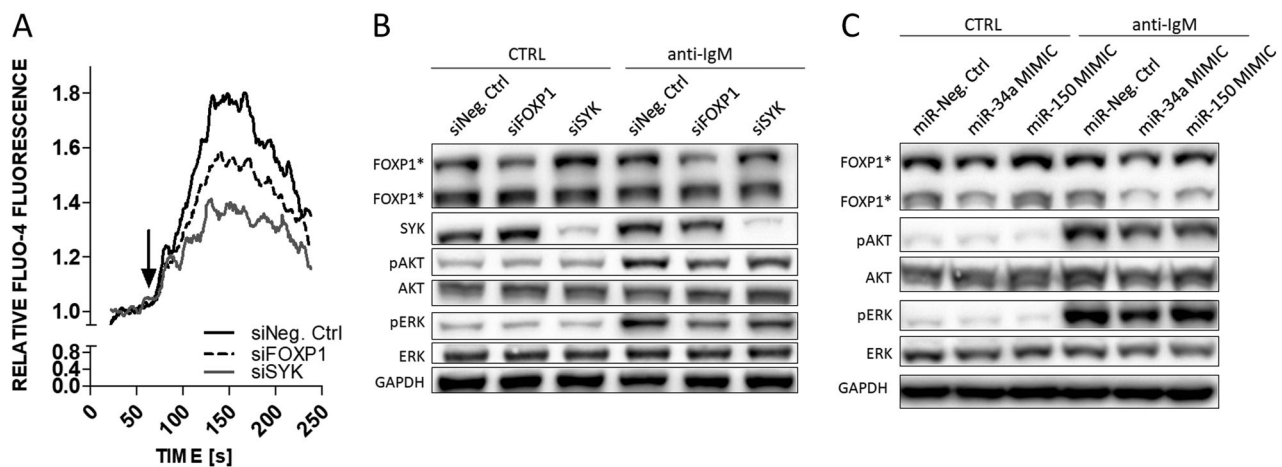


Fig. 4 *MiR-34a* limits BCR signalling during DNA damage response. **a** Representative result of the calcium flux in WSU-NHL cells transfected with siRNA against FOXP1 (siFOX P1), SYK (siSYK; as a positive control) or the negative control (siNeg. Ctrl) after BCR crosslinking with anti-IgM (10 μ g/mL, indicated by the arrow). The cells were cultured for 48 h after the transfection and then processed for calcium flux measurements. **b** Representative immunoblot of WSU-NHL cells transfected with siRNA against FOXP1 (siFOX P1), SYK (siSYK; as a positive control) or the negative control

(siNeg. Ctrl). Cells were collected after 48 h, divided in equal numbers and exposed to the vehicle (CTRL) or 5 μ g/mL of anti-IgM. **c** Representative immunoblot of WSU-NHL cells transfected with artificial miRNA mimics, *miR-34a* mimic (*miR-34a* MIMIC), *miR-150* mimic (*miR-150* MIMIC), or negative control (*miR-Neg. Ctrl*). Cells were collected after 48 h, divided in equal numbers and exposed to the vehicle (CTRL) or anti-IgM (5 μ g/mL). **b, c** The double bands identified by the anti-FOXP1 antibody represent 2 splicing variants of FOXP1 as described previously [15, 20, 25, 46]

We hypothesized that down-modulating FOXP1 during DDR might be responsible for the lower levels of phospho-AKT and phospho-ERK induced by anti-IgM during DDR. Indeed, the calcium signalling was reduced in WSU-NHL B cells transfected with siRNA against FOXP1 or SYK (positive control) following the BCR crosslinking with anti-IgM antibody (Fig. 4a), and similar results were obtained in primary CLL cells (Supplementary Figure 9). Similarly, FOXP1 silencing or *miR-34a* mimic transfection had a direct effect on the phospho-AKT and phospho-ERK levels after BCR crosslinking (Fig. 4b, c). The cell-surface IgM expression was not influenced by the FOXP1 silencing (Supplementary Figure 10). These data support the conclusion that FOXP1 down-modulation during DDR represents at least partially, a mechanism to limit signalling from BCR under relatively mild DDR conditions that do not induce immediate cell death.

In an analysis of previous studies describing potential FOXP1 transcriptional targets (see Supplementary Table 6), we identified 6 potential targets involved in BCR signalling (NFATC4; PIK3CG; CD22; PIK3R1; CD19; SYK). Their profiling in CLL samples with relatively low vs. high FOXP1 levels ($n = 30$) revealed that the mRNA levels for a BCR-inhibitory CD22 molecule [44] are significantly lower in cells with higher FOXP1 expression ($P = 0.01$; Supplementary Figure 11A). We also observed a statistically significant, but relatively small, upregulation in CD22 cell-surface levels following FOXP1 silencing in WSU-NHL cells (Supplementary Figure 11B), but no change in other tested BCR-related proteins (Supplementary Figures 10,

12). The mild siRNA effect against FOXP1 on CD22 levels might be due to relatively inefficient FOXP1 silencing (Supplementary Figure 11B). Importantly, we observed CD22 upregulation after DDR activation in WSU-NHL and CLL cells upon fludarabine or Nutlin-3a treatment (Supplementary Figure 11C). Altogether, these data show that FOXP1 down-modulation during DDR activation leads to a higher BCR signalling activation threshold and higher levels of BCR-inhibitory molecule CD22.

Low levels of *miR-34a* predict worse response to FCR therapy

The data above suggests that *miR-34a* is an important determinant of CLL response to DDR-based chemotherapy. We observed that *miR-34a* is induced in a prospectively collected cohort of 50 CLL patients receiving FCR therapy (Supplementary Table 5; Fig. 2e), and the basal *miR-34a* levels before FCR were very well correlated with the induced *miR-34a* levels on day 3 of FCR ($r^2 = 0.82$; Supplementary Figure 13A). This suggests that its basal and induced levels are regulated by the same mechanism (p53/DDR pathway) [30]. Importantly, the *miR-34a* levels were a significant predictor of progression free survival (PFS) after FCR (Fig. 5a; median 20 vs. 26.4 months; $P = 0.05$; HR: 2.31 [CI 1.0–5.38]). The low *miR-34a* levels associated with deleted and/or mutated *TP53* (Fig. 5b; Supplementary Figures 13B, C), but low *miR-34a* levels predicted shorter PFS also in the *TP53* wild-type subgroup ($n = 45$, $P = 0.04$; HR: 2.74 [CI 1.06–7.10]; Fig. 5c). Interestingly, the patients

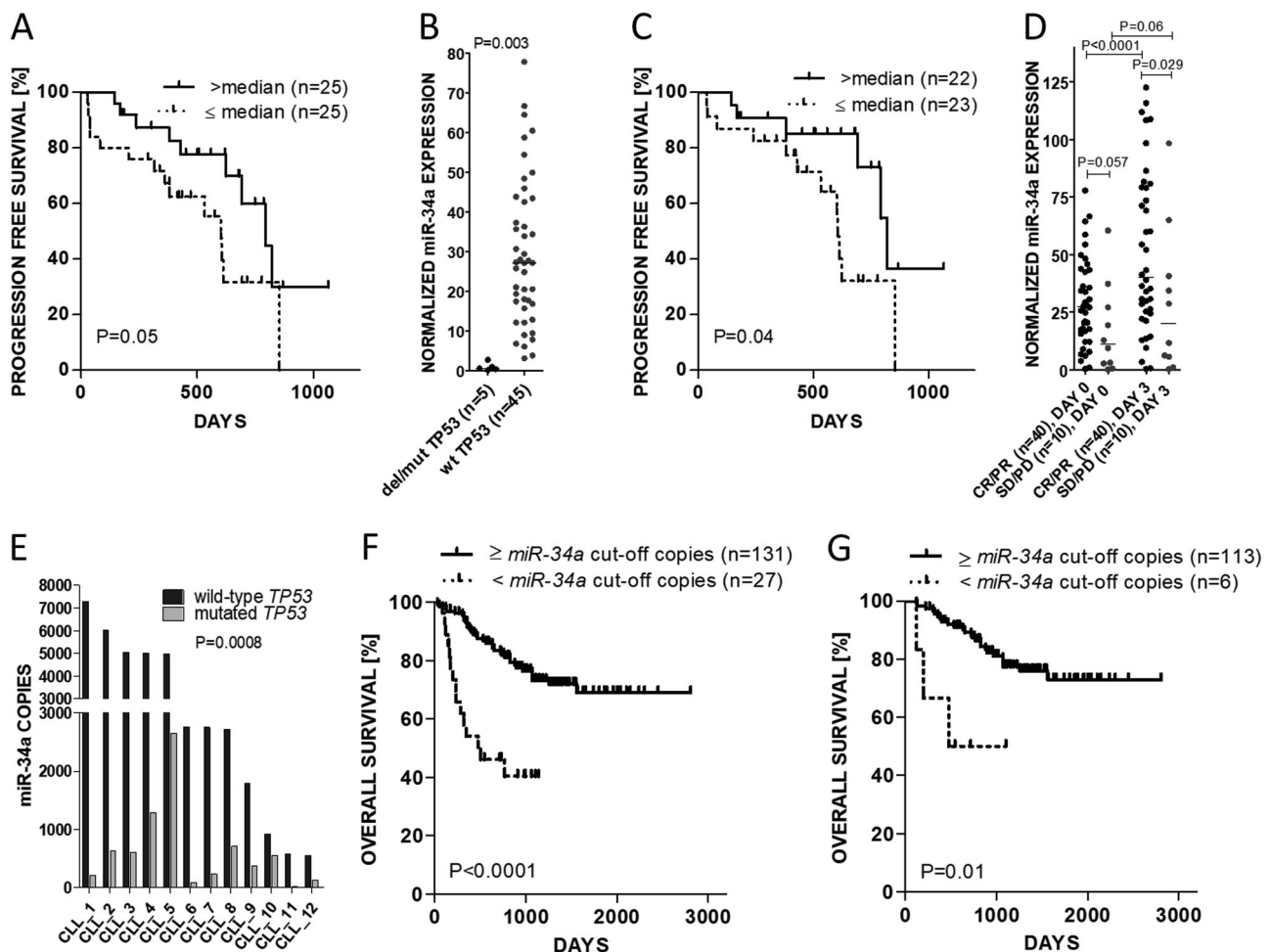


Fig. 5 Low levels of *miR-34a* associate with worse response to FCR therapy. **a** Progression free survival (from sampling) of FCR treated patients ($n = 50$, Supplementary Table 5) dichotomized according to the median of *miR-34a* expression on day 0 (before the therapy administration). **b** The *miR-34a* expression on day 0 before the therapy administration in cases with deleted and/or mutated *TP53* ($n = 5$) compared to wild-type *TP53* cases ($n = 45$). **c** Progression free survival (from sampling) of FCR treated patients dichotomized according to the median of *miR-34a* expression on day 0 in the *TP53* wild-type cohort ($n = 45$). **d** The *miR-34a* expression on day 0 and day 3 of FCR in patients that achieved CR/PR ($n = 40$) vs. SD/PD ($n = 10$). **e** The absolute *miR-34a* copies (per 10e6 copies of *RNU38B*) in CLL patients that developed a *TP53* mutation during the follow-up (CLL26-35; Supplementary Table 8; median follow-up 33.2 months). **f** The overall survival (from sampling) of patients ($n = 158$; Supplementary Table 6) dichotomized based on the cut-off of 2047 copies of *miR-34a* per 10e6 copies of *RNU38B* (OS: 15.8 months vs. not reached; $P < 0.0001$; HR: 4.05 [CI 2.17–7.57]). **g** The overall survival (from sampling) of the *TP53* wild-type CLL patients ($n = 119$) dichotomized based on the *miR-34a* cut-off (2047 copies; $P = 0.01$; HR: 5.00 [CI 1.48–16.96])

that only achieved a stable or progressive disease (SD/PD) had lower basal *miR-34a* levels than patients achieving partial or complete remission (PR/CR; Fig. 5d). Patients achieving PR/CR also induced higher *miR-34a* levels than patients with SD/PD (Fig. 5d); however, the *miR-34a* level on day 3 was an inferior PFS predictor than the one on day 0 (Supplementary Figures 13D, E). This is likely due to an averaging of the *miR-34a* qRT-PCR signal induced by DDR from the whole CLL cell population that might contain a subset of DDR-proficient cells with high *miR-34a* induction and a DDR/p53 deficient clone with low levels of *miR-34a*. Altogether, the results suggest that *miR-34a* expression can be used as a novel predictive biomarker for FCR response.

Absolute quantification of *miR-34a* copies and its prognostic value

It has been previously shown that low *miR-34a* levels associate with shorter treatment free survival [32] and mutated/deleted *TP53* [30, 31, 33, 45] in CLL. This was based on determining the relative expression of *miR-34a*, which however, does not allow standardization or a definition of cut-offs that could be used in other laboratories. This is a general issue in the attempts to use miRNAs as a biomarker in oncology [46]. To address this, we designed a qRT-PCR assay with standards to measure the total number of *miR-34a* molecules (Supplementary Figure 14A and Supplementary Methods) in a cohort

of well-characterized CLL samples ($n = 158$; Supplementary Table 7). The lowest number of *miR-34a* copies was observed in the *TP53* abnormal group (Supplementary Figure 14B), and no other clinico-biological characteristics correlated with *miR-34a* levels (Supplementary Figure 14C). We defined a cut-off for *miR-34a* copies that discriminated *TP53* aberrant cases with a relatively high specificity and sensitivity (74–89% and 73–89%, respectively, Supplementary Figure 15). Interestingly, this can not only be used to detect *TP53* aberrations at diagnosis, but to detect *TP53* mutation evolution over time (Fig. 5e; Supplementary Table 8). We also defined a *miR-34a* cut-off (<2047 copies) as being strongly associated with shorter OS (15.8 months vs. not reached; $P < 0.0001$; HR: 4.05 [CI 2.17–7.57]; Fig. 5f; Supplementary Table 9). The low *miR-34a* levels also predicted shorter OS in the *TP53* wild-type cohort ($P = 0.01$; HR: 5.01 [CI 1.48–16.96]; $n = 119$; Fig. 5g) and this was independent in a multivariate analysis ($P = 0.002$; HR: 9.68 [CI 2.31–40.66; Supplementary Tables 10A, B). Overall, the absolute *miR-34a* quantification can be used as a reliable biomarker for OS, prediction of response to FCR, and quick screening of *TP53* status.

Discussion

Here we describe that during the response to DNA damage, the transcription factor FOXP1 is downregulated by *miR-34a* induction in CLL cells. We provide evidence that FOXP1 acts as a positive BCR signalling regulator and its down-modulation during DDR represents a mechanism to limit the pro-survival/pro-proliferative signals from BCR (summarized in Supplementary Figure 16). We have also shown that higher levels of *miR-34a* associate with a deeper clinical response to FCR chemoimmunotherapy with longer PFS, and also longer OS in CLL patients.

We and others have suggested that high-FOXP1 levels positively affect the BCR signalling pathway and activation of malignant B cells, and showed that this associates with shorter OS in CLL and other mature B-cell malignancies [15–17, 20, 36, 37]. Here we describe that FOXP1 levels are down-modulated in B cells during DNA damage response (DDR) in vitro and also during FCR therapy in vivo. We have previously shown that the FOXP1 can be regulated by *miR-150* [15], however we determined that *miR-150* is not responsible for FOXP1 down-modulation during DDR. The screening of miRNAs affected by DDR activation in CLL cells revealed that three miRNAs were significantly induced; including *miR-34a*, which has 2 evolutionary conserved binding sites in the 3'-UTR region of FOXP1. We confirmed *miR-34a*

upregulation in samples obtained from CLL patients undergoing therapy with FCR and also in cells with DDR induced in vitro. We have further shown that *miR-34a* directly binds the FOXP1 3'-UTR and represses its expression in a luciferase assay. Importantly, the *miR-34a*'s ability to down-modulate FOXP1 was twofold greater than that of *miR-150*, which is a known FOXP1 regulator. The transfection of B-cell lines or primary CLL cells with *miR-34a* synthetic mimic led to FOXP1 down-modulation. The transfection with synthetic *miR-34a* (or siRNA against FOXP1) or DDR led to down-modulation of both major splicing variants of FOXP1, but with a different kinetics which likely reflect differences in their half-life as observed previously [15]. Importantly, the inhibition of *miR-34a* under DDR conditions led to sustained FOXP1 levels in comparison to cells with inhibited *miR-150* which suggests that *miR-34a* is a dominant FOXP1 level regulator in B cells responding to DNA damage. *miR-34a* is known to be upregulated through p53 protein [39], but we also observed some induction of *miR-34a* and repression of FOXP1 in B-cell lines with p53 mutations, or in syngeneic *TP53*-deficient cells. This shows that similar to other cell types, *miR-34a* regulation is partially independent of p53 (refs. [40, 41]) under specific cellular conditions, which is analogical to other canonical p53 transcriptional targets, e.g. p21 (refs. [42, 43]). The upregulation of *miR-34a* in cells with aberrant *TP53* might be caused by partial replacement of p53 function with its homologs p63/73 that have binding sites in *miR-34a* upstream region [47], residual activity of p53 protein in cases with *TP53* mutation [48] or some other mechanisms [49, 50]. Altogether, our data and the published studies suggest that under conditions with functional p53 protein this transcription factor governs upregulation of *miR-34a* during DDR, while *miR-34a* transcription is potentially regulated by alternative less potent mechanisms in the absence of p53. However, the dominant role of p53 in *miR-34a* regulation in the primary CLL cells is apparent in cases with deleted/mutated *TP53* which express the lowest levels of *miR-34a*, and such cells are inducing much lower levels of *miR-34a* than *TP53* wild-type CLL cells during DDR [30, 31].

It has been suggested previously that the basal *miR-34a* levels can affect the FOXP1 levels in MALT lymphomas [51]. However, in CLL the basal *miR-34a* levels are very low and do not correlate, in contrary to *miR-150*, with the basal levels of FOXP1. We showed that the prominent induction of *miR-34a* during DDR and its strong binding to FOXP1 mRNA allows for a meaningful stoichiometric concentration to affect the FOXP1 levels under DDR conditions. This is an important case of a context-dependent regulation of a mRNA targeted by multiple miRNAs [26, 52], when *miR-34a* “over-rules” the *miR-150* regulation under specific DDR conditions.

MiR-34a belongs to the family containing two other members, *miR-34b/c* located on another chromosome, but these are not detectable in CLL cells [30, 31] (see Supplementary Table 3), probably due to epigenetic silencing [53]. It has been reported in other cell types that *miR-34a* targets MDM4, SIRT1, AXL, and YY1, regulates cell cycle, autophagy, migration, metabolism, and multiple signalling pathways [54]. However, the miRNAs' targets largely depend on the cellular and functional context [26, 52]. The observation that *miR-34a* targets FOXP1 in CLL cells is critical to the understanding why *miR-34a* levels associate with disease aggressiveness in CLL, and what role its induction plays during DDR. However, it is likely that *miR-34a* also affects other targets. We demonstrated that FOXP1 down-modulation is at least partially responsible for the impaired response to BCR activation while B cells react to DNA damage and stabilize p53. FOXP1 repression impaired calcium flux as well as reduced phosphorylation of AKT and ERK induced by anti-IgM stimulation. This is of great interest since the BCR signalling propensity is an important determinant of CLL aggressiveness [7–10], but it is unknown how the p53 pathway regulates its activity or how is interconnected with it. Studies of how exactly FOXP1 regulates BCR signalling are ongoing in multiple laboratories, and hundreds (~300–900) of potential FOXP1 target genes have been identified [19, 20, 23–25] (Supplementary Table 6). We tested the expression of putative FOXP1 targets canonically involved in BCR signalling and found that CLL cells with lower FOXP1 levels have higher CD22 levels, FOXP1 silencing leads to CD22 upregulation, and CD22 is induced with DDR activation. CD22 serves as a key inhibitory molecule in BCR signalling, since it provides a docking site for phosphatases such as SHP-1 that limits BCR signalling [44], and FOXP1 was previously found to bind to its promoter [20]. The observed effect of FOXP1 on BCR signalling might be partially mediated through CD22 levels but identifying other BCR-relevant FOXP1 targets remains an ongoing effort in multiple laboratories. None of the other known *miR-34a* targets are direct regulators of BCR signalling, however we cannot exclude the possibility that *miR-34a* targets such as MYC or MYB [26, 40] might indirectly influence the BCR signalling by regulating expression of genes involved in this pathway.

The role of *miR-34a* in decreasing FOXP1 and BCR pathway activity might be of importance for the therapeutic effect of DNA damaging drugs. Indeed, CLL patients with low *miR-34a* levels have significantly shorter PFS after FCR. The patients with *TP53* mutations/deletions have low *miR-34a* [30, 45], but we also found low *miR-34a* levels in some patients harbouring intact *TP53*, and also in this group *miR-34a* remained a predictor of shorter PFS after FCR. To further explore the use of *miR-34a* as a biomarker, we

developed a standardized assay for its quantification that allows obtaining an exact number of *miR-34a* copies in the sample. We have defined specific cut-off values that can be used to screen for *TP53* mutations at diagnosis or their evolution during the course of the disease. This does not require the *miR-34a* assaying after DDR, since both the basal and induced *miR-34a* levels are low in p53-aberrant cases. This overcomes laborious DDR induction required for other available assays for functional p53 analysis [55–58], and the herein described absolute quantification technique makes it easy to standardize (standards available upon request). We have also defined a specific cut-off value that distinguishes CLL patients with a shorter OS, and this was independent of *TP53* status or other prognostic factors in a multivariate analysis ($P = 0.002$; HR: 9.68 [CI 2.31–40.66; Supplementary Table 9B]). It has been shown by others that low *miR-34a* levels are associated with shorter time to treatment in CLL [32], but for OS this is reported for the first time. Recently, the association of *miR-34a* levels with poor survival and therapeutic response in solid tumours led to pre-clinical studies and a clinical trial of *miR-34a* replacement therapy (MRX34, trial NCT01829971) [59, 60], and our data suggest that this might be of interest also in B-cell malignancies.

In summary, here we show *miR-34a*'s role as a FOXP1 regulator during activated DDR and its ability to limit BCR signalling during DDR, which represents a first direct description of such a regulatory loop in CLL. We have also demonstrated that *miR-34a* can be used as an independent predictive marker of response to FCR in CLL, and also as an independent prognostic marker for OS.

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Author contributions K.C. performed experiments, analysed data and wrote the paper; V.C., K.M. and G.P. performed experiments; V.S. interpreted the data; J.O. and L.R. analysed the data; M.A. and R.A.C. performed Illumina NGS sequencing; other co-authors provided patients' samples, clinical data and interpreted data, M.M. designed the study, interpreted data and wrote the paper. All authors edited and approved the paper for submission.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Byrd JC, Furman RR, Coutre SE, Flinn IW, Burger JA, Blum KA, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2013;369:32–42.
- Furman RR, Cheng S, Lu P, Setty M, Perez AR, Guo A, et al. Ibrutinib resistance in chronic lymphocytic leukemia. *N Engl J Med*. 2014;370:2352–4.
- Hallek M, Fischer K, Fingerle-Rowson G, Fink A, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet*. 2010;376:1164–74.
- Döhner H, Stilgenbauer S, Benner A, Leupolt E, Kröber A, Bullinger L, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med*. 2000;343:1910–6.
- Trbusek M, Smardova J, Malcikova J, Sebejova L, Dobes P, Svitakova M, et al. Missense mutations located in structural p53 DNA-binding motifs are associated with extremely poor survival in chronic lymphocytic leukemia. *J Clin Oncol*. 2011;29:2703–8.
- Malcikova J, Pavlova S, Kozubik KS, Pospisilova S. TP53 mutation analysis in clinical practice: lessons from chronic lymphocytic leukemia. *Hum Mutat*. 2014;35:663–71.
- D'Avola A, Drennan S, Tracy I, Henderson I, Chiecchio L, Larrazoy M, et al. Surface IgM expression and function associate with clinical behavior, genetic abnormalities and DNA methylation in CLL. *Blood*. 2016;128:816–26.
- Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. 2011;118:4313–20.
- Kipps TJ. The B-cell receptor and ZAP-70 in chronic lymphocytic leukemia. *Best Pract Res Clin Haematol*. 2007;20:415–24.
- Seda V, Mraz M. B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells. *Eur J Haematol*. 2015;94:193–205.
- Cui B, Chen L, Zhang S, Mraz M, Fecteau J-F, Yu J, et al. MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia. *Blood*. 2014;124:546–54.
- Nakade K, Zheng H, Ganguli G, Buchwalter G, Gross C, Wasyluk B. The tumor suppressor p53 inhibits Net, an effector of Ras/extracellular signal-regulated kinase signaling. *Mol Cell Biol*. 2004;24:1132–42.
- Stambolic V, MacPherson D, Sas D, Lin Y, Snow B, Jang Y, et al. Regulation of PTEN transcription by p53. *Mol Cell*. 2001;8:317–25.
- Elston R, Inman GJ. Crosstalk between p53 and TGF- β Signaling. *J Signal Transduct*. 2012;2012:1–10.
- Mraz M, Chen L, Rassenti LZ, Ghia EM, Li H, Jepsen K, et al. miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1. *Blood*. 2014;124:84.
- Barrans SL, Fenton JAL, Banham A, Owen RG, Jack AS. Strong expression of FOXP1 identifies a distinct subset of diffuse large B-cell lymphoma (DLBCL) patients with poor outcome. *Blood*. 2004;104:2933–5.
- Sagaert X, de Paepe P, Libbrecht L, Vanhentenrijk V, Verhoef G, Thomas J, et al. Forkhead box protein P1 expression in mucosa-associated lymphoid tissue lymphomas predicts poor prognosis and transformation to diffuse large B-cell lymphoma. *J Clin Oncol*. 2006;24:2490–7.
- Brown P, Marafioti T, Kusec R, Banham AH. The FOXP1 transcription factor is expressed in the majority of follicular lymphomas but is rarely expressed in classical and lymphocyte predominant hodgkin's lymphoma. *J Mol Histol*. 2005;36:249–56.
- van Keimpema M, Grüneberg LJ, Mokry M, van Boxtel R, Koster J, Coffey PJ, et al. FOXP1 directly represses transcription of proapoptotic genes and cooperates with NF- κ B to promote survival of human B cells. *Blood*. 2014;124:3431–40.
- Dekker JD, Park D, Shaffer AL, Kohlhammer H, Deng W, Lee B-K, et al. Subtype-specific addiction of the activated B-cell subset of diffuse large B-cell lymphoma to FOXP1. *Proc Natl Acad Sci USA*. 2016;113:E577–E586.
- van Keimpema M, Grüneberg LJ, Schilder-Tol EJM, Oud MECM, Beuling EA, Hensbergen PJ, et al. The small FOXP1 isoform predominantly expressed in activated B cell-like diffuse large B-cell lymphoma and full-length FOXP1 exert similar oncogenic and transcriptional activity in human B cells. *Haematologica*. 2017;102:573–83.
- Walker MP, Stopford CM, Cederlund M, Fang F, Jahn C, Rabinowitz AD, et al. FOXP1 potentiates Wnt/ β -catenin signaling in diffuse large B cell lymphoma. *Sci Signal*. 2015;8:ra12. nor
- Sagardoy A, Martinez-Ferrandis JJ, Roa S, Bunting KL, Aznar MA, Elemento O, et al. Downregulation of FOXP1 is required during germinal center B-cell function. *Blood*. 2013;121:4311–20.
- van Boxtel R, Gomez-Puerto C, Mokry M, Eijkelenboom A, van der Vos KE, Nieuwenhuis EE, et al. FOXP1 acts through a negative feedback loop to suppress FOXO-induced apoptosis. *Cell Death Differ*. 2013;20:1219–29.
- Flori M, Schmid CA, Sumrall ET, Tzankov A, Law CW, Robinson MD, et al. The hematopoietic oncoprotein FOXP1 promotes tumor cell survival in diffuse large B-cell lymphoma by repressing S1PR2 signaling. *Blood*. 2016;127:1438–48.
- Musilova K, Mraz M. MicroRNAs in B-cell lymphomas: how a complex biology gets more complex. *Leukemia*. 2015;29:1004–17.
- Mraz M, Kipps TJ. MicroRNAs and B cell receptor signaling in chronic lymphocytic leukemia. *Leuk Lymphoma*. 2013;54:1836–9.
- Vigorito E, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, et al. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity*. 2007;27:847–59.
- Mraz M, Dolezalova D, Plevova K, Stano Kozubik K, Mayerova V, Cerna K, et al. MicroRNA-650 expression is influenced by immunoglobulin gene rearrangement and affects the biology of chronic lymphocytic leukemia. *Blood*. 2012;119:2110–3.
- Mraz M, Malinova K, Kotaskova J, Pavlova S, Tichy B, Malcikova J, et al. miR-34a, miR-29c and miR-17-5p are down-regulated in CLL patients with TP53 abnormalities. *Leukemia*. 2009;23:1159–63.
- Zenz T, Mohr J, Eldering E, Kater AP, Bühler A, Kienle D, et al. miR-34a as part of the resistance network in chronic lymphocytic leukemia. *Blood*. 2009;113:3801–8.
- Asslaber D, Pinon JD, Seyfried I, Desch P, Stocher M, Tinhofer I, et al. microRNA-34a expression correlates with MDM2 SNP309 polymorphism and treatment-free survival in chronic lymphocytic leukemia. *Blood*. 2010;115:4191–7.
- Dufour A, Palermo G, Zellmeier E, Mellert G, Duchateau-Nguyen G, Schneider S, et al. Inactivation of TP53 correlates with disease progression and low miR-34a expression in previously treated chronic lymphocytic leukemia patients. *Blood*. 2013;121:3650–7.
- Chen L, Widhopf G, Huynh L, Rassenti L, Rai KR, Weiss A, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. 2002;100:4609–14.
- Mottok A, Jurinovic V, Farinha P, Rosenwald A, Leich E, Ott G, et al. FOXP1 expression is a prognostic biomarker in follicular lymphoma treated with rituximab and chemotherapy. *Blood*. 2018;131:226–35.

36. Banham AH, Connors JM, Brown PJ, Cordell JL, Ott G, Sreenivasan G, et al. Expression of the FOXP1 transcription factor is strongly associated with inferior survival in patients with diffuse large B-cell lymphoma. *Clin Cancer Res J Am Assoc Cancer Res*. 2005;11:1065–72.
37. Brown PJ, Ashe SL, Leich E, Burek C, Barrans S, Fenton JA, et al. Potentially oncogenic B-cell activation-induced smaller isoforms of FOXP1 are highly expressed in the activated B cell-like subtype of DLBCL. *Blood*. 2008;111:2816–24.
38. Cejkova S, Rocnova L, Potesil D, Smardova J, Novakova V, Chumchalova J, et al. Presence of heterozygous ATM deletion may not be critical in the primary response of chronic lymphocytic leukemia cells to fludarabine. *Eur J Haematol*. 2009;82:133–42.
39. He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, et al. A microRNA component of the p53 tumour suppressor network. *Nature*. 2007;447:1130–4.
40. Christoffersen NR, Shalgi R, Frankel LB, Leucci E, Lees M, Klausen M, et al. p53-independent upregulation of miR-34a during oncogene-induced senescence represses MYC. *Cell Death Differ*. 2010;17:236.
41. Vinall RL, Ripoll AZ, Wang S, Pan C-X, deVere White RW. MiR-34a chemosensitizes bladder cancer cells to cisplatin treatment regardless of p53-Rb pathway status. *Int J Cancer*. 2012;130:2526–38.
42. Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, et al. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes Dev*. 1995;9:935–44.
43. Galanos P, Vougas K, Walter D, Polyzos A, Maya-Mendoza A, Haagensen EJ, et al. Chronic p53-independent p21 expression causes genomic instability by deregulating replication licensing. *Nat Cell Biol*. 2016;18:777–89.
44. Nitschke L, Carsetti R, Ocker B, Köhler G, Lamers MC. CD22 is a negative regulator of B-cell receptor signalling. *Curr Biol*. 1997;7:133–43.
45. Zenz T, Habe S, Denzel T, Mohr J, Winkler D, Buhler A, et al. Detailed analysis of p53 pathway defects in fludarabine-refractory chronic lymphocytic leukemia (CLL): dissecting the contribution of 17p deletion, TP53 mutation, p53-p21 dysfunction, and miR34a in a prospective clinical trial. *Blood*. 2009;114:2589–97.
46. Bartels CL, Tsongalis GJ. MicroRNAs: novel biomarkers for human cancer. *Clin Chem*. 2009;55:623–31.
47. Antonini D, Russo MT, De Rosa L, Gorrese M, Del Vecchio L, Missero C. Transcriptional repression of miR-34 family contributes to p63-mediated cell cycle progression in epidermal cells. *J Invest Dermatol*. 2010;130:1249–57.
48. Kato S, Han S-Y, Liu W, Otsuka K, Shibata H, Kanamaru R, et al. Understanding the function–structure and function–mutation relationships of p53 tumor suppressor protein by high-resolution missense mutation analysis. *Proc Natl Acad Sci USA*. 2003;100:8424–9.
49. Pulikkan JA, Peramangalam PS, Dengler V, Ho PA, Preudhomme C, Meshinchi S, et al. C/EBP regulated microRNA-34a targets E2F3 during granulopoiesis and is down-regulated in AML with CEBPA mutations. *Blood*. 2010;116:5638–49.
50. Wang LQ, Kwong YL, Wong KF, Kho CSB, Jin DY, Tse E, et al. Epigenetic inactivation of mir-34b/c in addition to mir-34a and DAPK1 in chronic lymphocytic leukemia. *J Transl Med*. 2014;12:52.
51. Craig VJ, Cogliatti SB, Imig J, Renner C, Neuenschwander S, Rehauer H, et al. Myc-mediated repression of microRNA-34a promotes high-grade transformation of B-cell lymphoma by dysregulation of FoxP1. *Blood*. 2011;117:6227–36.
52. Jens M, Rajewsky N. Competition between target sites of regulators shapes post-transcriptional gene regulation. *Nat Rev Genet*. 2015;16:113–26.
53. Deneberg S, Kanduri M, Ali D, Bengtzen S, Karimi M, Qu Y, et al. *microRNA-34b/c* on chromosome 11q23 is aberrantly methylated in chronic lymphocytic leukemia. *Epigenetics*. 2014;9:910–7.
54. Bader AG. miR-34 – a microRNA replacement therapy is headed to the clinic. *Front Genet*. 2012;3:120.
55. Le Garff-Tavernier M, Blons H, Nguyen-Khac F, Pannetier M, Brissard M, Gueguen S, et al. Functional assessment of p53 in chronic lymphocytic leukemia. *Blood Cancer J*. 2011;1:e5–e5.
56. Pozzo F, Dal Bo M, Peragine N, Bomben R, Zucchetto A, Rossi FM, et al. Detection of TP53 dysfunction in chronic lymphocytic leukemia by an in vitro functional assay based on TP53 activation by the non-genotoxic drug Nutlin-3: a proposal for clinical application. *J Hematol Oncol J Hematol Oncol*. 2013;6:83.
57. te Raa GD, Malcikova J, Mraz M, Trbusek M, Garff-Tavernier L, Merle-Beral H, et al. Assessment of TP53 functionality in chronic lymphocytic leukaemia by different assays; an ERIC-wide approach. *Br J Haematol*. 2014;167:565–9.
58. te Raa GD, Moerland PD, Leeksa AC, Derks IA, Yigittop H, Laddach N, et al. Assessment of p53 and ATM functionality in chronic lymphocytic leukemia by multiplex ligation-dependent probe amplification. *Cell Death Dis*. 2015;6:e1852–e1852.
59. Urgard E, Brjalin A, Langel Ü, Pooga M, Rebane A, Annilo T. Comparison of peptide- and lipid-based delivery of miR-34a-5p Mimic into PPC-1 Cells. *Nucleic Acid Ther*. 2017;27:295–302.
60. Trang P, Wiggins JF, Daige CL, Cho C, Omotola M, Brown D, et al. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Mol Ther*. 2011;19:1116–22.

Novel CHK1 inhibitor MU380 exhibits significant single-agent activity in TP53-mutated chronic lymphocytic leukemia cells

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ABSTRACT

Introduction of small-molecule inhibitors of B-cell receptor signaling and BCL2 protein significantly improves therapeutic options in chronic lymphocytic leukemia. However, some patients suffer from adverse effects mandating treatment discontinuation, and cases with *TP53* defects more frequently experience early progression of the disease. Development of alternative therapeutic approaches is, therefore, of critical importance. Here we report details of the anti-chronic lymphocytic leukemia single-agent activity of MU380, our recently identified potent, selective, and metabolically robust inhibitor of checkpoint kinase 1. We also describe a newly developed enantioselective synthesis of MU380, which allows preparation of gram quantities of the substance. Checkpoint kinase 1 is a master regulator of replication operating primarily in intra-S and G₂/M cell cycle checkpoints. Initially tested in leukemia and lymphoma cell lines, MU380 significantly potentiated efficacy of gemcitabine, a clinically used inducer of replication stress. Moreover, MU380 manifested substantial single-agent activity in both *TP53*-wild type and *TP53*-mutated leukemia and lymphoma cell lines. In chronic lymphocytic leukemia-derived cell lines MEC-1, MEC-2 (both *TP53*-mut), and OSU-CLL (*TP53*-wt) the inhibitor impaired cell cycle progression and induced apoptosis. In primary clinical samples, MU380 used as a single-agent noticeably reduced the viability of unstimulated chronic lymphocytic leukemia cells as well as those induced to proliferate by anti-CD40/IL-4 stimuli. In both cases, effects were comparable in samples harboring p53 pathway dysfunction (*TP53* mutations or *ATM* mutations) and *TP53*-wt/*ATM*-wt cells. Lastly, MU380 also exhibited significant *in vivo* activity in a xenotransplant mouse model (immunodeficient strain NOD-*scid* IL2R γ^{null}) where it efficiently suppressed growth of subcutaneous tumors generated from MEC-1 cells.

Introduction

Significant progress has been achieved in the therapy of chronic lymphocytic leukemia (CLL) with the introduction of small-molecule inhibitors targeting the B-cell receptor (BCR) signaling^{1,2} and BCL2 protein.³ Drugs like ibrutinib, idelalisib or venetoclax are currently changing clinical practice in CLL treatment. The most extensive data are related to the BCR inhibitor ibrutinib, and besides many positive aspects revealed also that: (i) the drug possesses a specific toxicity profile enforcing treatment



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discontinuation in a proportion of patients;⁴ (ii) some patients progress during therapy to the stage of highly adverse diffuse large B-cell lymphoma (Richter transformation);⁵ and (iii) relapsed/refractory patients harboring 17p deletion (*TP53* defect) experience relatively short progression-free survival and overall survival after the single-agent ibrutinib treatment.⁶⁷ Nevertheless, the clinical efficacy of ibrutinib is substantially better compared to chemioimmunotherapy, which has been found to be unsuitable for *TP53*-defective patients.⁸

Replication is a vital process for each cancer cell, and the proteins controlling its course represent interesting targets for anti-cancer therapy. The checkpoint kinase 1 (CHK1) supervises replication through the intra-S and G₂/M cell cycle checkpoints, where it stabilizes stalled replication forks after DNA damage and participates in DNA repair by homologous recombination process.^{9,10} CHK1 is an important member of the DNA damage response (DDR) pathway, which represents a fundamental anti-cancer barrier.¹¹ Central to DDR are two signaling cascades: ATR→CHK1 and ATM→CHK2→p53. While the latter is frequently mutated in tumors, the activity of *ATR* and *CHK1* genes is essential for cell survival.^{12,13} In line with this, *CHK1* was found to be an essential gene for 557 out of 558 cancer cell lines, according to the DepMap database (depmap.org) (Online Supplementary Figure S1).

Numerous structurally diverse CHK1 inhibitors have been developed as potentiating agents, i.e. for combination with chemotherapy. Nevertheless, some of them showed interesting pre-clinical single-agent activity against diverse cancer types, including breast and ovarian cancer,¹⁴ small-cell lung cancer,¹⁵ colorectal cancer,¹⁶ neuroblastoma,¹⁷ melanoma,¹⁸ MYC driven lymphoma,¹⁹ and leukemia.^{20,21} Currently, several CHK1 inhibitors are undergoing evaluation in clinical trials focusing on solid tumors and hematologic malignancies. In CLL, CHK1 inhibition represents a potentially attractive concept for the following reasons: (i) CHK1 is essential for normal B-cell development and lymphomagenesis;²² (ii) leukemia and lymphoma cells are particularly vulnerable to CHK1 depletion;²⁰ and (iii) CLL cells are sensitive to manipulation with the level of replication stress (RS), as shown in experiments inhibiting ATR, a CHK1 upstream kinase.²³

In our previous study,²⁴ we employed one of the most selective CHK1 inhibitors, SCH900776,²⁵ and showed that it significantly potentiates activity of fludarabine in *TP53*-mutated CLL cells, as well as in a CLL *TP53*-wt mouse model. Subsequently, we developed compound MU380, a non-trivial analog of SCH900776 which contains unusual N-trifluoromethylpyrazole moiety protecting the molecule from oxidative dealkylation and thus improving its metabolic stability.²⁶ With our current study, we present a robust enantioselective synthesis of MU380, and demonstrate its single-agent efficacy in lymphoid cancer cells. Significantly, to the best of our knowledge, we have for the first time demonstrated the potential for CHK1 inhibition to affect high-risk CLL cells with *TP53* defects.

Methods

CHK1 inhibitors

Compound SCH900776 (Merck; MK-8776) was prepared in-house using previously described procedure.²⁴ Compound MU380 was also prepared in-house using our newly developed

enantioselective synthesis (see Results section and Online Supplementary Appendix). These inhibitors were stored at room temperature as 10 mM stock solutions dissolved in DMSO.

Cell lines and primary chronic lymphocytic leukemia cells

Leukemia and lymphoma cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and cultured in accordance with DSMZ recommendations. *TP53* mutation status was verified by sequencing, and was in accordance with the International Agency for Research on Cancer database.²⁷ The origin of non-cancerous cell lines is provided in Online Supplementary Appendix. Primary CLL samples consisting of peripheral blood mononuclear cells (PBMNC) with >90% leukemic cells were obtained from patients treated at the Department of Internal Medicine, Hematology and Oncology of the University Hospital Brno. Written informed consent was signed by all patients, and the study was approved by the Ethics Committee of the University Hospital Brno (Project n. 15-33999A). After thawing, CLL cells were cultured in RPMI-1640 medium with 10% FBS and penicillin/streptomycin. Genetic characterization of the samples is described in Online Supplementary Table S3.

Pro-proliferative stimulation of chronic lymphocytic leukemia cells

Pro-proliferative stimulation of CLL cells was made using the anti-CD40 + IL-4 system developed by Patten *et al.*²⁸ and used by us previously.²⁴ Here we made the following modifications: the ratio of CLL cells to murine fibroblasts (irradiated by 50 Gy) was 20:1 and length of stimulation was ten days; fresh medium (half volume), and fresh mAb anti-CD40 and IL-4 (full doses of 200 ng/mL and 10 ng/mL, respectively) were added on days 3 and 7. On day 10, CLL cells were gently removed and cultured for an additional three hours (h) to allow residual fibroblasts to attach. For viability testing, CLL cells were transferred to 96-well plates and treated with MU380 or DMSO (mock control).

Transfection of chronic lymphocytic leukemia cells

The cells were transfected by electroporation using Neon Transfection System (Thermo Fisher Scientific) according to the manufacturer's instructions. Detailed information is provided in the Online Supplementary Appendix.

Cell viability assays, immunoblotting, real-time polymerase chain reaction, analyses of cell cycle, apoptosis and mitotic cells

Detailed information on all methodologies is provided in the Online Supplementary Appendix.

Xenograft experiments

Experiments were approved by the Ethics Committee of the Faculty of Medicine of Masaryk University (n. 47499/2013-8) and performed in accordance with the international ARRIVE guidelines.²⁹ Localized tumors were established in immunodeficient NOD-*scid* IL2Rγ^{null} mice strain³⁰ (Charles River Laboratories, Cologne, Germany) using a subcutaneous injection of MEC-1 cell line (5×10⁶ cells per animal). Mice were matched according to initial tumor size and randomized to treatment with MU380 in 20% aqueous Kolliphor solution (single inhibitor dose 20 mg/kg) or 20% Kolliphor alone. Additional information is included in the Online Supplementary Appendix.

Statistical analyses

Significance level was set as: **P*<0.05; ***P*<0.01; ****P*<0.001; = : not significant. The standard level of statistical significance was

$P < 0.05$. Detailed information is available in the *Online Supplementary Appendix*.

Results

Enantioselective synthesis of MU380

In order to prepare sufficient quantities of MU380 for *in vivo* studies, we developed its enantioselective synthesis from commercially available *N*-Boc-(*R*)-nipecotic acid **1** (Figure 1). Briefly, acid **1** was converted into the Weinreb amide **2**, whose treatment with deprotonated acetonitrile at low temperature afforded the required β -ketonitrile **3** with high optical purity [99% ee, determined by high performance liquid chromatography (HPLC) on chiral stationary phase]. Subsequent cyclization of **3** with 3-aminopyrazole proved to be quite challenging when, under a variety of reaction conditions including alcoholic solvents used in analogous synthesis of the CHK1 inhibitor SCH900776,³¹ we observed significant loss of stereochemical integrity. Finally, we found neat acetic acid to be the optimal solvent: cyclization was rapid and provided the desired pyrazolo[1,5-*a*]pyrimidine intermediate

4 in high yield (95%) and optical purity (96% ee). Using part of the sequence we had previously reported,²⁶ compound **4** was converted into advanced intermediate **11**, utilizing the in-house prepared boronate **9**. Optical purity of **11** (96% ee) was again determined by HPLC on chiral stationary phase, confirming that no loss of stereochemical integrity was associated with the post-cyclization steps of the sequence. Subsequent Boc-protection followed by regioselective bromination and deprotection afforded the target compound MU380 (overall yield 33% over 10 steps). Final recrystallization from acetonitrile afforded optically pure MU380 (> 99% ee) on gram scale. For detailed procedure see the *Online Supplementary Appendix*.

MU380 effectively inhibits CHK1 kinase and sensitizes lymphoid tumor cells to gemcitabine

Our novel inhibitor showed satisfactory target-specific effects in a pilot study using cell lines established from solid tumors.²⁶ Herein, we first explored whether MU380 also efficiently inhibits CHK1 in lymphoid tumor cells. We treated NALM-6 and MEC-1 cell lines with gemcitabine, a potent inducer of RS, and analyzed impact of

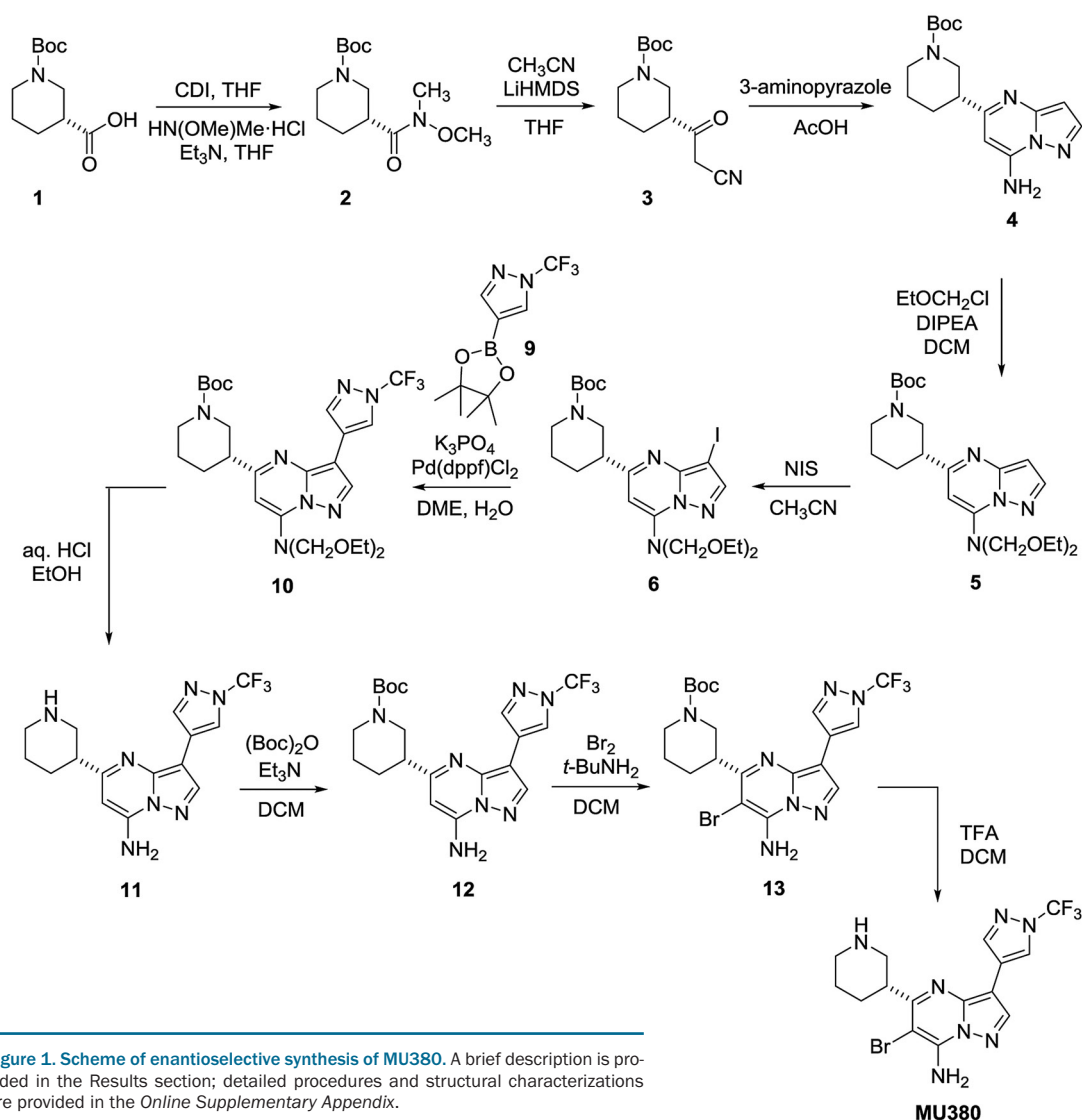


Figure 1. Scheme of enantioselective synthesis of MU380. A brief description is provided in the Results section; detailed procedures and structural characterizations are provided in the *Online Supplementary Appendix*.

the inhibitor on CHK1 protein. MU380 effectively blocked CHK1 activation (autophosphorylation pS296), while at the same time enhancing signaling from (presumably) ATR kinase towards the CHK1 (pS317 and pS345) reflecting RS potentiation (Figure 2A). MU380 also impacted the CHK1 downstream activity, which was demonstrated by reduced level of total CDC25A and CDC25C, one pS216 CDC25C isoform, pY15 CDK1, cyclin B1, and cyclin E1 (Figure 2B). The effect was more pronounced when MU380 was combined with gemcitabine.

Since effective CHK1 inhibitors should sensitize cancer cells to RS inductors, we tested the potential of MU380 in combination with gemcitabine using 10 cell lines harboring *TP53* gene disruption and 7 *TP53*-wt cell lines. MU380 (100 nM) significantly potentiated gemcitabine's efficacy in all tested cell lines (median half-maximal inhibitory concentration (IC_{50}) = 20.5 nM for gemcitabine vs. 6.5 nM for gemcitabine + MU380) (Table 1, Figure 2C, *Online*

Supplementary Table S1 and *Online Supplementary Figure S2*). As expected, MU380 enhanced the chemotherapy-induced DNA damage level, as evidenced by pS139 H2AX (γ H2AX) accumulation (Figure 2D). Altogether, MU380 effectively inhibited CHK1 in lymphoid cancer cells.

MU380 manifests single-agent activity in both p53-wt and p53-mutated cell lines

Certain cancer cell lines including those of hematopoietic origin have been shown to be sensitive to single-agent CHK1 inhibition.^{20,32} Along these lines, we tested MU380 alone in 10 leukemia and 9 lymphoma cell lines; an additional four non-cancerous cell cultures were also tested. The cancer cell lines responded with concentration-dependent viability decrease, which was similar in the *TP53*-wt (n=8) and *TP53*-mutated (n=11) samples (median IC_{50} = 330 and 392 nM, respectively) (Table 1 and Figure 3A). Interestingly, leukemia cell lines were significantly more sensitive than lymphoma lines (median IC_{50} = 238 and 401

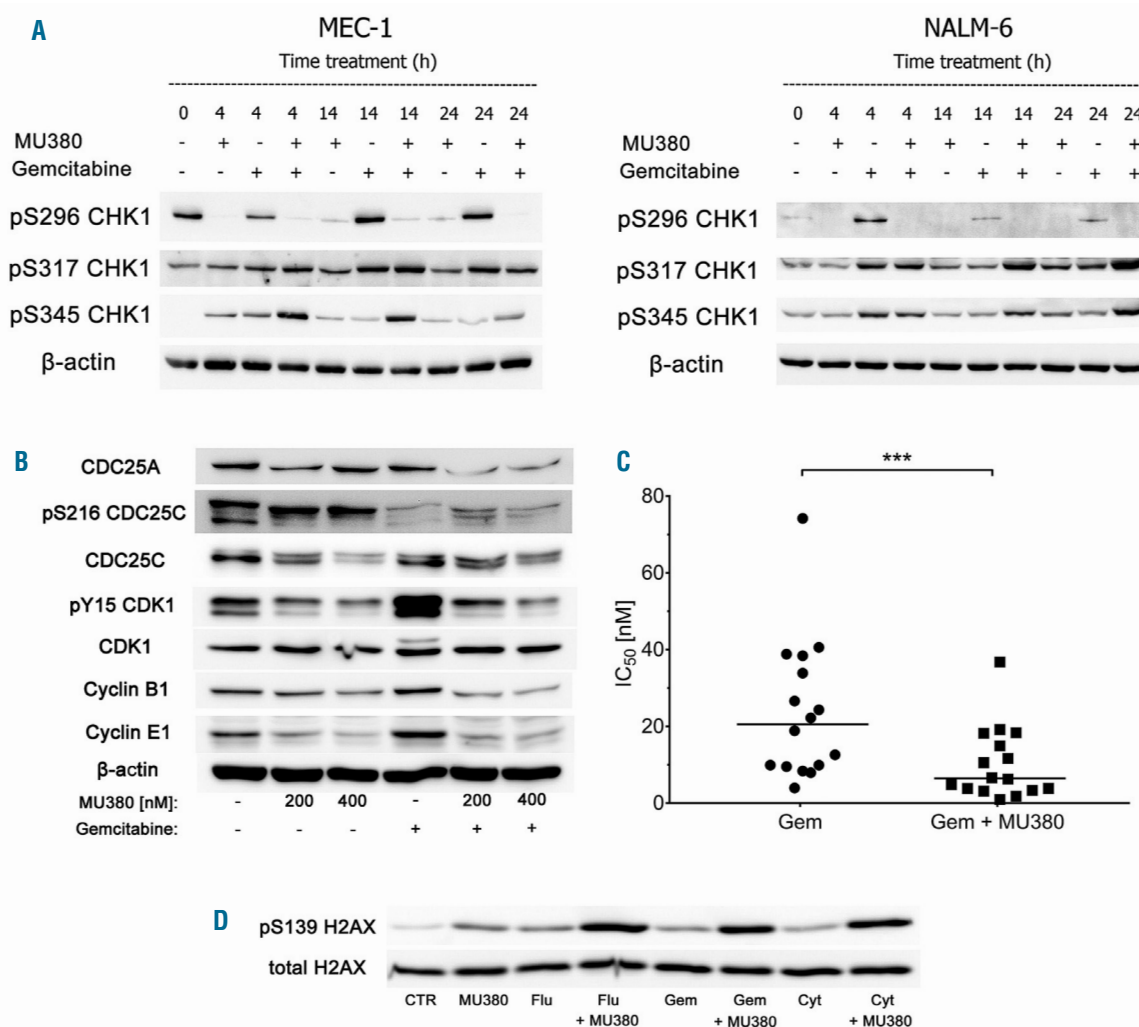


Figure 2. MU380 is effective in lymphoid tumor cells. (A) Effects on the phosphorylation status of CHK1. The cells were treated for the indicated time with MU380 (200 nM), gemcitabine (MEC-1 cell line: 10 ng/mL; NALM-6 cell line: 5 ng/mL) or combination of the agents. (B) Blocking of CHK1 downstream targets after 24 hours (h) treatment with MU380. Gemcitabine: 10 ng/mL. (C) Synergy with gemcitabine. The combined treatment of MU380 (100 nM) with gemcitabine affected viability (measured by WST-1) of the cell lines significantly more than gemcitabine alone ($P < 0.001$). Note: the graph does not involve the Jeko-1 cell line, in which IC_{50} for gemcitabine alone was not reached. *** $P < 0.001$. (D) MU380 (200 nM; 24 h) potentiates DNA damage in MEC-1 cells treated with nucleoside analogs. Fludarabine (Flu): 5 μ g/mL; gemcitabine (Gem): 5 ng/mL; cytarabine (Cyt): 100 ng/mL; CTR: untreated control.

nM, respectively) (Figure 3B). Four cell lines were tested within the project of DepMap and lethal phenotype with the CHEK1 elimination using CRISPR was noted (*Online Supplementary Figure S4*). Although some cell lines manifested a high baseline RS level (see phosphorylated CHK1 isoforms and/or γ H2AX in *Online Supplementary Figure S3A*), we did not observe an apparent correlation to the viability decrease (*Online Supplementary Figure S3B*).

Essentially, non-cancerous cells were much less sensitive to MU380, specifically: immortalized epithelial cells (RPE-1 cell line) ($IC_{50} > 10 \mu M$), immortalized bone

marrow/stromal fibroblasts (HS-5 cell line) ($IC_{50} = 3.7 \mu M$), primary skin fibroblasts ($IC_{50} = 3.7 \mu M$), and primary skin fibroblasts from a patient with ataxia telangiectasia harboring complete *ATM* gene inactivation ($IC_{50} > 10 \mu M$) (*Online Supplementary Figure S3*).

To elucidate MU380 mechanistic effects, we analyzed cell cycle profile and apoptosis in six selected cell lines. The inhibitor (400 nM, 24 h treatment) significantly affected the cell cycle profile in CLL-derived cell lines: *TP53*-mutated MEC-1 and MEC-2 exhibited profound S-phase accumulation together with G_2/M phase decrease, while

Table 1. Effects of MU380 in cancer cell lines and non-cancerous cells.

Cell lines / Cells	Cancer	<i>TP53</i> status	Other genetics	Synergy with Gem	Single-agent MU380 (IC_{50} ; μM)
MEC-1	CLL/PL	c.949dupC p.Q317fs	NA	+++	0.20
MEC-2	CLL/PL	c.949dupC p.Q317fs	NA	+++	0.41
BL-41	BL	c.743G>A p.R248Q	t(8;14) (<i>MYC/IGH</i>)	+++	0.38
SU-DHL-4	DLBCL	c.817C>T p.R273C	t(14;18) (<i>IGH/BCL2</i>)	+++++	0.40
JEKO-1	MCL	c.173delC p.P58X	t(11;14) (<i>CCND1/IGH</i>)	++++	0.39
NALM-16	ALL	c.868_869insTC p.R290fs	NA	++++	0.20
RAJI	BL	c.638G>A; p.R213Q c.700T>C; p.Y234H	t(8;14) (<i>MYC/IGH</i>)	+++	0.50
REC-1	MCL	c.734G>A; p.G245D c.949C>T; p.Q317X	t(11;14) (<i>CCND1/IGH</i>)	+++	0.48
REH	ALL	c.541C>T p.R181C	t(12;21) (<i>TEL/AML1</i>)	ND	0.25
MAVER-1	MCL	c.843C>G p.D281E	t(11;14) (<i>CCND1/IGH</i>)	++++	0.25
MINO	MCL	c.440T>G p.V147G	t(11;14) (<i>CCND1/IGH</i>)	+++	0.46
OSU-CLL	CLL	WT	NA	+++	0.22
JVM-2	B-PLL	WT	t(11;14) (<i>CCND1/IGH</i>)	+++	0.22
JVM-3	B-PLL	WT	NA	+++	0.34
WSU-NHL	DLBCL	WT	t(14;18) (<i>IGH/BCL2</i>)	++++	0.32
DOHH-2	FL	WT	t(8;14;18) (<i>MYC/IGH/BCL2</i>)	+++	0.43
NALM-6	ALL	WT	t(5;12) (<i>ETV6/PDGFRB</i>)	+++	0.38
GRANTA-519	MCL	WT (del 17p)	t(11;14) (<i>CCND1/IGH</i>)	+++	ND
OCI-AML3	AML	WT	NA	ND	0.34
MOLM-13	AML	WT	(<i>MLL/AF9</i>)	ND	0.14
RPE-1	Non-cancerous	NA	NA	ND	>10
HS-5	Non-cancerous	NA	NA	ND	3.7
Fibroblasts normal	Non-cancerous	NA	NA	ND	3.7
Fibroblasts AT	Non-cancerous	NA	NA	ND	>10

Genetic characteristics of the cell lines were adopted from the German Collection of Microorganisms and Cell Cultures (DSMZ). Statistical evaluation of the synergy between MU380 and gemcitabine (Gem) was done by Chou-Talalay test; +++++ very strong synergism; ++++ strong synergism; +++ synergism; NA: not applicable; ND: not determined. CLL/PL: chronic lymphocytic leukemia in prolymphocytoid transformation; BL: Burkitt lymphoma; DLBCL: diffuse large B-cell lymphoma; MCL: mantle cell lymphoma; ALL: acute lymphoblastic leukemia; B-PLL: B-cell prolymphocytic leukemia; FL: follicular lymphoma; AML: acute myeloid leukemia. Characterization of the non-cancerous cells and cell lines is provided in *Online Supplementary Appendix*.

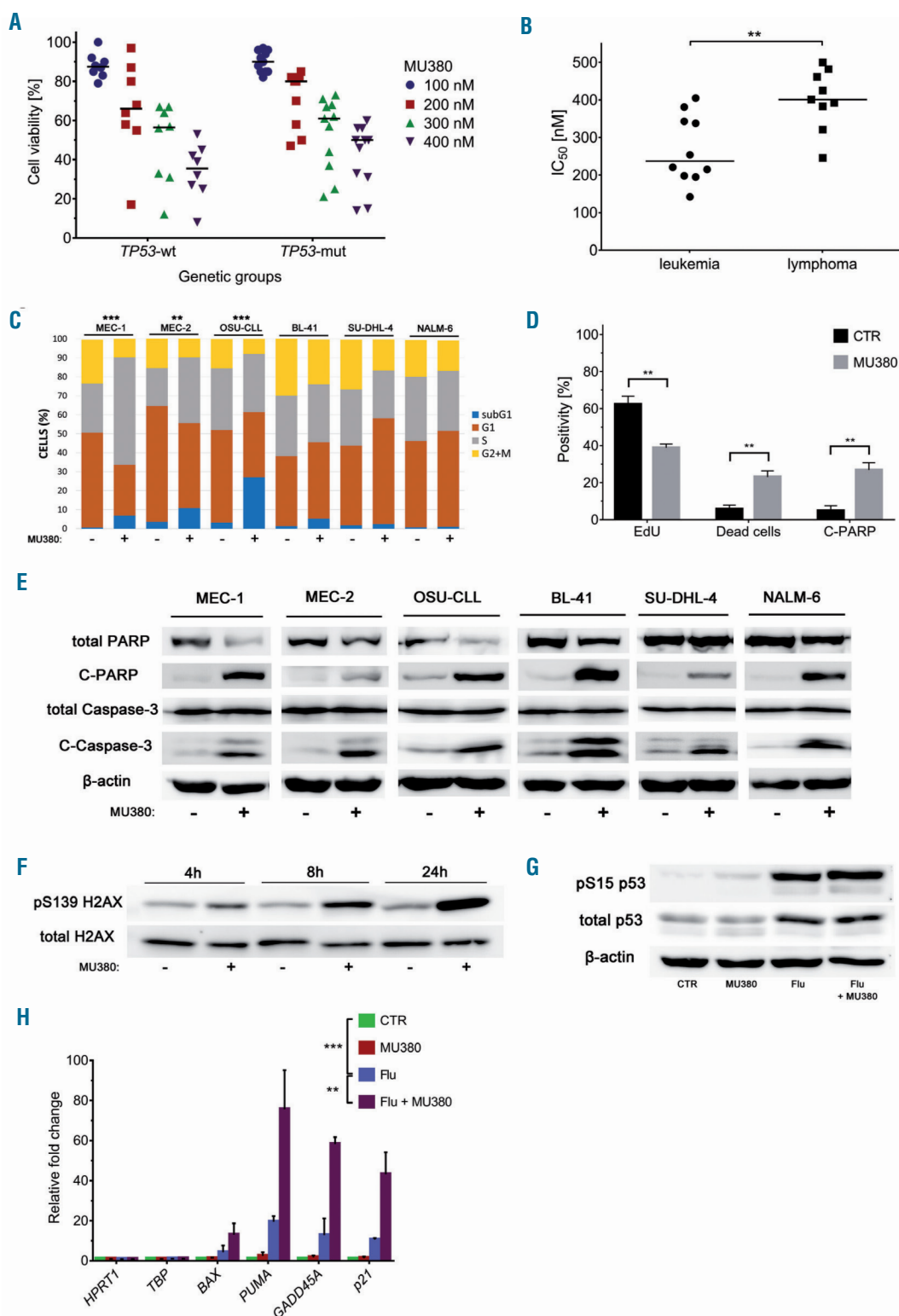


Figure 3. Effects of MU380 (single-agent) on leukemia and lymphoma cell lines. (A) Cell viability was reduced similarly in the TP53-wt and TP53-mutated cell lines ($P=0.257$) after 72 hours (h) treatment. (B) Distribution of IC₅₀ values in leukemia and lymphoma cell lines ($P=0.004$). (C) MU380 (400 nM; 24 h) significantly changed the cell cycle profile in MEC-1 ($P<0.001$), MEC-2 ($P=0.010$) and OSU-CLL ($P<0.001$) cell lines; the other three cell lines showed insignificant differences. (D) MEC-1 cells treated with MU380 (400 nM; 24 h) exhibited significantly reduced DNA synthesis rate compared to control untreated cells (lower EdU incorporation, $P=0.001$) and consequently manifested extensive apoptosis as evidenced by the PARP protein cleavage ($P=0.001$). The cell death was also confirmed using labeling with Live/Dead Red agent ($P=0.002$). Graph summarizes results of three independent experiments. (E) MU380 (400 nM; 48 h) elicited apoptosis in all tested cell lines as evidenced through the cleaved PARP (C-PARP) and caspase-3 (C-Caspase-3) proteins. Note: the cell lines were exposed individually on UVITEC detection instrument; hence, intensity of the bands among the cell lines cannot be mutually compared. (F) The time-dependent γ H2AX accumulation reflects gradually increasing RS after treatment with MU380 (400 nM); the cells were harvested at indicated time points. (G) MU380 (400 nM; 24 h) does not change the p53 protein level in p53-wt NALM-6 cell line, in contrast to fludarabine (2.7 μ M; positive control). (H) MU380 (400 nM; 24 h) induces negligible expression of p53 target genes BAX, PUMA, GADD45A, and CDKN1A (p21), in contrast to fludarabine (2.7 μ M; positive control). The fold change is related to the untreated control (CTR). The graph summarizes results of two independent real-time polymerase chain reaction analyses. Error bars represent standard deviation. ** $P<0.01$; *** $P<0.001$.

TP53-wt OSU-CLL cells manifested extensive sub-G1 peak (Figure 3C and *Online Supplementary Figure S5*). In MEC-1 cells, we also recorded greatly reduced DNA synthesis rate and concurrently apparent apoptosis induction (cleavage of PARP protein) after MU380 treatment (Figure 3D and *Online Supplementary Figure S6*). Apoptosis was also detected in all other tested cell lines using western blot analysis of cleaved PARP and Caspase-3 proteins (Figure 3E). In contrast, no significant apoptosis induction was observable in non-cancerous human cell lines and primary fibroblasts (*Online Supplementary Figure S7*). Cell death was likely a consequence of enhanced DNA damage as evidenced by γ H2AX accumulation in MU380-treated cells (Figure 3F).

All aforementioned results indicate that MU380 activity is not dependent on p53 status, which is further supported by absence of p53 protein accumulation after treating the p53-wt NALM-6 cell line with MU380 (Figure 3G), as well as by negligible induction of p53-downstream target genes *CDKN1A* (*p21*), *PUMA*, *BAX*, and *GADD45* in this cell line; interestingly, the inhibitor further increased the expression elicited by fludarabine (Figure 3H).

MU380-mediated CHK1 inhibition affects transition of MEC-1 cells into mitosis

CHK1 protein inhibition abrogates the intra-S and G2/M cell cycle checkpoints.^{33,34} In p53-deficient cells lacking a functional G1/S checkpoint, CHK1 suppression can result in premature mitosis involving unrepaired DNA damage.³⁴ We hence employed a TP53-mutated MEC-1 cell line, in which MU380 significantly affected the cell cycle profile, and analyzed mitoses, specifically mitotic index (MI) and integrity of mitotic chromosomes. In addition to CHK1 inhibition, we also examined parallel blocking of ATR to assess contribution of this closely co-operating kinase to the studied phenotypes; in a recent study, ATR/CHK1 co-inhibition exhibited a surprising synergy in cancer cells, which was attributed to accentuated replication collapse.³⁵ For ATR depletion, we used selective inhibitor VE-821,³⁵ which blocked ATR-mediated CHK1 phosphorylations (pS317/pS345) in MEC-1 cells (*Online Supplementary Figure S8*).

The results of two independent experiments are summarized in Table 2. After treatment with MU380 alone,

the MI sharply decreased, and a proportion of mitotic cells manifested chromosome damage in some cells resembling pulverization (*Online Supplementary Figure S9*). Remarkably, with cells co-treated by CHK1 and ATR inhibitors we measured a higher MI compared to the CHK1 inhibitor alone. In line with this observation, we also recorded a higher proportion of cells with chromosome damage.

MU380 induces cell death in dividing and non-dividing primary chronic lymphocytic leukemia lymphocytes

We subsequently tested MU380 single-agent activity in primary CLL cells using vitally frozen clinical samples. Since CLL lymphocytes obtained from patient peripheral blood manifest only weak ATR/CHK1 pathway activity,^{23,36} we initially stimulated proliferation of CLL cells using the anti-CD40/IL-4 system.^{24,28} This stimulation shifted a significant part of the CLL cells to post-G1 phases of the cell cycle, which was apparent from both the DNA content analysis and enhanced expression of proliferation markers, *MKI67* and *BIRC5* (coding survivin) (*Online Supplementary Figure S10*). The stimulation also results in upregulation of activated CHK1 protein as we previously reported²⁴ and in enhanced anti-apoptotic signaling.³⁷

In the stimulated CLL cells, MU380 effectively elicited RS (pS345 CHK1) and abrogated CHK1 activation (pS296) (Figure 4A). The impact on cell viability was tested in 13 stimulated CLL cultures harboring adverse genetic features including TP53 mutations, ATM mutations, and/or complex karyotype (*Online Supplementary Table S2*). Notably, MU380 reduced viability of these samples with different genetic background to a similar extent; the IC₅₀ value was approximately 1 μ M in all but one sample (Figure 4B). Cell death mechanism included apoptosis as evidenced by the PARP protein cleavage (Figure 4C).

Consequently, we investigated non-stimulated primary CLL cells, which manifest a low but detectable CHK1 protein level (Figure 5A). Historically, the ATR pathway was considered to be inactive in quiescent lymphocytes,³⁸ such as those from CLL patients. However, a recent study³⁶ reported that ATR is active in primary CLL cells, and accordingly we detected a rise in pS345 CHK1 level after treatment with fludarabine (Figure 5B). We also confirmed that MU380 leads to blockade of CHK1 autophosphorylation at S296 (Figure 5C). Moreover, we noticed reduction of the CDC25C protein level; however, it was not detectable in all tested samples (*Online Supplementary Figure S11*).

To address MU380 impact on cell viability, we tested 96 non-stimulated CLL cultures (*Online Supplementary Table S3*). A vast majority of these non-dividing CLL cells responded to the inhibitor (100-400 nM) by clear concentration-dependent viability decrease, with insignificant differences among studied genetic groups: TP53-mutated samples, mean IC₅₀ = 337 nM; ATM-mutated samples 385 nM; 11q- deleted samples (the other ATM allele intact) 355 nM; and ATM-wt/TP53-wt samples 414 nM; healthy PBMNC cultures were virtually inert to MU380 (Figure 5D). Apoptosis induction was already apparent at 100 nM MU380 (Figure 5E). We also recorded similar response when our CLL cultures were clustered according to the presence of *SF3B1* mutations, *NOTCH1* mutations, *IGHV* status, complex karyotype presence, or their therapy status (*Online Supplementary Figure S12*).

Table 2. Cytogenetic analysis in MEC-1 cell line.

Experiment 1	Mitotic index (%)	Analyzed mitoses	Cells with breaks
Control	11.5	50	0
CHK1i	3.0	50	7
ATRi	10.8	50	7
CHK1i + ATRi	5.7	50	19

Experiment 2	Mitotic index (%)	Analyzed mitoses	Cells with breaks
Control	18.0	50	0
CHK1i	2.2	54	4
ATRi	18.5	55	19
CHK1i + ATRi	3.9	52	27

The cells were treated for 24 hours with the following agents: Experiment 1: 200 nM MU380 (CHK1i); 1 μ M VE-821 (ATRi); respective combination. Experiment 2: 400 nM MU380, 2 μ M VE-821; respective combination.

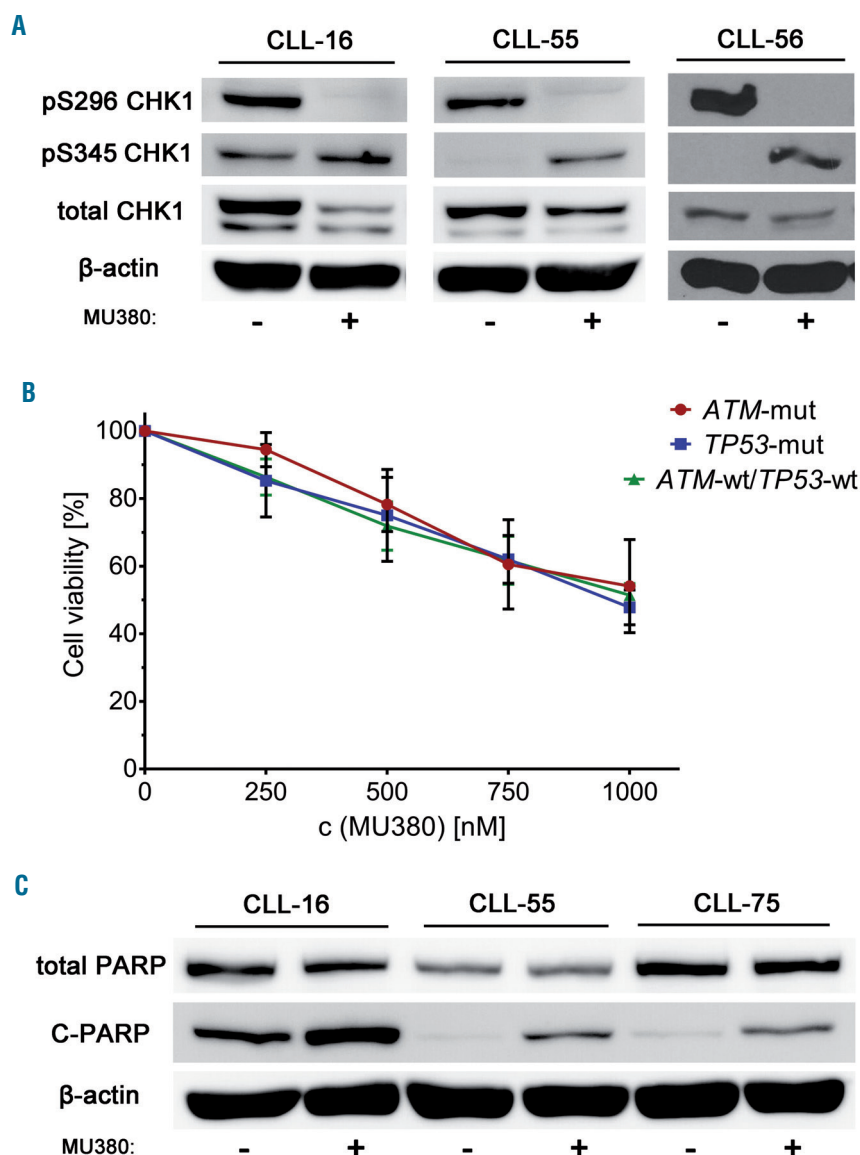


Figure 4. Effects of MU380 in chronic lymphocytic leukemia (CLL) cells pre-treated with pro-proliferative stimuli. Primary CLL cells were cultured in the presence of pro-proliferative stimuli for ten days and subsequently treated with MU380. (A) MU380 [1 μ M; 24 hours (h)] enhanced RS (pS345) and abrogated CHK1 protein activation (pS296). (B) The 72-h treatment with MU380 reduced viability of all tested samples; TP53-mutated (n=7), ATM-mutated (n=3) and TP53-wt/ATM-wt (n=3). The effect was similar (IC_{50} approx. 1 μ M) with the exception of sample CLL-75 harboring complete ATM inactivation (viability 66% at 1 μ M MU380). Error bars represent standard deviation. (C) The 48 h treatment with 1 μ M MU380 led to cleavage of PARP protein (C-PARP) in the tested samples.

Importantly, we also confirmed a decrease in viability in CLL cells after transfection with siRNA targeting CHEK1 (Figure 5F and *Online Supplementary Figure S13*). Moreover, to rule out the possibility of compound-specific MU380 effects, we confirmed a decrease in viability in non-stimulated CLL cells using structurally different CHK1 inhibitor CHIR-124³⁹ (*Online Supplementary Figure S14*). The mechanism of cell death caused by MU380 treatment included apoptosis (PARP protein cleavage was detected in 19 of 22 tested samples), which was probably a consequence of DNA damage accumulation (γ H2AX rise in 8 of 10 samples). Concerning MU380 impact on apoptosis-associated proteins, we noted a frequent decrease in MCL1 (7 of 9 samples) and NF- κ B (7 of 12 samples), whilst there was no change in the level of BCL2 (12 samples tested). MU380 also reduced the MYC protein level in 4 of 6 samples and total CHK1 level in 17 of 23 samples. Western blots for all proteins listed above are presented in *Online Supplementary Figure S15*.

Similarly to cell lines, we observed no accumulation of p53 protein or its downstream target genes after treatment of primary (p53-wt) CLL samples with MU380; in contrast, inhibitor did not further increase the expression elicited by fludarabine (Figure 5G and H).

MU380 suppresses growth of TP53-mutated subcutaneous tumors *in vivo*

Finally, we also tested the activity of MU380 *in vivo* using immunodeficient mice strain NOD-*scid* IL2R γ^{null} with subcutaneous tumors generated from MEC-1 cells similarly as reported by Attianese *et al.*⁴⁰ In line with our previous study,⁴¹ subcutaneous tumors were readily visible on day +14 post transplant; the tumors consisted of proliferating MEC-1 cells (Ki-67- and CD20-positive) (Figure 6A). In experiment I, we administered seven doses of MU380 between days +14 and +28 post transplant, and the sequential measurement of tumor volume revealed significantly suppressed growth in the inhibitor group (n = 7

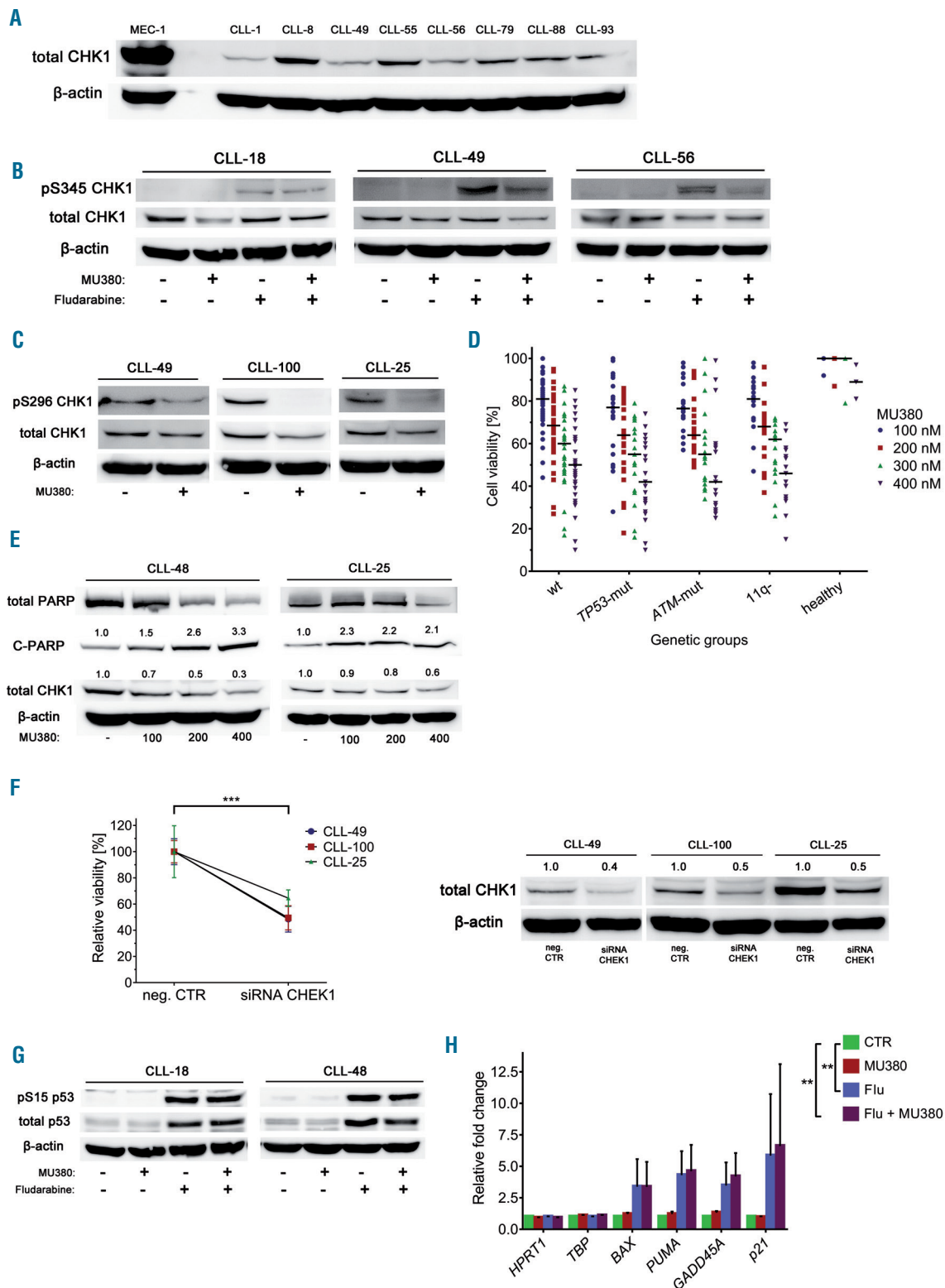


Figure 5. CHK1 protein level and effects of MU380 in non-stimulated chronic lymphocytic leukemia (CLL) cells. (A) The CHK1 protein was detectable in all tested CLL samples using the sensitive detection kit. (B) The treatment with fludarabine [10 μ M; 24 hours (h)] resulted in phosphorylation of the CHK1 protein on Ser345 residue indicating its activation. (C) Reduction of pS296 autophosphorylation after MU380 treatment (400 nM, 24 h). (D) The 72 h treatment with MU380 (100-400 nM) decreased viability of most CLL samples, with insignificant differences among the studied samples; wt-ATM/wt-TP53 (wt) versus TP53-mut $P=0.199$; versus ATM-mut $P=0.964$; versus 11q- (the other ATM allele intact) $P=0.849$. The healthy peripheral blood mononuclear cell samples ($n=3$) were substantially less affected ($P<0.001$). (E) MU380 elicited apoptosis as evidenced by the cleaved PARP (C-PARP) protein. The values indicate densitometric analysis set to 1.0 in control. (F, left) Viability decrease in CLL cells transfected with siRNA targeting CHEK1. (F, right) Decrease in the CHK1 protein level after transfection with siRNA targeting CHEK1. (G) MU380 (400 nM; 24 h) did not change the p53 protein level in TP53-wt samples, in contrast to fludarabine (10 μ M; positive control). (H) MU380 (400 nM; 24 h) did not induce expression of p53-downstream target genes BAX, PUMA, GADD45A, and CDKN1A (p21), in contrast to fludarabine (10 μ M; positive control). The fold change is related to untreated control (CTR). The graph summarizes results of real-time polymerase chain reaction analyses in three samples (CLL-58, CLL-77, CLL-83). Error bars represent standard deviation. *** $P<0.001$; ** $P<0.01$.

mice) compared to control animals ($n = 8$ mice) on average by approximately 44% (Figure 6B and C). In experiment II, we administered ten doses of MU380 between days +14 and +25 post-transplant (inhibitor group $n = 15$ mice; control group $n = 8$ mice), which again resulted in pronounced tumor growth suppression on average by ~ 61% in this case (Figure 6D).

The tumor cells from mice treated with MU380 exhibited a significantly increased RS level as evidenced by accumulation of γ H2AX (Figure 6E and F) and pS345 CHK1 (Figure 6F), while also manifesting moderately increased apoptosis (Figure 6E).

No apparent adverse effects were observed in either experiment.

Discussion

Chronic lymphocytic leukemia has long been considered a disease caused by gradual accumulation of malignant B lymphocytes with disabled apoptosis induction. However, this static view changed dramatically when Messmer *et al.*⁴² reported data on CLL cell kinetics *in vivo*, revealing that malignant cell turnover is much higher than appreciated, and may reach over 1% of a total clone per day. Lymph nodes are the primary site of CLL cell proliferation *in vivo*;^{43,44} approximately one-fourth of leukemic cells in this compartment possess proliferative potential.⁴⁵ Beside a simple cell renewal, proliferation is a prerequisite for clonal selection of new genetic variants, and this phenomenon is currently well-documented in CLL.⁴⁶ Furthermore, CLL genome is characterized by deregulated expression of genes involved in DNA replication, repair and recombination.⁴⁷ Taking these observations into account, we surmised that targeting dividing leukemic cells may represent a therapeutic strategy in CLL and therefore hypothesized that CHK1 kinase, which is essential for DNA replication and recombination-based repair, can be a suitable target.

We initially focused on obtaining sufficient quantities of the metabolically robust CHK1 inhibitor MU380. Although structurally related clinical candidate SCH900776 can certainly be considered one of the most specific CHK1 inhibitors with excellent selectivity for CHK1 over CHK2 or cyclin-dependent kinases,^{24,25} its metabolic profile may not be optimal. Specifically, SCH900776 contains the N-methylpyrazole motif, which undergoes oxidative demethylation resulting in the formation of significantly less selective metabolite and a rapid decrease of active concentration in plasma.²⁶ In contrast, MU380 contains highly unusual N-trifluoromethylpyrazole pharmacophore, which provides substantially better metabolic robustness and pharmacokinetic profile.²⁶

The newly developed enantioselective synthesis of MU380 described here provides access to gram quantities of enantiomerically pure substance, which enables thorough *in vivo* testing of the compound.

In our *in vivo* experiments with xenotransplanted MEC-1 cells, MU380 elicited strong and reproducible tumor growth suppression that was accompanied by an adequate molecular phenotype, namely the RS accumulation. Although the induction of apoptosis was rather modest, encouraging *in vivo* activity of MU380 opens up further opportunities to test more intense administration of the

compound and/or its combination with additional appropriate agents.

MU380 exhibited interesting single-agent activity in tested leukemia and lymphoma cell lines that responded *via* viability decrease with IC_{50} values between 142 and 500 nM. By virtue of this relatively uniform good reaction, we were not able to find determinants that would further stratify the response, except that leukemia cell lines were more sensitive than lymphoma ones. Although we hypothesized that a distinct RS level could justify this observation, baseline CHK1 phosphorylations and γ H2AX, standard markers of RS, did not correlate with the leukemia/lymphoma status.

Throughout our study, we focused on MU380 effects in TP53-mutated lymphoid cells. Hypothetically, CHK1 inhibition should be effective in a p53-deficient background due to dysfunction of all major cell cycle checkpoints and consequently complete impairment of cell cycle control potentially resulting in mitotic catastrophe. Nevertheless, our study indicates that this concept of “inducing death by releasing the breaks”⁷³⁴ may not be completely straightforward. Initially, certain p53 mutated cells surprisingly manifest G1-phase accumulation upon CHK1 inhibition, which we consistently observed, for example, in SU-DHL-4 cell line. Moreover, even the cells responding to CHK1 inhibition by more forthcoming S-phase accumulation and G2/M phase decrease are probably equipped with relevant mitotic entry control. This was apparent from the co-inhibition of ATR with CHK1, which resulted in increased MI and chromosome damage compared to the sole CHK1 inhibition. In this respect, recently recorded synergy between CHK1 and ATR inhibition may not only be a consequence of more pronounced replication collapse,³⁵ but of increased mitotic damage as well.

Beside the proliferative fraction, the CLL cell population also consists of non-dividing cells arrested either at the G_0 (quiescent cells) or G_1 phase of the cell cycle.⁴⁵ Intuitively, such cells might not respond to CHK1 inhibition due to low ATR^{23,38} and CHK1 levels.^{11,48} Nevertheless, a recent study⁴⁹ reported apoptosis induction in non-dividing CLL cells caused by treatment with a dual CHK1/CHK2 inhibitor AZD7762. Moreover, we found that *CHK1* is targetable in CLL cells using siRNA transfection. Another recent work by Beyaert *et al.*³⁶ concluded that ATR, despite its low level, is active in quiescent CLL cells and phosphorylates downstream targets upon DNA damage induction. Here, to our knowledge for the first time, we document that non-stimulated CLL cells also phosphorylate CHK1 upon DNA damage, despite the fact that their CHK1 level is low. Notably, we also observed significant MU380 single-agent activity in non-dividing CLL cells. Although the set of samples was enriched by those with therapeutically unfavorable genetics, only a few were resistant to our inhibitor. In fact, only 5 of 96 samples showed viability $\geq 80\%$ after 72 h treatment. Interestingly, 3 of 5 samples harbored complete *ATM* inactivation (2 others were *ATM*-wt/*TP53*-wt). Thus, although the *ATM*-mutated samples on average did not manifest resistance, some of them were particularly refractory. It is intriguing that non-cancerous cells with *ATM* inactivation (fibroblasts from *ataxia-telangiectasia* patient) also manifested strong resistance to MU380.

Overall, our results support the concept that CHK1 is a critical protein for B-cell lymphomagenesis and that even

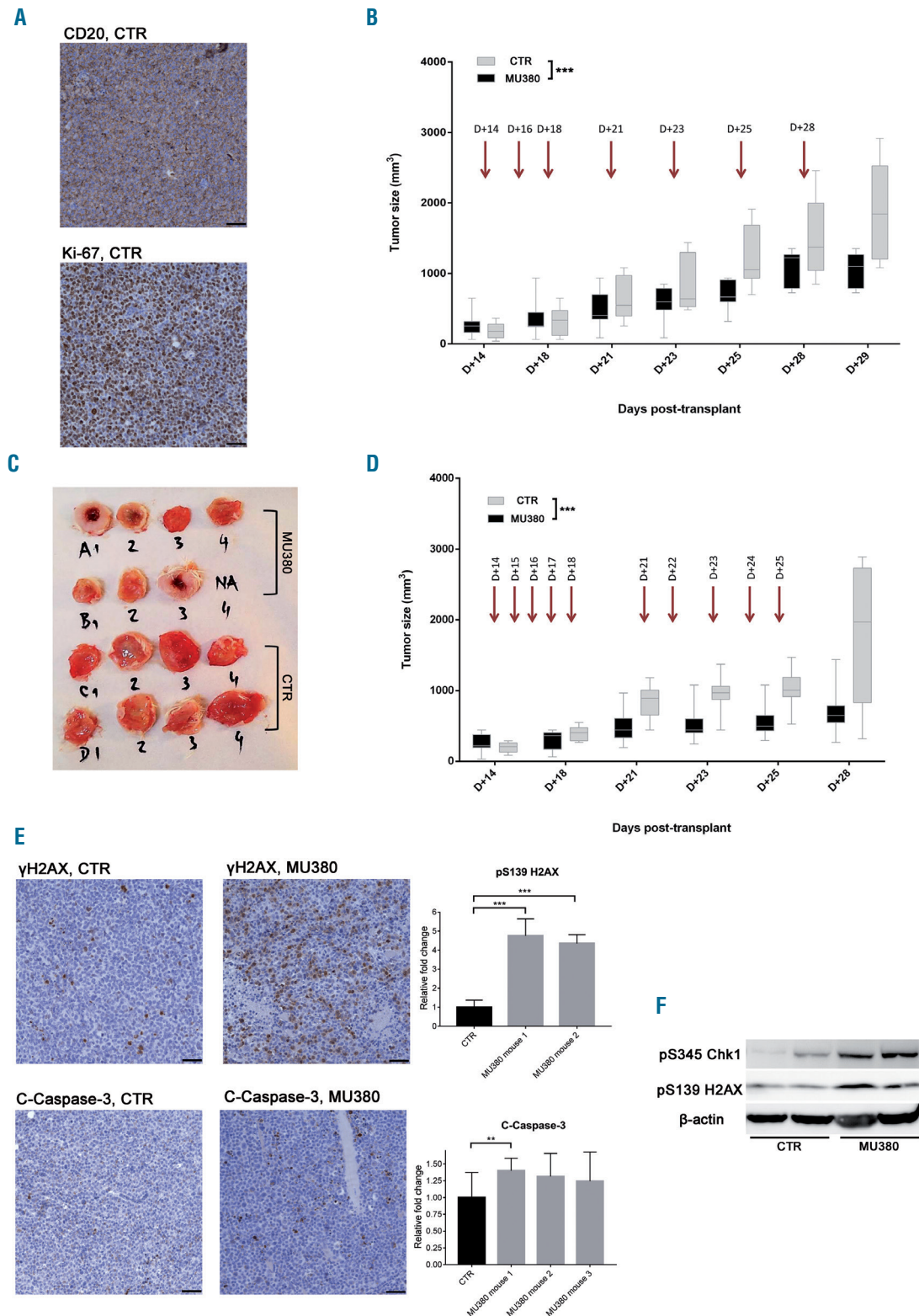


Figure 6. MU380 suppresses tumor growth *in vivo*. (A) Immunohistochemical analysis of tumors. The tumors consisted dominantly of MEC-1 cells expressing the B-cell specific antigen CD20. The cells were proliferating showing the high Ki-67 positivity. Bars represent 50 μ m. (B) Growth of the tumors in experiment I. Mean tumor volume at day (D)+29 was 1897 mm³ in the control group (CTR) and 1072 mm³ in the inhibitor group ($P < 0.001$). Arrows mark the administration of MU380 (20 mg/kg) or 20% aqueous Kolliphor alone (mock control). (C) The tumors extracted at D+29 post transplant in Experiment I. MU380: tumors from mice treated with the inhibitor; CTR: tumors from control mice. (D) Growth of the tumors in experiment II. Mean tumor volume at D+28 was 1771 mm³ in the control group and 695 mm³ in the inhibitor group ($P < 0.001$). Arrows mark the administration of MU380 (20 mg/kg) or 20% aqueous Kolliphor alone (mock control). (E, top left) The replication stress was significantly increased in tumor cells from mice treated with MU380 compared to those from control animals (CTR) ($P < 0.001$). (E, bottom left) In the same comparison, apoptosis increase was modest. Samples were collected 24 h after the last administration of MU380. Bars represent 50 μ m. (E, right) Quantitative evaluation of the immunohistochemical analysis. (F) Western blot analysis of replication stress markers in tumors from control and treated mice. Samples were collected 24 h after the last administration of MU380. Tumors from two control (CTR) and two treated (MU380) mice are shown. Error bars represent standard deviation. *** $P < 0.001$; ** $P < 0.01$.

resting B cells are vulnerable to CHK1 depletion.²²

The MU380 single-agent activity is noteworthy, especially in the light of the fact that CLL is typically resistant to therapy based on a single drug and that most current therapeutic regimens consist of several agents with combined mechanisms of action. In any case, it will be worthwhile analyzing potential synergy between CHK1 inhibition and current state-of-the-art CLL therapeutics targeting BCR signaling or BCL2 protein. Such analysis was not within the scope of this pilot study, but preliminary data we obtained with MEC-1 cells indicate an approximate additive effect of MU380 combined with ibrutinib (Boudny et al., 2019, unpublished observation). Furthermore, a recent study focusing on molecular analysis of druggable pathways in blood cancers⁴⁹ determined that the CLL cell response to SCH900776 is distinct from that of BCR pathway inhibitors. Concerning BCL2, at least the total level of this protein is unaffected by MU380. It might, therefore, be interesting to test a combination of MU380 with BH3-mimetics, e.g. venetoclax. Since MU380 reduces important anti-apoptotic proteins MCL1 and NF- κ B activated by

BCR signaling in CLL cells,^{43,50} response to combination treatment could, indeed, be synergistic.

In summary, we have demonstrated that our novel CHK1 inhibitor MU380 effectively affects both dividing and non-dividing CLL cells harboring TP53 mutations. Consequently, CHK1 inhibition may represent an attractive therapeutic option for high-risk CLL.

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References

- Seda V, Mraz M. B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells. *Eur J Haematol*. 2015;94(3):193-205.
- Thompson PA, Burger JA. Bruton's tyrosine kinase inhibitors: first and second generation agents for patients with Chronic Lymphocytic Leukemia (CLL). *Expert Opin Investig Drugs*. 2018;27(1):31-42.
- Daniel C, Mato AR. BCL-2 as a therapeutic target in chronic lymphocytic leukemia. *Clin Adv Hematol Oncol*. 2017;15(3):210-218.
- Mato AR, Nabhan C, Thompson MC, et al. Toxicities and outcomes of 616 ibrutinib-treated patients in the United States: a real-world analysis. *Haematologica*. 2018;103(5):874-879.
- Maddocks KJ, Ruppert AS, Lozanski G, et al. Etiology of Ibrutinib Therapy Discontinuation and Outcomes in Patients With Chronic Lymphocytic Leukemia. *JAMA Oncol*. 2015;1(1):80-87.
- O'Brien S, Furman RR, Coutre S, et al. Single-agent ibrutinib in treatment-naïve and relapsed/refractory chronic lymphocytic leukemia: a 5-year experience. *Blood*. 2018;131(17):1910-1919.
- Jones J, Mato A, Coutre S, et al. Evaluation of 230 patients with relapsed/refractory deletion 17p chronic lymphocytic leukaemia treated with ibrutinib from 3 clinical trials. *Br J Haematol*. 2018;182(4):504-512.
- Stilgenbauer S, Schnaiter A, Paschka P, et al. Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. *Blood*. 2014;123(21):3247-3254.
- Thompson R, Eastman A. The cancer therapeutic potential of Chk1 inhibitors: how mechanistic studies impact on clinical trial design: Therapeutic potential of Chk1 inhibitors. *Br J Clin Pharmacol*. 2013;76(3):358-369.
- González Besteiro MA, Gottifredi V. The fork and the kinase: a DNA replication tale from a CHK1 perspective. *Mutat Res Rev*. 2015;763:168-180.
- Bartkova J, Horejsí Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*. 2005;434(7035):864-870.
- de Klein A, Muijtjens M, van Os R, et al. Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice. *Curr Biol*. 2000;10(8):479-482.
- Takai H, Tominaga K, Motoyama N, et al. Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. *Genes Dev*. 2000;14(12):1439-1447.
- Bryant C, Rawlinson R, Massey AJ. Chk1 inhibition as a novel therapeutic strategy for treating triple-negative breast and ovarian cancers. *BMC Cancer*. 2014;14:570.
- Sen T, Tong P, Stewart CA, et al. CHK1 Inhibition in Small-Cell Lung Cancer Produces Single-Agent Activity in Biomarker-Defined Disease Subsets and Combination Activity with Cisplatin or Olaparib. *Cancer Res*. 2017;77(14):3870-3884.
- Manic G, Signore M, Sistigu A, et al. CHK1-targeted therapy to deplete DNA replication-stressed, p53-deficient, hyperdiploid colorectal cancer stem cells. *Gut*. 2018;67(5):903-917.
- Lowery CD, VanWye AB, Dowless M, et al. The Checkpoint Kinase 1 Inhibitor Prexasertib Induces Regression of Preclinical Models of Human Neuroblastoma. *Clin Cancer Res*. 2017;23(15):4354-4363.
- Oo ZY, Stevenson AJ, Proctor M, et al. Endogenous Replication Stress Marks Melanomas Sensitive to CHEK1 Inhibitors In Vivo. *Clin Cancer Res*. 2018;24(12):2901-2912.
- Walton MI, Eve PD, Hayes A, et al. The clinical development candidate CCT245737 is an orally active CHK1 inhibitor with preclinical activity in RAS mutant NSCLC and Eµ-MYC driven B-cell lymphoma. *Oncotarget*. 2016;7(3):2329-2342.
- Bryant C, Scriven K, Massey AJ. Inhibition of the checkpoint kinase Chk1 induces DNA damage and cell death in human Leukemia and Lymphoma cells. *Mol Cancer*. 2014;13:147.
- Iacobucci I, Di Rorà AGL, Falzaccappa MVV, et al. In vitro and in vivo single-agent efficacy of checkpoint kinase inhibition in acute lymphoblastic leukemia. *J Hematol Oncol*. 2015;8:125.
- Schuler F, Weiss JG, Lindner SE, et al. Checkpoint kinase 1 is essential for normal B cell development and lymphomagenesis. *Nat Commun*. 2017;8(1):1697.
- Kwok M, Davies N, Agathangelou A, et al. ATR inhibition induces synthetic lethality and overcomes chemoresistance in TP53- or ATM-defective chronic lymphocytic leukemia cells. *Blood*. 2016;127(5):582-595.
- Zemanova J, Hylse O, Collakova J, et al. Chk1 inhibition significantly potentiates activity of nucleoside analogs in TP53-mutated B-lymphoid cells. *Oncotarget*. 2016;7(38):62091-62106.
- Guzi TJ, Paruch K, Dwyer MP, et al. Targeting the replication checkpoint using SCH 900776, a potent and functionally selective CHK1 inhibitor identified via high content screening. *Mol Cancer Ther*. 2011;10(4):591-602.
- Samadder P, Suchánková T, Hylse O, et al. Synthesis and Profiling of a Novel Potent Selective Inhibitor of CHK1 Kinase Possessing Unusual N-trifluoromethylpyrazole Pharmacophore Resistant to Metabolic N-dealkylation. *Mol Cancer Ther*. 2017;16(9):1831-1842.
- Petitjean A, Mathe E, Kato S, et al. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Hum Mutat*. 2007;28(6):622-629.
- Patten PEM, Chu CC, Albesiano E, et al. IGHV-unmutated and IGHV-mutated chronic lymphocytic leukemia cells produce activation-induced deaminase protein with a full range of biologic functions. *Blood*. 2012;120(24):4802-4811.

29. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* 2010;8(6):e1000412.
30. Shultz LD, Lyons BL, Burzenski LM, et al. Human Lymphoid and Myeloid Cell Development in NOD/LtSz-scid IL2R γ null Mice Engrafted with Mobilized Human Hemopoietic Stem Cells. *J Immunol.* 2005;174(10):6477-6489.
31. Labroli MA, Dwyer MP, Poker C, Keertikar KM, Rossman R, Guzi TJ. A convergent preparation of the CHK1 inhibitor MK-8776 (SCH 900776). *Tetrahedron Lett.* 2016;57(24):2601-2603.
32. Davies KD, Humphries MJ, Sullivan FX, et al. Single-agent inhibition of Chk1 is antiproliferative in human cancer cell lines in vitro and inhibits tumor xenograft growth in vivo. *Oncol Res.* 2011;19(7):349-363.
33. Kawabe T. G2 checkpoint abrogators as anticancer drugs. *Mol Cancer Ther.* 2004;3(4):513-519.
34. Ma CX, Janetka JW, Piwnicka-Worms H. Death by releasing the breaks: CHK1 inhibitors as cancer therapeutics. *Trends Mol Med.* 2011;17(2):88-96.
35. Sanjiv K, Hagenkoort A, Calderón-Montaña JM, et al. Cancer-Specific Synthetic Lethality between ATR and CHK1 Kinase Activities. *Cell Rep.* 2016;14(2):298-309.
36. Beyaert M, Starczewska E, Pérez ACG, et al. Reevaluation of ATR signaling in primary resting chronic lymphocytic leukemia cells: evidence for pro-survival or pro-apoptotic function. *Oncotarget.* 2017;8(34):56906-56920.
37. Natoni A, Murillo LS, Kliszczak AE, et al. Mechanisms of action of a dual Cdc7/Cdk9 kinase inhibitor against quiescent and proliferating CLL cells. *Mol Cancer Ther.* 2011;10(9):1624-1634.
38. Jones GG, Reaper PM, Pettitt AR, Sherrington PD. The ATR-p53 pathway is suppressed in noncycling normal and malignant lymphocytes. *Oncogene.* 2004;23(10):1911-1921.
39. Tse AN, Rendahl KG, Sheikh T, et al. CHIR-124, a novel potent inhibitor of Chk1, potentiates the cytotoxicity of topoisomerase I poisons in vitro and in vivo. *Clin Cancer Res.* 2007;13(2 Pt 1):591-602.
40. Giordano Attianese GMP, Marin V, Hoyos V, et al. In vitro and in vivo model of a novel immunotherapy approach for chronic lymphocytic leukemia by anti-CD23 chimeric antigen receptor. *Blood.* 2011;117(18):4736-4745.
41. Verner J, Trbusek M, Chovancova J, et al. NOD/SCID IL2R γ -null mouse xenograft model of human p53-mutated chronic lymphocytic leukemia and ATM-mutated mantle cell lymphoma using permanent cell lines. *Leuk Lymphoma.* 2015;56(11):3198-3206.
42. Messmer BT, Messmer D, Allen SL, et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *J Clin Invest.* 2005;115(3):755-764.
43. Herishanu Y, Pérez-Galán P, Liu D, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood.* 2011;117(2):563-574.
44. Herndon TM, Chen S-S, Saba NS, et al. Direct in vivo evidence for increased proliferation of CLL cells in lymph nodes compared to bone marrow and peripheral blood. *Leukemia.* 2017;31(6):1340-1347.
45. Obermann EC, Went P, Tzankov A, et al. Cell cycle phase distribution analysis in chronic lymphocytic leukaemia: a significant number of cells reside in early G1-phase. *J Clin Pathol.* 2007;60(7):794-797.
46. Landau DA, Tausch E, Taylor-Weiner AN, et al. Mutations driving CLL and their evolution in progression and relapse. *Nature.* 2015;526(7574):525-530.
47. Grgurevic S, Berquet L, Quillet-Mary A, et al. 3R gene expression in chronic lymphocytic leukemia reveals insight into disease evolution. *Blood Cancer J.* 2016;6(6):e429.
48. Kaneko YS, Watanabe N, Morisaki H, et al. Cell-cycle-dependent and ATM-independent expression of human Chk1 kinase. *Oncogene.* 1999;18(25):3673-3681.
49. Dietrich S, Oleś M, Lu J, et al. Drug-perturbation-based stratification of blood cancer. *J Clin Invest.* 2018;128(1):427-445.
50. Petlickovski A, Laurenti L, Li X, et al. Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells. *Blood.* 2005;105(12):4820-4827.

7. TCGA. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med.* 2013;368:2059–74.
8. Yadav B, Wennerberg K, Aittokallio T, Tang J. Searching for drug synergy in complex dose–response landscapes using an interaction potency model. *Comput Struct Biotechnol J.* 2015;13:504–13.
9. Karjalainen R, Pemovska T, Popa M, Liu M, Javarappa KK, Majumder MM, et al. JAK1/2 and BCL2 inhibitors synergize to counteract bone marrow stromal cell-induced protection of AML. *Blood.* 2017;130:789–802.
10. Senkevitch E, Li W, Hixon JA, Andrews C, Cramer SD, Pauly GT, et al. Inhibiting Janus Kinase 1 and BCL-2 to treat T cell acute lymphoblastic leukemia with IL7-Ralpha mutations. *Oncotarget.* 2018;9:22605–17.
11. DiNardo CD, Pratz KW, Letai A, Jonas BA, Wei AH, Thirman M, et al. Safety and preliminary efficacy of venetoclax with decitabine or azacitidine in elderly patients with previously untreated acute myeloid leukaemia: a non-randomised, open-label, phase 1b study. *Lancet Oncol.* 2018;19:216–28.
12. Zhang M, Mathew Griner LA, Ju W, Dubeau DY, Guha R, Petrus MN, et al. Selective targeting of JAK/STAT signaling is potentiated by Bcl-xL blockade in IL-2-dependent adult T-cell leukemia. *Proc Natl Acad Sci USA.* 2015;112:12480–5.
13. Waibel M, Solomon VS, Knight DA, Rallia RA, Kim SK, Banks KM, et al. Combined targeting of JAK2 and Bcl-2/Bcl-xL to cure mutant JAK2-driven malignancies and overcome acquired resistance to JAK2 inhibitors. *Cell Rep.* 2013;5:1047–59.
14. Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin Cancer Res.* 2002;8:945–54.
15. Ertel F, Nguyen M, Roulston A, Shore GC. Programming cancer cells for high expression levels of Mcl1. *EMBO Rep.* 2013;14:328–36.
16. Glaser SP, Lee EF, Trounson E, Bouillet P, Wei A, Fairlie WD, et al. Anti-apoptotic Mcl-1 is essential for the development and sustained growth of acute myeloid leukemia. *Genes Dev.* 2012;26:120–5.

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Chronic lymphocytic leukemia

Rituximab primarily targets an intra-clonal BCR signaling proficient CLL subpopulation characterized by high CD20 levels

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The use of the anti-CD20 antibody rituximab has improved the outcome of patients with chronic lymphocytic leukemia

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(CLL). Rituximab was shown to act via various mechanisms, such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), direct apoptosis, or sensitization to chemotherapy (reviewed in ref. [1]). However, the biological function of CD20 and the reasons for the impressive activity of rituximab and other anti-CD20 antibodies remain elusive. It has been suggested, but remains controversial, whether CD20 functions as a calcium channel, couples with CD40 and MHCII, or B-cell receptor (BCR), and whether it has a role in T cell-dependent and -independent immunity [1–4]. This is also of great clinical interest for the design of combinatorial treatment of rituximab with BCR inhibitors, DNA damaging or immunomodulatory drugs, or CAR T cells. We have previously shown that microenvironmental interactions upregulate the CD20 levels on CLL cells through the CXCR4/SDF-1 axis [5]. Here we describe that higher CD20 expression has a direct role in the BCR signaling in CLL cells, and a BCR-proficient intra-clonal CLL cell

subpopulation is more efficiently eliminated by rituximab *in vivo* due to higher CD20 levels.

We and others have described that CLL cells that have exited the lymph node microenvironment to the peripheral blood express low levels of chemokine receptor CXCR4 and high cell-surface levels of CD5 [5, 6]. Recently, we have shown that these CXCR4^{dim}CD5^{bright} CLL cells also have the highest CD20 levels within the CLL cells' population (both at cell-surface and mRNA level) [5]. This is due to CD20 being directly transcriptionally upregulated by the CXCR4 activation by its ligand SDF-1 (CXCL12) in the CLL microenvironment [5]. We hypothesized that the higher levels of CD20 on CXCR4^{dim}CD5^{bright} cells, which is irrespective of their other clinico-biological characteristics (Fig. 1a, Suppl. Figs 1, 2), make these CLL cells the primary target of rituximab since it has been shown that rituximab-mediated effects such as CDC depend on CD20 levels [7, 8]. To test this, we obtained peripheral blood samples from CLL patients before and 24 h after rituximab administration (375 mg/m², single agent; administered as a part of FCR regimen). This showed that rituximab primarily and potently eliminates the CXCR4^{dim}CD5^{bright} CLL cell subpopulation corresponding to the cells with the highest cell-surface CD20 levels ($N = 17$, $P < 0.0001$; Fig. 1b, c), irrespective of other clinico-biological characteristics (Suppl. Fig. 3). The preferential elimination of CXCR4^{dim}CD5^{bright} cells was also maintained at day 3 in patients treated by FCR ($P < 0.01$; Suppl. Fig. 4). Notably, we were also able to replicate the preferential elimination of CXCR4^{dim}CD5^{bright} subpopulation *in vitro* by treating CLL cells with rituximab in the presence of human complement (Suppl. Fig. 5), suggesting that CDC is at least partially responsible for the disappearance of CXCR4^{dim}CD5^{bright} subpopulation *in vivo*. However, it is also possible that other mechanisms such as ADCC or antibody-dependent cellular-phagocytosis might play a role *in vivo* [9]. It is also important to note that rituximab treatment *in vitro* without complement did not affect transcription of CXCR4/CD5 (Suppl. Fig. 6), and we did not see any rituximab effect on the CXCR4^{dim}CD5^{bright} subpopulation in samples obtained from a patient whose cells did not express cell-surface CD20 (Suppl. Fig. 7). Overall, rituximab was *in vivo* ~9-fold more efficient (range 2.1–128.1-fold) in eliminating CXCR4^{dim}CD5^{bright} CLL cells than CXCR4^{bright}CD5^{dim} cells ($P = 0.03$). It is tempting to speculate that CXCR4^{dim}CD5^{bright} cells are constantly eliminated after they exit the lymph nodes if sufficient concentration of rituximab (half-life ~1 week) and complement and/or effector cells are present in the peripheral blood. It remains unclear to what extent and how CLL cells survive in lymph nodes in the presence of rituximab. Published studies suggest that stromal cell-adhesion protects malignant B cells

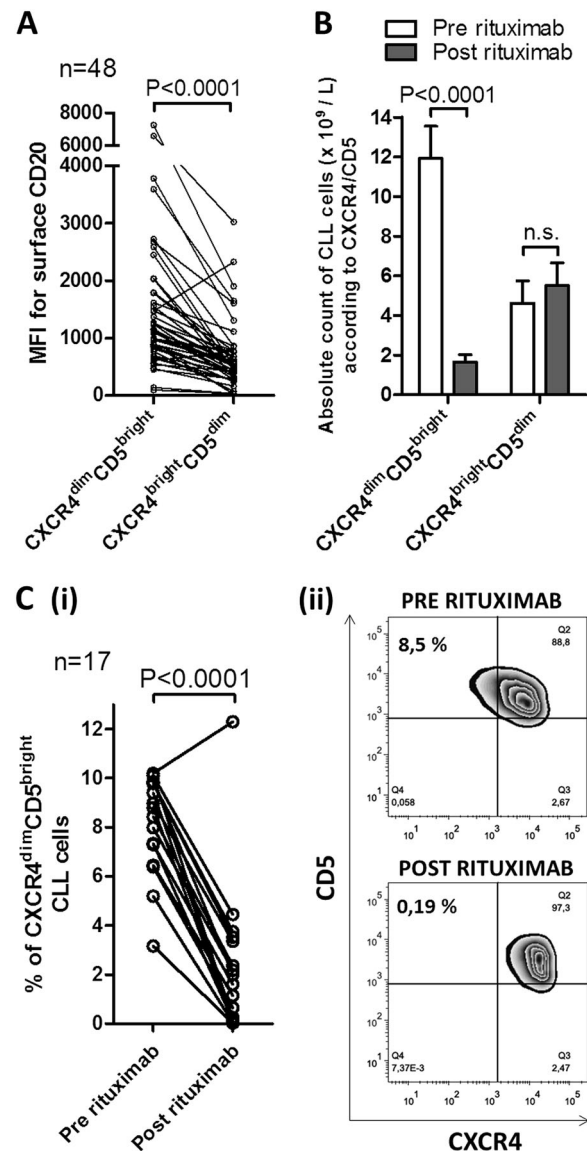


Fig. 1 The CXCR4^{dim}CD5^{bright} CLL cell subpopulation is preferentially eliminated by rituximab *in vivo*. **a** The surface CD20 expression on CXCR4/CD5 CLL cells subpopulations. For patients' characteristics see Suppl. Table 1. MFI mean fluorescence intensity. **b** Absolute count of CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} CLL cells before (pre rituximab) and 24 h after rituximab (post rituximab) infusion ($N = 17$ pairs). The absolute count of CLL cells was determined from the fraction of CXCR4/CD5 subpopulations (flow cytometry), and the number of CLL cells in the peripheral blood (determined using flow cytometry and hematology analyzer). For patients' characteristics see Suppl. Table 3: CLL FCR 1–17. **c** (i) The percentage of CXCR4^{dim}CD5^{bright} CLL cells before (pre rituximab) and 24 h after rituximab (post rituximab) infusion to CLL patients ($N = 17$ pairs). For patients' characteristics see Suppl. Table 3: CLL FCR 1–17 (ii) A representative example of flow cytometric analysis of CXCR4/CD5 subpopulations in the peripheral blood obtained from CLL patients before and 24 h after rituximab therapy *in vivo*

from rituximab effects (apoptosis and CDC) even when CD20 is highly expressed [10, 11].

The rituximab-mediated pressure to eliminate the CXCR4^{dim}CD5^{bright} cells led us to further characterize these

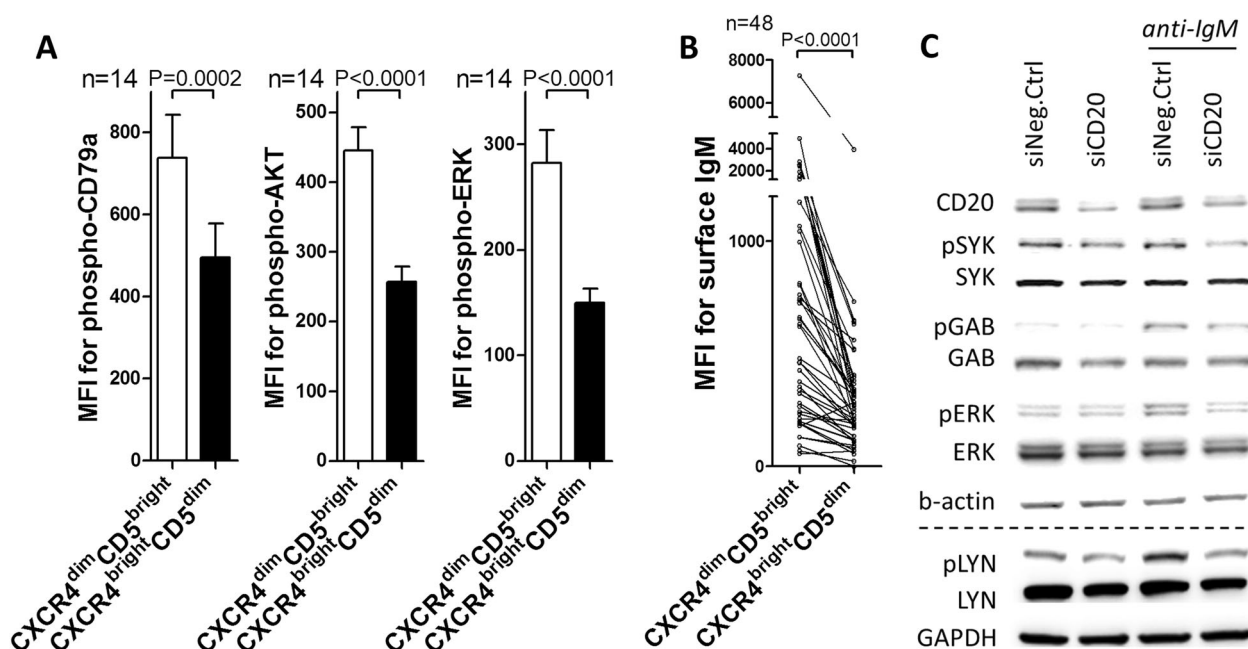


Fig. 2 CD20 supports BCR signaling and is associated with the proliferative phenotype of an intra-clonal subpopulation of CLL cells. **a** MFI for phospho-CD79a, phospho-Akt, and phospho-Erk measured in CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} CLL cells ($N = 14$, for patients' characteristics see Suppl. Table 2: CLL 4-13, 19, 21, 23, 24). **b** MFI for surface IgM on CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} CLL cells ($N = 48$; for patients' characteristics see Suppl. Table 1). **c** A representative immunoblot of CD20's silencing (siCD20) effect on phosphorylation of proteins involved in BCR signaling as compared

with negative control (siNeg. Ctrl). Cells were harvested 48 h after transfection for stimulation using anti-IgM (serum free media, 10 μ g/ml) for 1 min (pLYN) or 3 min (pSYK, pGAB1, pERK) followed by immunoblot analyses (for statistical analysis see Suppl. Fig. 16). The shorter time for pLYN analyses was selected because it is the first kinase activated after BCR crosslinking. Silencing of CD20 had no effect on the expression of cell-surface Ig or the viability of cells (Suppl. Fig. 17 and 18)

cells. We performed RNAseq (Illumina) profiling of mRNA expression in highly purified CLL subpopulations sorted according to the CXCR4/CD5 expression. This showed that 1103 genes are differentially expressed between CXCR4^{dim}CD5^{bright}(CD20^{bright}) and CXCR4^{bright}CD5^{dim}(CD20^{dim}) subpopulations, and these are enriched for the genes involved in BCR signaling ($P = 6 \times 10^{-5}$), MAPK kinase activation ($P = 1 \times 10^{-6}$), migration ($P = 7 \times 10^{-15}$), and actin cytoskeleton organization ($P = 9 \times 10^{-17}$) (Suppl. Fig. 8, 9; Suppl. Tab. 4). Furthermore, we analyzed the phosphorylation of the intracellular molecules involved in BCR signaling and cell activation. The CXCR4^{dim}CD5^{bright}(CD20^{bright}) cells displayed higher levels of phosphorylated CD79a ($P = 0.0002$), which is directly related to BCR activation, pAKT ($P < 0.0001$) and pERK ($P < 0.0001$) (Fig. 2a). The overall activated status of the CXCR4^{dim}CD5^{bright}(CD20^{bright}) cells is evidenced by strong enrichment in proliferative Ki67-positive cells ($P < 0.0001$; Suppl. Fig. 10, 11). This has been reported previously, and authors hypothesized that the entry of CLL cells to the lymph nodes, which induces proliferation, is a random re-circulation process [6]. Indeed, our analysis of the telomere length in the CXCR4/CD5 subpopulations suggests that the cells' re-circulation to the lymph nodes and their exit to the peripheral blood is random within the cell population

(Suppl. Fig. 12). Additionally, the analysis of variant allele frequency from CXCR4/CD5 subpopulations confirmed no difference in the frequency of genetic variants between these two subpopulations (difference smaller than 1.5% in all cases) including genes recurrently mutated in CLL such as *TP53*, *SF3B1*, *ATM* (Suppl. Fig. 13). These data together with the telomere length analysis support the conclusion that the difference in cell-surface markers and gene expression pattern reflect the CLL cells' re-circulation and interaction in the immune microenvironment rather than a genetic difference that would predispose specific cells to migrate preferentially to lymph nodes.

Based on the findings above and the fact that CD20 is upregulated in the microenvironment, we hypothesized that higher levels of CD20 might be of direct importance for BCR signaling. Notably, we have observed that CXCR4^{dim}CD5^{bright} CLL cells not only have a 2-fold higher cell-surface CD20 levels, but also have a 2.2-fold higher cell-surface immunoglobulin (sIg) expression than CXCR4^{bright}CD5^{dim} cells from the same patient ($N = 48$, $P < 0.0001$; Fig. 2b). This was also coupled with higher responsiveness of CXCR4^{dim}CD5^{bright}(CD20^{bright}) cells to BCR crosslinking with anti-IgM as measured by calcium flux ($P = 0.0015$, Suppl. Fig. 14). Further, we asked whether CD20 is of direct functional importance for BCR

signaling, since CLL cells with high CD20 have also higher sIg levels (see Suppl. Fig. 15). Silencing CD20 in MEC1-cells revealed that CD20 affects the phosphorylation of multiple BCR-associated kinases and proteins (LYN, SYK, GAB1, and ERK) after Ig-ligation (Fig. 2c, Suppl. Fig. 16). Silencing CD20 also impaired LYN kinase phosphorylation, which is the first kinase phosphorylated in response to BCR ligation. This suggests that upregulation of CD20 in the microenvironment is required for proper activity of BCR, and that CD20 is very “intimately” connected with BCR. This is also supported by the impaired BCR-induced calcium flux in MEC1 cells with silenced CD20 (Suppl. Fig. 19), and by a similar phenotype in mice deficient for CD20 in B cells [12]. Our data show that the subpopulation of CLL cells that interact in the immune niches (CXCR4^{dim}CD5^{bright}CD20^{bright} cells) represents the “aggressive” cells with the capacity for BCR signaling [13]. Interestingly, we observed that the effect of CD20 on phosphorylation of BCR-associated kinases is not entirely dependent on its calcium-channel function [2, 12], since the effect of CD20 silencing was present also in calcium-free media (Suppl. Fig. 20).

Overall, here we show that the high CD20 expression on BCR signaling proficient CLL cells makes them the primary rituximab target in vivo and leads to their more efficient elimination. This can be replicated also in vitro (see Suppl. Fig. 5) and suggests a rapid elimination by CDC in vivo. Interestingly, the shrinking of the CXCR4^{dim}CD5^{bright}CD20^{bright} subpopulation in peripheral blood is also prominent during ibrutinib therapy in vivo [5], which is due to the inhibition of chemokine signaling and redistribution of the cells [5, 14]. These two different drugs lead indirectly to the elimination of the same CXCR4^{dim}CD5^{bright}CD20^{bright} CLL subpopulation. Moreover, we provide a first mechanistic demonstration that CD20 is directly involved in the BCR signaling, and contributes to the activated status of an intra-clonal “aggressive” subpopulation of CLL cells (summarized in Suppl. Fig. 21). This could also explain some previous observations suggesting that rituximab binding to CD20 interferes with the BCR signaling cascade [15]. It is tempting to speculate that rituximab’s clinical success is at least partially attributed to the preferential elimination of the intra-clonal proliferative subpopulation of CLL cells with activated BCR signaling (with the consequence of indirect “BCR inhibition”), which is of interest for combinatorial therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Cartron G, Watier H, Golay J, Solal-Celigny P. From the bench to the bedside: ways to improve rituximab efficacy. *Blood*. 2004;104:2635–42.
2. Bubien JK, Zhou LJ, Bell PD, Frizzell RA, Tedder TF. Transfection of the CD20 cell surface molecule into ectopic cell types generates a Ca²⁺ conductance found constitutively in B lymphocytes. *J Cell Biol*. 1993;121:1121–32.
3. Morsy DED, Sanyal R, Zaiss AK, Deo R, Muruve DA, Deans JP. Reduced T-dependent humoral immunity in CD20-deficient mice. *J Immunol*. 2013;191:3112–18.
4. Kuijpers TW, Bende RJ, Baars PA, Grummels A, Derks IAM, Dolman KM, et al. CD20 deficiency in humans results in impaired T cell-independent antibody responses. *J Clin Invest*. 2010;120:214–22.
5. Pavlasova G, Borsky M, Seda V, Cerna K, Osickova J, Doubek M, et al. Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis. *Blood*. 2016;128:1609–13.
6. Calissano C, Damle RN, Marsilio S, Yan X, Yancopoulos S, Hayes G, et al. Intracolon complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. *Mol Med*. 2011;17:74–1382.
7. Golay J, Lazzari M, Facchinetti V, Bernasconi S, Borleri G, Barbui T, et al. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood*. 2001;98:3383–89.
8. van Meerten T, van Rijn RS, Hol S, Hagenbeek A, Ebeling SB. Complement-induced cell death by rituximab depends on CD20 expression level and acts complementary to antibody-dependent cellular cytotoxicity. *Clin Cancer Res*. 2006;12:4027–35.
9. Zent CS, Elliott MR. Maxed out macs: physiologic cell clearance as a function of macrophage phagocytic capacity. *FEBS J*. 2017;284:1021–39.
10. Buchner M, Brantner P, Stickel N, Prinz G, Burger M, Bär C, et al. The microenvironment differentially impairs passive and active immunotherapy in chronic lymphocytic leukaemia – CXCR4 antagonists as potential adjuvants for monoclonal antibodies. *Br J Haematol*. 2010;151:167–78.
11. Mraz M, Zent CS, Church AK, Jelinek DF, Wu X, Pospisilova S, et al. Bone marrow stromal cells protect lymphoma B-cells from rituximab-induced apoptosis and targeting integrin α 4 β 1 (VLA-4) with natalizumab can overcome this resistance. *Br J Haematol*. 2011;155:53–64.
12. Uchida J, Lee Y, Hasegawa M, Liang Y, Bradney A, Oliver JA, et al. Mouse CD20 expression and function. *Int Immunol*. 2004;16:119–29.
13. D’Avola A, Drennan S, Tracy I, Henderson I, Chiecchio L, Larrayoz M, et al. Surface IgM expression and function are associated with clinical behavior, genetic abnormalities, and DNA methylation in CLL. *Blood*. 2016;128:816–26.
14. Chen S-S, Chang BY, Chang S, Tong T, Ham S, Sherry B, et al. BTK inhibition results in impaired CXCR4 chemokine receptor surface expression, signaling and function in chronic lymphocytic leukemia. *Leukemia*. 2016;30:833–43.
15. Kheirallah S, Caron P, Gross E, Quillet-Mary A, Bertrand-Michel J, Fournié J-J, et al. Rituximab inhibits B-cell receptor signaling. *Blood*. 2010;115:985–94.

LYMPHOID NEOPLASIA

miR-150 downregulation contributes to the high-grade transformation of follicular lymphoma by upregulating FOXP1 levels

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KEY POINTS

- *miR-150* is downmodulated with transformation of FL leading to upregulation of FOXP1 protein.
- Upregulation of MYC protein is responsible for the repression of *miR-150* in transformed FL.

Follicular lymphoma (FL) is a common indolent B-cell malignancy with a variable clinical course. An unfavorable event in its course is histological transformation to a high-grade lymphoma, typically diffuse large B-cell lymphoma. Recent studies show that genetic aberrations of *MYC* or its overexpression are associated with FL transformation (tFL). However, the precise molecular mechanisms underlying tFL are unclear. Here we performed the first profiling of expression of microRNAs (miRNAs) in paired samples of FL and tFL and identified 5 miRNAs as being differentially expressed. We focused on one of these miRNAs, namely *miR-150*, which was uniformly downmodulated in all examined tFLs (~3.5-fold), and observed that high levels of MYC are responsible for repressing *miR-150* in tFL by binding in its upstream region. This MYC-mediated repression of *miR-150* in B cells is not dependent on LIN28A/B proteins, which influence the maturation of *miR-150* precursor (*pri-miR-150*) in myeloid cells. We also demonstrated that low *miR-150* levels in tFL lead to upregulation of its

target, namely FOXP1 protein, which is a known positive regulator of cell survival, as well as B-cell receptor and NF-κB signaling in malignant B cells. We revealed that low levels of *miR-150* and high levels of its target, FOXP1, are associated with shorter overall survival in FL and suggest that *miR-150* could serve as a good biomarker measurable in formalin-fixed paraffin-embedded tissue. Overall, our study demonstrates the role of the MYC/*miR-150*/FOXP1 axis in malignant B cells as a determinant of FL aggressiveness and its high-grade transformation. (Blood. 2018;132(22):2389-2400)

Introduction

Follicular lymphoma (FL) is the second most common indolent B-cell malignancy, with a variable clinical course. An adverse event in the course of FL is histological transformation to a high-grade lymphoma, which is associated with clinically aggressive behavior and poor patient prognosis.¹⁻⁴ FL most frequently transforms to diffuse large B-cell lymphoma (DLBCL), with the continuous risk of 2% to 3% per year.^{2,5} The effort to understand and predict histological transformation in FL is an active area of research, and several aberrations have been described as potential drivers of this process.⁶⁻¹⁶ The most common genomic aberrations acquired during transformation include genetic lesions activating *MYC* protooncogene and mutations resulting in

the inactivation of the cell-cycle regulators CDKN2A/B, p53, and MDM2.^{6,7,10,15,17} Changes in malignant B-cell gene expression and/or lymphoma microenvironment have also been associated with FL progression and transformation.^{10,17-21} Gene expression profiling studies revealed that a MYC pathway “proliferation signature” is associated with >70% of high-grade FL transformations.^{10,17} However, the precise molecular mechanisms underlying the transformation of FL to DLBCL remain largely unknown.

Factors that might regulate the expression of protein-coding genes involved in FL transformation are microRNAs (miRNAs). These short noncoding RNAs can posttranscriptionally regulate expression of variety of genes and thus fine tune essential cell

functions. We and others have shown that this is needed for the survival and normal maturation of B cells, immunoglobulin production, and B-cell receptor (BCR) signaling regulation.²²⁻²⁵ Aberrations in such miRNA-mediated regulation directly influence malignant B-cell biology, as well as the prognosis of patients (reviewed by Musilova and Mraz²⁴). The miRNA role in lymphomagenesis was directly demonstrated in studies generating miRNA mouse models, with an impact on B-cell lymphoma development.^{24,26-28} However, the role of miRNA deregulation in tFL is unclear.

Here we performed the first complex profiling of miRNA expression in paired samples of FL before and after its transformation to DLBCL. We identified 5 miRNAs as being differentially expressed between FL and transformed FL (tFL). In our study, we focused on one of these miRNAs, namely *miR-150*, which was downmodulated in tFL. We show that low levels of *miR-150* in tFL lead to upregulation of FOXP1 protein, which is a direct target of *miR-150*. We have previously shown that high-level expression of FOXP1 increases BCR signaling propensity in chronic lymphocytic leukemia (CLL).²² FOXP1 has been extensively studied in DLBCL, where it is known to regulate survival and aggressiveness of B cells (reviewed by Gascoyne and Banham²⁹).³⁰⁻³³ High FOXP1 levels are associated with worse prognosis in DLBCL,³⁴⁻³⁷ and FOXP1 is used in numerous algorithms (such as Choi, Tally, and Visco-Young) that distinguish the non-germinal center (non-GC) vs GC subtypes of DLBCL.^{29,38-43} We further show that upregulation of MYC protein is responsible for the repression of *miR-150* in tFL. Additionally, we reveal that *miR-150* and its target FOXP1 are strong prognostic markers in FL, which further supports the relevance of the MYC/*miR-150*/FOXP1 axis in malignant B-cell biology.

Methods

Patient samples and lymphoma cell lines

Formalin-fixed paraffin-embedded (FFPE) tissue or freshly frozen lymph node biopsy material was obtained from the contributing institutions, and the study was approved by the respective institutional review boards. The samples were obtained with written informed consent. Patient characteristics are described in supplemental Methods and supplemental Tables 1 and 2, available on the *Blood* Web site. The samples were processed for gene expression analysis and immunohistochemistry (IHC; supplemental Methods).

RNA isolation and mRNA/miRNA expression analyses

Total RNA was isolated from 10- to 20- μ m FFPE tissue sections using a High Pure miRNA Isolation Kit (Roche) according to the manufacturer's protocol. Total RNA from freshly frozen tissue or cell lines was isolated with TRI-Reagent (Sigma-Aldrich) as described previously.⁴⁴ MiRNA expression profiling was performed using TaqMan Array Human MicroRNA A Cards v2.0 (377 miRNAs; Thermo Fisher Scientific) and individual TaqMan microRNA Assays (detailed description provided in supplemental Methods).

Luciferase assay

Luciferase reporter assay was performed using psiCHECK2 reporter plasmid and the Promega Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer's protocol (supplemental Methods).

Transient miRNA or siRNA transfection and cell-cycle analysis

A WSU-NHL B-cell line was obtained from the German Collection of Microorganisms and Cell Cultures and cultured in RPMI 1640 supplemented with 10% fetal bovine serum in 5% carbon dioxide at 37°C. B cells (0.75×10^6) were electroporated (Neon Transfection System; Thermo Fisher Scientific; program 1500 V per 20 ms per 1 pulse) with a short artificial *miR-150* (1000 nM; MISSION miRNA Mimic; Sigma-Aldrich), control miRNA (1000 nM; MISSION miRNA Mimic Negative Control), short interfering RNA (siRNA; 1000 nM; On-TargetPlus Smartpool siRNAs for FOXP1; GE Dharmacon; Silencer Select Pre-Designed siRNA for MYC; Thermo Fisher Scientific; or 1000 nM; On-Target Plus Smartpool Negative Control). The WSU-NHL cell line was used for the transfection experiments because of its FL-like features (GC origin and presence of t[14;18]), relatively low-level expression of *miR-150*, relatively high levels of FOXP1 protein, and sufficient silencing efficiency and viability after transfection. After the indicated period of time, all cells in the well were harvested for cell-cycle and viability analyses and immunoblotting (supplemental Methods).

Statistical analysis

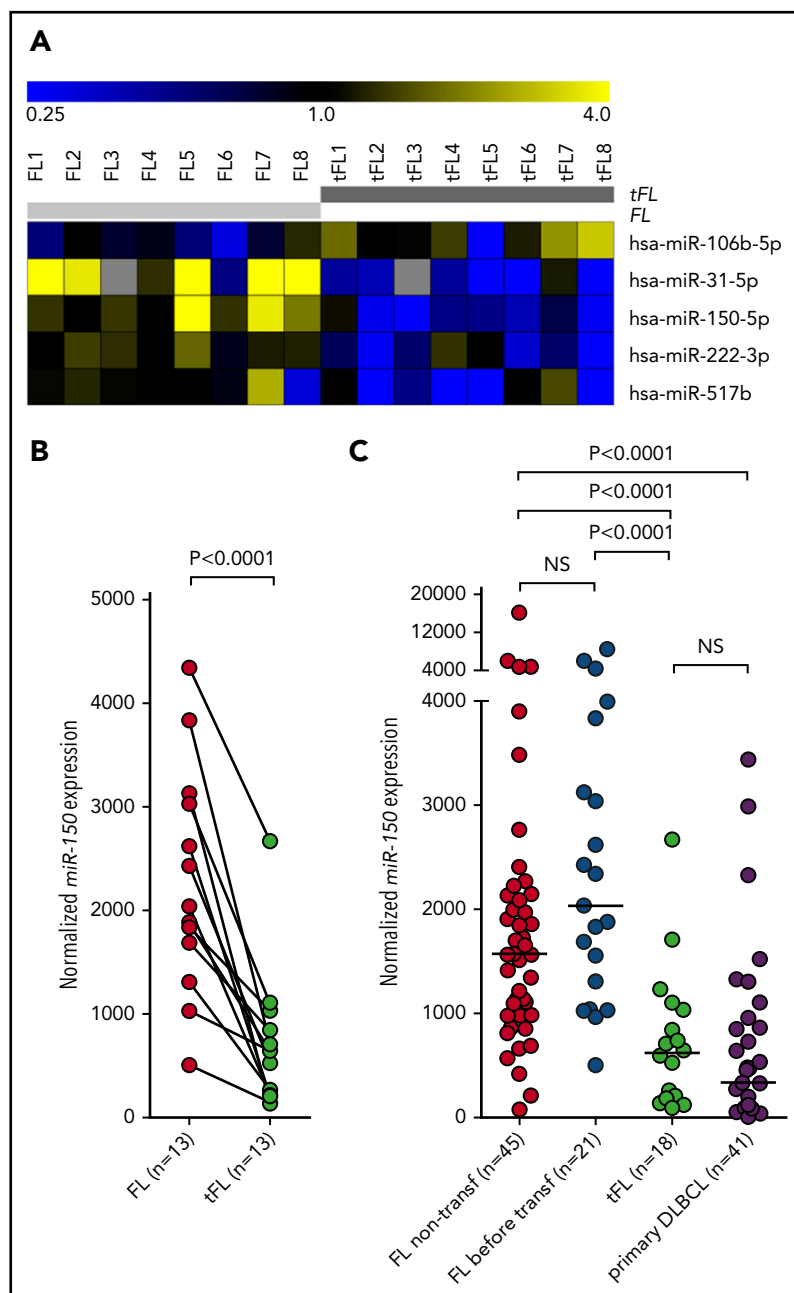
Univariate and multivariate Cox regression analyses of overall survival (OS) were computed using Statistica 13.2 (TIBCO Software Inc.; supplemental Methods). All other statistical analyses were performed with GraphPad Prism software v5.0 (GraphPad Software). All statistical tests were 2 sided, and *P* values < .05 were considered significant.

Results

miR-150 is downregulated after transformation of FL to DLBCL

To identify miRNAs that are changed upon FL transformation, we analyzed the global expression of 377 miRNAs in 8 pairs of samples (*n* = 16) from patients with FL that subsequently transformed to DLBCL. The analyzed miRNAs contained most of the miRNAs known to be associated with normal or malignant B-cell biology (supplemental Table 3). This identified 4 miRNAs (*miR-150*, *miR-31*, *miR-222*, and *miR-517b*) as downregulated and 1 miRNA (*miR-106b*) as upregulated in tFL (*P* < .05; fold-change ≥ 2 ; Figure 1A; supplemental Table 3). We further focused on *miR-150* studies, because we have previously shown that *miR-150* plays an important role in CLL biology by regulating transcription factor FOXP1 known to be of biological relevance in multiple B-cell lymphomas.^{22,29,34,35,37} *miR-150* was the most constantly changed miRNA, with ~ 3.5 -fold downmodulation in FL (Figure 1A). To validate this, we analyzed *miR-150* expression by individual qRT-PCRs in an extended cohort of 26 paired FL-tFL samples (13 pairs, including the original 8 pairs) and confirmed reduced *miR-150* levels in all examined tFL samples (Figure 1B; supplemental Figure 1A). Additional *miR-150* expression analyses in an extended cohort of tFLs (*n* = 18), non-transformed FL (*n* = 66), and de novo DLBCLs (*n* = 41) validated that *miR-150* is consistently less expressed in tFL and DLBCL than in FL (Figure 1C; supplemental Figure 2). The non-B cells in the non-Hodgkin lymphoma samples (eg, T cells or stromal cells) did not significantly affect the measurement of *miR-150* levels (supplemental Figure 3A), which was further supported by lack of any correlation between *miR-150* levels and the percentage of T-cell infiltration in FL samples (supplemental Figure 3B).

Figure 1. MiRNAs are differentially expressed in tFL. (A) miRNAs were quantified in paired FL-tFL samples from 8 patients (analyzed by TaqMan Array Human MicroRNA Cards; supplemental Methods). Expression of each miRNA was centered on the median miRNA expression in all samples and visualized in a heatmap (MeV software). Lower expression is indicated in blue, and higher expression in yellow (linear scale). The differences in miRNA expression between FL vs tFL were tested by paired t test ($P < .05$; fold change ≥ 2). Patient characteristics are listed in supplemental Table 1 (FL patients 1-8). All tFLs were transformations (transf) to histologically verified DLBCL. All RNA samples in the analyses were isolated from FFPE tissue. (B) Validation of *miR-150* levels in paired FL and tFL samples from 13 patients. The same 16 samples from panel A and additional 10 samples (5 pairs) were analyzed by individual quantitative reverse transcription polymerase chain reaction (qRT-PCR; tested by paired t test; patient characteristics listed in supplemental Table 1 [FL patients 1-13]). (C) *miR-150* expression in patients who did not experience transformation during follow-up (FL non-transf) and those with FL before transformation (FL before transf; patient later transformed to DLBCL), tFL (at sampling), and primary DLBCL (tested by Mann-Whitney test). The median follow-up for patients in the non-transforming FL group was 131 months (range, 2-276 months), the median follow-up for those in the FL before transformation group was 95 months (range, 25-231 months), and the median follow-up for those in the tFL group was 98 months (range, 5-231 months). NS, not significant.



We also analyzed *miR-150* levels in FL patient samples that later developed transformation ($n = 21$) and those FL samples that did not develop transformation during follow-up ($n = 45$). This showed that there is no difference in *miR-150* levels in cases that later develop transformation (Figure 1C), and *miR-150* is only downmodulated after transformation to DLBCL. The *miR-150* levels also did not significantly decrease with disease progression in FL (diagnosis vs relapse [transformation excluded]; supplemental Figure 4). Altogether these data demonstrate that *miR-150* is consistently and specifically downmodulated in tFL when compared with FL.

High levels of MYC repress *miR-150* expression in transformed FL

We further investigated the possible mechanisms for *miR-150* downmodulation in tFL. MYC was previously reported

to downregulate *miR-150* in acute myeloid leukemia,⁴⁵ and MYC mutations/translocations (~40% of tFL cases) and overexpression are among the most frequent aberrations in tFL.^{7,10,17} We detected reduced *miR-150* expression in splenic B cells from transgenic MYC-overexpressing mice (MYC transgene controlled by the immunoglobulin α heavy-chain enhancer) compared with wild-type mice (Figure 2A-B). Furthermore, the factors known to induce MYC and activate B cells such as CD40 and interleukin-4 signaling,^{46,47} also lead to downmodulation of *miR-150* levels in malignant B cells (supplemental Figure 5A-B). Importantly, silencing MYC in B cells by siRNA led to upregulation of *miR-150* expression in B cells (Figure 2C).

We next assessed MYC protein levels by IHC in paired FL-tFL samples from 14 patients for whom material was available. tFL

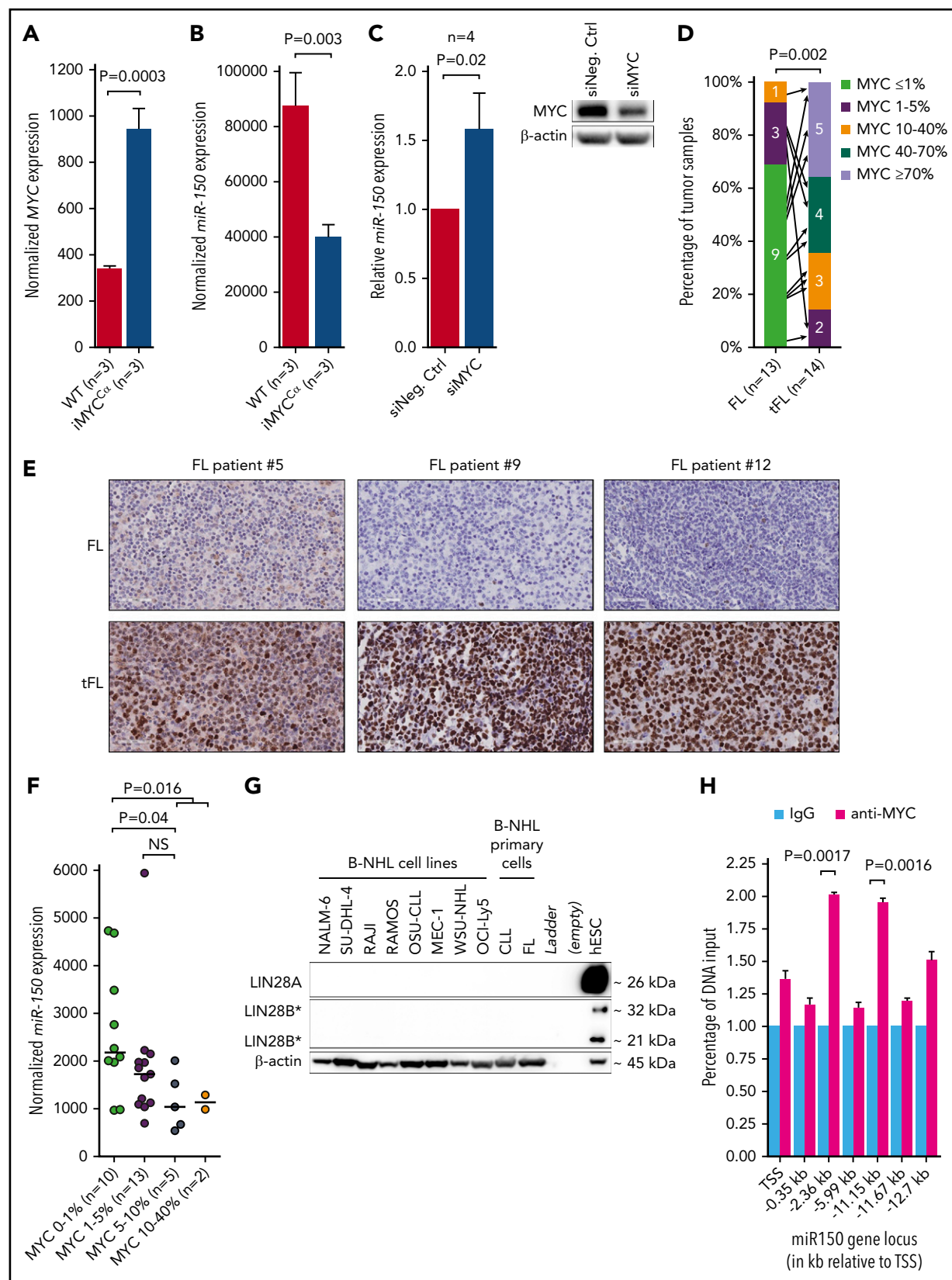


Figure 2. MYC represses miR-150 expression in tFL. (A-B) The differences in MYC messenger RNA (mRNA) and miR-150 levels in B cells from transgenic iMYC^{Ca} mice (MYC transgene controlled by the immunoglobulin [Ig] α heavy-chain enhancer) compared with wild-type (WT) mice. The splenic B cells were purified from MYC hemizygous and control (ctrl) spleens of relatively young mice before the development of any malignancy, which was verified by flow cytometry together with the sample purity (>95% B cells postpurification; supplemental Methods). The differences between groups were tested by unpaired t test. (C) miR-150 expression in WSU-NHL B-cell line transfected with siRNA against MYC (siMYC) or siRNA negative control (siNeg. Ctrl) (n = 4). Statistical differences were tested by paired t test. (D) Intensity of MYC IHC staining in paired FL-tFL samples

samples were in great majority strongly positive for MYC staining in a majority of B cells, and all had lower *miR-150* levels compared with FL samples, which were mostly weakly positive for MYC ($\leq 5\%$ MYC⁺ cells); (Figures 1 and 2D-E; supplemental Figure 6A-B). Study of additional 30 FL samples using tissue microarrays showed that the MYC⁺ (>5% positive cells) FL cases, although rare ($n = 7$), had significantly lower *miR-150* levels ($P < .05$; Figure 2F). Similarly, IHC staining of de novo DLBCL showed significantly lower *miR-150* expression in highly MYC⁺ cases in comparison with MYC⁻ cases (supplemental Figure 8). Additionally, the study of a cohort of rare samples of CLL cells with MYC aberration ($n = 29$) also showed significantly lower *miR-150* levels compared with CLL samples that did not harbor MYC aberration (supplemental Figure 5C). These data suggest that *miR-150* is transcriptionally repressed by MYC and that this could be an important feature of high-grade transformation of FL.

***miR-150* is directly repressed by MYC and its maturation is not regulated by LIN28A/B proteins**

It has previously been shown in AML cells that MYC upregulates LIN28, which then inhibits the maturation of *miR-150* from its precursor.⁴⁵ However, the regulation of *miR-150* expression could be cell type and context specific. Therefore, we analyzed the expression of LIN28 family members (*LIN28A* and *LIN28B*) in FL/tFL. The *LIN28B* mRNA was undetectable by qRT-PCR in most FL specimens (94% of cases had cycle threshold [Ct] value >40), and *LIN28A* mRNA had median Ct value of 38.0 and was not reliably detectable in most FL specimens. There was no anticorrelation of *LIN28A* mRNA with *miR-150* levels (supplemental Figure 9). Immunoblot analysis of LIN28A/B protein in B-cell lymphoma cell lines and primary FL and CLL samples revealed the absence of this protein in B cells (Figure 2G). The expression of primary *miR-150* transcript (*pri-miR-150*) in FL and DLBCL strongly correlated with mature *miR-150* levels (supplemental Figure 10). *pri-miR-150* was expressed at relatively low levels (median Ct value, 32.7) when compared with mature *miR-150* (median Ct value, 21.7), suggesting that *miR-150* is efficiently processed from its precursor (*pri-miR-150*) in FL B cells. We also did not find any association between the *miR-150* levels in B cells and functionality of p53 protein (supplemental Figure 11), which is frequently affected by aberrations in tFL. We hypothesized that MYC regulates *miR-150* expression by binding directly to its upstream regions. Indeed, our chromatin immunoprecipitation data for MYC have shown

that MYC directly binds in the region upstream of MIR150 transcription start site in malignant B cells (Figure 2H; supplemental Figure 12).

Low levels of *miR-150* lead to high levels of FOXP1 transcription factor

We have previously shown that *miR-150* regulates the levels of FOXP1 transcription factor in CLL cells.²² Similarly to CLL cells, the transfection of the non-Hodgkin lymphoma B-cell line WSU-NHL with synthetic *miR-150* reduced the FOXP1 levels by ~33% ($P < .05$; Figure 3A). A luciferase assay with cloned 3'UTR region of FOXP1 gene containing the putative *miR-150* binding site confirmed a direct physical binding of *miR-150* to FOXP1, and this was interrupted by introducing mutations in the region encoding the *miR-150* binding site (Figure 3B). This suggests that FOXP1 is a relevant target for *miR-150* in FL/tFL.

We further examined FOXP1 protein levels by IHC in FL and tFL. The immunostaining of these paired FL-tFL samples showed significantly stronger FOXP1 staining in tFL ($P < .05$; Figure 3C-D; supplemental Figure 6C). We also assessed the protein levels of FOXP1 in the other 30 FL samples (in a tissue microarray format). This revealed significantly lower *miR-150* levels in FL samples with high FOXP1 positivity (Figure 3E). These data suggest that *miR-150* contributes to the regulation of FOXP1 levels in both FL and tFL.

Impact of *miR-150* on the cell cycle

We and others have previously shown that FOXP1 can directly influence normal and malignant B-cell BCR signaling,²² positively regulate NF- κ B signaling activity,³³ and bind ~300 gene promoters in malignant B cells.⁴⁸ These data suggest that the *miR-150*/FOXP1 axis might influence malignant B-cell behavior on multiple levels. Additionally, several publications have reported the role of FOXP1 in the proliferation and survival of activated B-cell (ABC) DLBCL B cells.³³ To examine this, we transfected B-cell line WSU-NHL with artificial *miR-150* or siRNA against its target FOXP1 (Figure 3A). Transfection with *miR-150* or siRNA against FOXP1 did not have any direct impact on cell viability (supplemental Figure 15). However, the *miR-150* transfection resulted in a significantly increased percentage of cells in the G1 phase and a reduced percentage of cells in the S phase, which corresponded to reduced cell proliferation (Figure 3F; supplemental Figure 16). However, we did not observe changes in cell cycle or viability upon transfecting B cells with

Figure 2 (continued) from all 14 patients with available material (patient characteristics listed in supplemental Table 1 [FL patients 5-10 and 12-19]). Percentages of FL-tFL samples are shown, which were scored as MYC⁻ (0% to 1% positive cells), mostly MYC⁻ (1% to 5% positive cells), MYC⁺ (10% to 40% positive cells), strongly MYC⁺ (40% to 70% positive cells), and very strongly MYC⁺ (>70% positive cells). The number in each segment of the column indicates the number of samples with the corresponding intensity of IHC staining. Each arrow indicates the change in the IHC staining category in each of the analyzed pairs (note that the angle of the line does not show the trend of increase/decrease in percentage of positive cells). Representative images of the scoring of MYC staining in FL are shown in panel E and in supplemental Figure 7. The statistical differences between 13 paired FL-tFL samples were tested by Wilcoxon matched-pairs test. For 1 patient, only the material from the tFL sample was available for IHC (included in the graph but not in the paired statistical analysis). (E) Representative examples of differences in IHC staining for MYC between paired samples of FL (upper) and tFL (lower) from 3 patients. Original magnification $\times 400$. (F) The *miR-150* expression levels in FL samples scored as MYC⁻ (0% to 1% positive cells), mostly MYC⁻ (1% to 5% positive cells), weakly MYC⁺ (5% to 10% positive cells), or MYC⁺ (10% to 40% positive cells) for MYC staining. *P* values were assessed by unpaired *t* test. Representative images of the scoring of MYC staining in FL are shown in supplemental Figure 7 (MYC staining performed as tissue microarray blocks; supplemental Methods). (G) Immunoblot analysis of LIN28 protein (LIN28A and LIN28B) in B-cell non-Hodgkin lymphoma (B-NHL) cell lines and primary samples. The membrane was overexposed while detecting immunocomplexes, and no band(s) appeared in any B-NHL samples. Protein lysates from human embryonic stem cells (hESCs) were used as a positive control for LIN28A/B. *Two bands identified by the anti-LIN28B antibody represent two isoforms of LIN28B protein. (H) ChIP analysis for MYC binding in the region upstream of the MIR150 transcription start site in WSU-NHL cells. DNA from immunoprecipitates was assayed by qPCR on the independent amplicons upstream of the MIR150 gene (−12.7 to −0.35 kb relative to the MIR150 transcription start site [TSS]; supplemental Figure 12). The results are represented as percentage of DNA input; the error bars indicate the standard error of the mean. Enrichment quantitation of immunoprecipitates with control IgG antibody was calculated to equal 1, and MYC IP was normalized accordingly. NS, not significant.

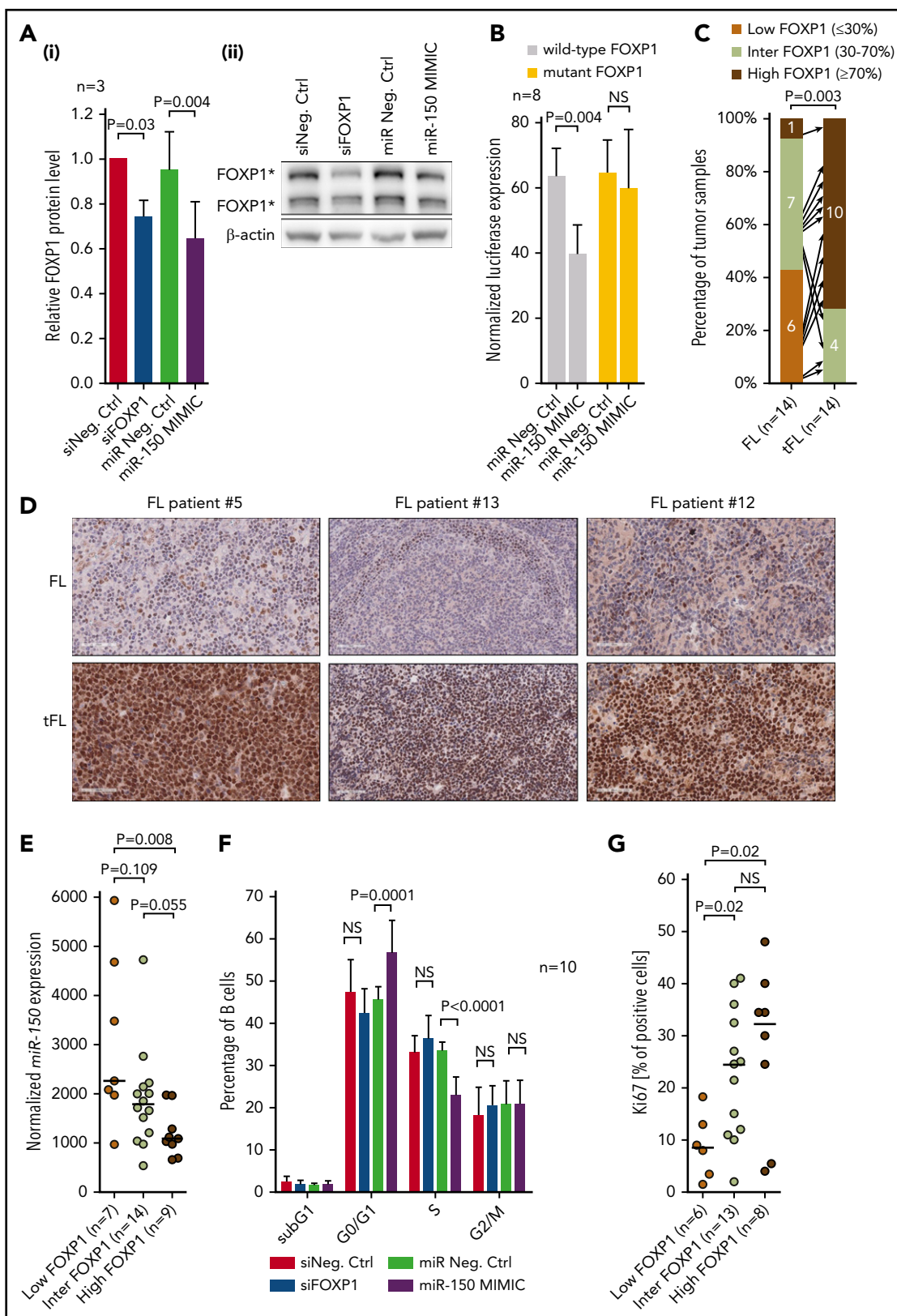


Figure 3. miR-150 regulates FOXP1 levels in FL/tFL. (Ai) Statistical analysis of the effect of synthetic *miR-150* on FOXP1 levels in WSU-NHL cells (48 hours; n = 3). WSU-NHL cells were transfected with siRNA against FOXP1 (siFOXP1) or siRNA negative control (siNeg. Ctrl) or *miR-150* mimic (*miR-150* MIMIC) or *miR-150* negative control (*miR-150* Neg. Ctrl) and harvested after 48 hours. The siRNA against FOXP1 serves as a positive control. The blot images were quantified with UVitec Alliance 4.7, and the FOXP1/β-actin ratio in the first sample (siNeg. Ctrl) was arbitrarily set at 1. The differences were tested by paired t test. (Aii) Representative immunoblot of FOXP1 levels in WSU-NHL cells transfected with siRNA against FOXP1 or *miR-150* mimic as described in panel Ai. *Two bands identified by the anti-FOXP1 antibody represent two isoforms of FOXP1 protein. (B) The luciferase activity in HEK293T cells cotransfected with *miR-150* mimic (400 nM) and luciferase reporter construct (15 ng), containing the 3'UTR region of wild-type or mutated FOXP1

siRNA for *FOXP1* (Figure 3F; supplemental Figures 15 and 16). This suggests that *miR-150* might regulate other targets besides *FOXP1* that are influencing the cell cycle independently or together with *FOXP1*. This is also in line with other reports showing that *FOXP1* affects cell proliferation and survival in ABC DLBCL, but not GC DLBCL, cell lines such as WSU-NHL used in our study.^{29,30,33} The effect of *FOXP1* on cell proliferation is likely to depend on the specific context of other signaling pathways in individual patients with FL. Importantly, primary FL samples with high *FOXP1* levels have a higher percentage of Ki67⁺ B cells (Figure 3G), suggesting *FOXP1* relevance in proliferation in vivo.

Prognostic significance of *miR-150* and *FOXP1* in FL

Altogether our data suggest that *miR-150* might play a role not only in tFL but also in the behavior of FL B cells. Indeed, the expression level of *miR-150* is associated with characteristics related to malignant B-cell biology and the outcome of patients with FL. *miR-150* expression was reduced in FL samples with high Ki67 positivity (>30%; $P = .01$; Figure 4A) and in patients with high FLIPI score (3-5; $P = .03$; Figure 4B). To assess the potential prognostic impact of *miR-150* levels in FL, we analyzed its expression in a cohort of FFPE samples and freshly frozen FL samples that were available (to increase the size of the cohort). The expression levels for *miR-150* obtained by qRT-PCR from both sample types (ie, FFPE and freshly frozen tissues) were highly concordant, which allowed us to include both sample types in the analyses; $r = 0.82$; $P = .006$; $n = 10$ pairs of FFPE and freshly frozen samples from the same biopsy; data not shown). The patients with FL with relatively low *miR-150* levels (divided by median) had a trend toward a shorter OS than those with relatively high *miR-150* levels ($P = .07$; $n = 85$; Figure 4C). The difference in OS was more pronounced and statistically significant when we compared patients with the lowest and the highest *miR-150* levels based on the terciles (median survival, 9.1 years vs not reached; $P = .008$; hazard ratio [HR], 3.5; confidence interval [CI], 1.3-9.2; Figure 4D) or quartiles of its expression (median survival, 9.1 years vs not reached; $P = .006$; HR, 4.9; CI, 1.6-15.4; Figure 4E). To determine whether *miR-150* is an independent predictor of OS, we performed a multivariate analysis, which included several known prognostic markers (age, sex, FLIPI, hemoglobin levels, lactate dehydrogenase levels, number of lymph node areas involved, Ann Arbor stage, presence of B symptoms, and *miR-150* levels). This showed that *miR-150* levels retained independent predictive power for OS (supplemental Table 4; $P = .02$). The prognostic value of low-level *miR-150* expression was also retained ($P < .05$) when the patients who experienced transformation during follow-up ($n = 8$; 9.4%) were excluded from the OS analysis. Interestingly, *miR-150* was also

significantly less expressed in patients who experienced early death within 3 years from diagnosis or biopsy (transformation to tFL excluded), and similar data were obtained for occurrence of early relapse (<24 months), which suggests a potential to identify the ~20% of patients with FL with the most aggressive disease⁴⁹ (Figure 4F; supplemental Figure 17). This also supports the overall biological relevance of *miR-150* for the behavior of malignant FL cells. However, this will require further analysis in a cohort of patients treated homogeneously and prospectively. It has previously been shown that high-level expression of *miR-155* is associated with ABC DLBCL⁵⁰⁻⁵² and shorter survival in CLL⁵³; however, we did not find any association of *miR-155* levels with OS or high-grade transformation of FL (supplemental Figure 18).

In univariate analyses, high-level *FOXP1* expression was associated with a significantly shorter OS in FL (median survival, 33 months vs not reached; $P = .03$; HR, 5.0; CI, 1.1-22.6; Figure 4G). Altogether, the adverse prognostic impact of low-level *miR-150* expression and the reverse trend for *FOXP1* provide support for the relevance of these genes to the biology of neoplastic FL and tFL cells.

Discussion

We have shown that the process of high-grade transformation of indolent FL to a more aggressive lymphoma is associated with a uniform downregulation of miRNA *miR-150*. We have demonstrated that an increase in *MYC* levels represses *miR-150* levels in tFL. Downmodulating *miR-150* leads to the upregulation of *FOXP1* transcription factor, which is a direct target of *miR-150* and an important regulator of B-cell activation. Additionally, some FL cases have relatively low *miR-150* levels and therefore higher levels of the *FOXP1*, which is strongly associated with shorter OS in FL.

The histological transformation of FL to a high-grade lymphoma, usually DLBCL, is associated with clinically aggressive behavior and poor patient prognosis.¹⁻⁴ Recently, several genetic lesions acquired during the transformation process were identified, such as *MYC* aberrations and *CDKN2A/B* or *TP53* loss,^{6,7,15} and high AID expression was reported to increase the risk of tFL.⁵⁴ However, the role of miRNAs in this process is largely unknown. Here we report the first complex profiling of miRNA expression in paired samples of FL and tFL. A recent profiling of selected miRNAs in FL-tFL (only 2 pairs of samples) suggested that 2 miRNAs (*miR-17-5p* and *miR-31*) change upon tFL.⁵⁵ We have identified miRNAs *miR-150*, *miR-31*, *miR-106b*, *miR-222*, and *miR-517b* as differentially expressed in tFL. This partially validates and extends the previous findings, because we also

Figure 3 (continued) (supplemental Methods). As a negative control, we used a control miRNA mimic (miR Neg. Ctrl; 400 nM). Luciferase activity was measured 24 hours posttransfection and compared by paired *t* test. (C) Statistical analysis of *FOXP1* staining in paired FL-tFL samples from all 14 patients with available material (patient characteristics listed in supplemental Table 1 [FL patients 5-10 and 12-19]). Percentages of samples with low ($\leq 30\%$), intermediate (inter; 30%-70%), and high ($\geq 70\%$) percentages of *FOXP1*⁺ cells are shown. The number in each segment of the column indicates the number of samples with the corresponding intensity of IHC staining. Each arrow indicates the change in the IHC staining category in each of the analyzed pairs (note that the angle of the line does not show the trend of increase/decrease in percentage of positive cells). The statistical differences were tested by Wilcoxon matched-pairs test. Representative images of the scoring of *FOXP1* staining in FL are shown in panel D and in supplemental Figure 14. (D) Representative examples of *FOXP1* IHC staining between paired samples of FL (upper) and tFL (lower). Original magnification $\times 400$. (E) Differences in *miR-150* expression levels between FL samples with low ($\leq 30\%$), intermediate (30%-70%), and high ($\geq 70\%$) percentages of *FOXP1*⁺ cells from 30 FL patients with available material (*FOXP1* staining performed as tissue microarray blocks; supplemental Methods). *P* values were assessed by unpaired *t* test. Representative images of the scoring of *FOXP1* staining in FL are shown in supplemental Figure 14. (F) Cell-cycle analysis of WSU-NHL cells transfected with synthetic *miR-150* mimic or siRNA against *FOXP1* or negative controls for miRNA or siRNA. Cells were harvested after 24 hours ($n = 10$ independent experiments) and analyzed for cell cycle (supplemental Methods; supplemental Figure 16). *P* values were assessed by paired *t* test. (G) The Ki67 proliferation index in FL samples with low ($\leq 30\%$), intermediate (30%-70%), or high ($\geq 70\%$) percentage of *FOXP1*⁺ cells from 27 FL patients with available material (*FOXP1* staining performed as tissue microarray blocks; supplemental Methods). *P* values were assessed by unpaired *t* test. NS, not significant.

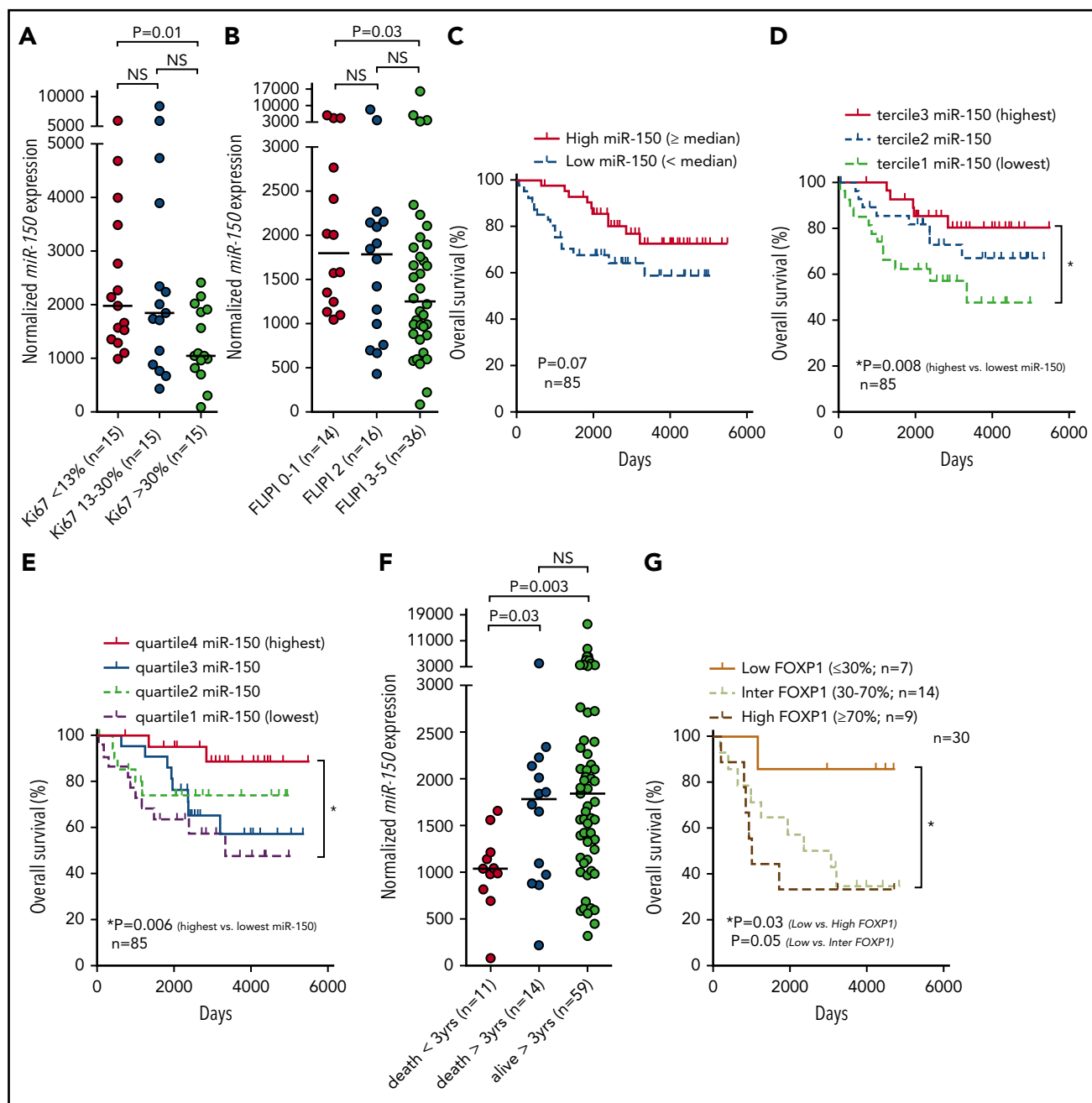
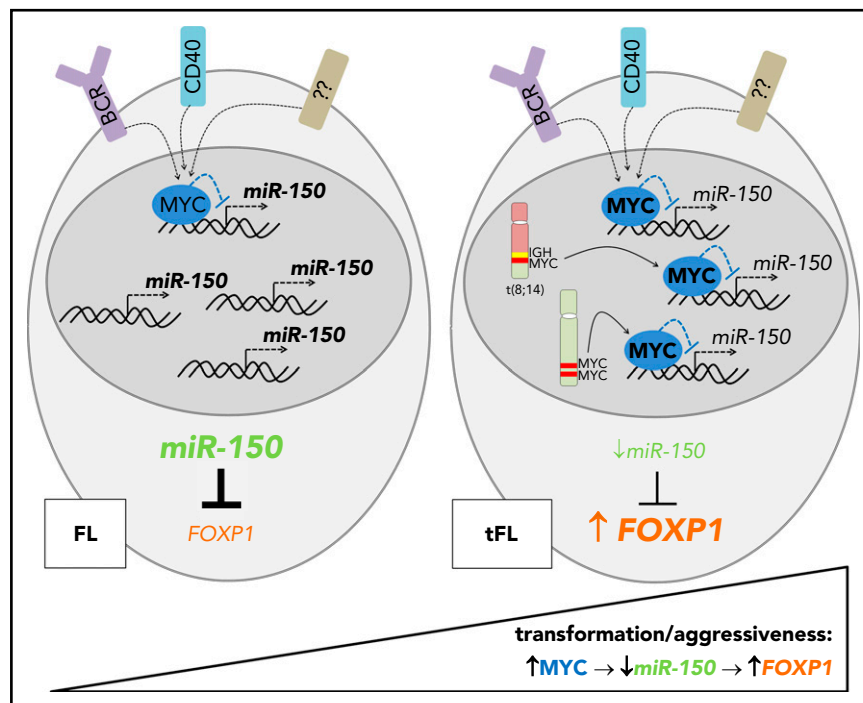


Figure 4. The association of *miR-150* expression with biological characteristics of FL and survival of patients. (A) *miR-150* expression in FL patients divided according to tertiles of Ki67 proliferation index (the differences were tested by Mann-Whitney test). All samples with available data on Ki67 were used in the analysis. (B) *miR-150* expression in FL patients divided according to Follicular Lymphoma International Prognostic Index (FLIPI) score (the differences were tested by Mann-Whitney test). All samples with available data on FLIPI were used in the analysis. (C-E) Association of *miR-150* expression with OS in FL. The OS is depicted using the Kaplan-Meier curves (with log-rank test) in the FL cohort divided by median *miR-150* expression (C) or by dividing the cohort into 3 (D) or 4 (E) groups based on tertiles or quartiles of *miR-150* expression, respectively. For all analyses, only patients with histologically verified FL at the time of sampling (with no histopathological signs of transformation to DLBCL) were included. Patient characteristics are listed in supplemental Table 2. (F) Differences in *miR-150* levels between FL patients who experienced early death within 3 years from biopsy (death <3 years) or who died later (death >3 years) or are still alive for longer than 3 years (alive >3 years) from biopsy. One patient with a follow-up time <3 years was excluded from the analysis. Similar data were obtained when only samples obtained at diagnosis were included in the analysis (54 of 84 samples; supplemental Figure 17A) or in patients experiencing early relapse (<24 months; supplemental Figure 17B). For all analyses, only patients with histologically verified FL at the time of sampling (with no histopathological signs of transformation to DLBCL) were included. The differences in *miR-150* levels between individual groups were tested by Mann-Whitney test. (G) The OS in FL patients divided according to immunostaining for FOXP1 (30 FL patients with material available as tissue microarray blocks; supplemental Methods). The patients were divided into 3 groups of samples with low ($\leq$ 30%), intermediate (inter; 30%-70%), or high ($\geq$ 70%) percentage of FOXP1⁺ cells. For all analyses, only patients with histologically verified FL at the time of sampling (with no histopathological signs of transformation to DLBCL) were included. Representative images of the scoring of FOXP1 staining in FL are shown in supplemental Figure 14. NS, not significant.

observed downmodulation of *miR-31* and upregulation of *miR-106b*, which is part of the cluster homologous to *miR-17-92* on chromosome 7. It has previously been described that miRNA

expression profiles in tFL (DLBCL) and primary DLBCL are not identical,⁵¹ but *miR-150* had a similar expression pattern in both entities when compared with FL (Figure 1C). Notably, none of

Figure 5. A schematic overview of the MYC/miR-150/FOXP1 role in the aggressiveness and high-grade transformation of FL. MYC overexpression caused by genomic aberrations (eg, translocations, mutations, or duplications) or B-cell activation down-regulates *miR-150* expression during the transformation of FL to DLBCL or in aggressive FL. Low levels of *miR-150* lead to the upregulation of its target, FOXP1.



the significantly changed miRNAs are located in regions with known genomic aberrations in FL/tFL.¹⁵

We focused on studying *miR-150* because it was consistently downmodulated in every tFL sample examined (fold change, ~3.5; Figure 1B). We have previously described low *miR-150* levels in aggressive CLL,²² and low levels of *miR-150* have been described in aggressive non-Hodgkin lymphomas.⁵⁶⁻⁵⁸ Here we show that MYC protein represses *miR-150* expression in tFL, which is compatible with the observed gain of MYC-activating aberrations and expression in tFL.^{7,10,17} In our experiments, MYC silencing by siRNA increased *miR-150* levels, and *miR-150* levels were low in B cells from transgenic MYC-overexpressing mice. Additionally, CLL samples with activating MYC aberrations had significantly lower *miR-150* levels. MYC is known to directly suppress the expression of large number of miRNAs⁵⁹ and activate expression of the *miR-17-92* cluster (and its homologs *miR-106b-25/miR-106a-92*).⁶⁰ In accordance with previous findings,^{15,61} most of our FL samples had weak MYC expression as assessed by immunostaining. In contrast, paired tFL samples were strongly MYC⁺ compared with the pretransformation samples that were MYC⁻ or showed only weak staining in a small number of cells. However, we did observe higher MYC levels (>5% positive cells) with lower *miR-150* expression in a few FL samples from patients who did not have tFL.

It has recently been shown that MYC upregulates LIN28 expression in myeloid cells, which inhibits *miR-150* maturation from its precursor *pri-miR-150*.⁴⁵ We have shown that LIN28A/B is not expressed in a panel of B-cell lines and FL samples. Additionally, *pri-miR-150* was effectively processed in B cells. These data suggest that the decrease in *miR-150* levels in tFL and some cases of FL is mediated by MYC through a mechanism that does not involve LIN28. This conclusion is supported by the results of the MYC–chromatin immunoprecipitation experiments

identifying MYC binding sites in the upstream region of *miR-150* gene in B cells.

We further investigated the effect of low *miR-150* levels on the expression of its direct target in B cells, namely FOXP1.²² We demonstrated that FOXP1 is upregulated with the transformation of FL to tFL, and its levels are regulated by *miR-150* in this context and in FL. FOXP1 has been extensively studied in lymphoid malignancies, because its high levels are known to be associated with worse prognosis in DLBCL and MALT lymphoma,^{34,35,37} and FOXP1-staining is used in IHC algorithms (eg, Choi, Tally, and Visco-Young) that distinguish the non-GC vs GC DLBCL subtypes.^{29,38-43} FOXP1 belongs to a family of forkhead box transcription factors and has been shown to be required for early B-cell development and GC reaction.^{48,62} In DLBCL cells, FOXP1 acts as a key transcription factor that regulates expression of a large number of genes, including those involved in cell survival, lymphocyte activation, chemotaxis, cell cycle, and plasmacytic differentiation.^{29,30,33,48,63,64} We and others have shown that FOXP1 positively affects BCR signaling and NF-κB pathway and Wnt signaling in malignant B cells^{22,33,65} and contributes to immune escape in DLBCL by suppressing MHCII expression.⁶³ Altogether, it has been shown that FOXP1 contributes to lymphomagenesis in vivo by affecting a number of proliferative and prosurvival genes and in ABC-DLBCL by disrupting terminal B-cell differentiation.²⁹ However, in vitro in DLBCL cell lines, FOXP1 silencing can have an effect on cell cycle and survival, or only on one or none of these cell characteristics.^{30-33,66} This is likely to depend on the context of other factors determining certain aspects of FOXP1 functionality (extensively discussed by Gascoyne and Banham²⁹), which could also explain why FOXP1 alone was insufficient to cause lymphomagenesis in mice.⁴⁸ In WSU-NHL cells, transfection with *miR-150* resulted in significant reduction of S phase, but this was not observed upon silencing FOXP1, suggesting that *miR-150* regulates other targets that are influencing the cell cycle, alone

or together with FOXP1. However, this does not exclude the possibility that FOXP1 is involved in proliferation of primary FL cells *in vivo*, and its known role in the activity of NF- κ B and BCR signaling suggests that it is plausible that it has some role in B-cell proliferation. Importantly, we observed a significantly higher percentage of Ki67⁺ cells in FL samples with high FOXP1 protein levels, which is in accordance with other studies.^{30,33,67}

Recently, FOXP1 upregulation was associated with a process of high-grade transformation of MALT lymphoma to aggressive gastric DLBCL.⁶⁶ In their report, the authors describe that downmodulation of *miR-34a* in gastric DLBCL leads to upregulation of FOXP1, because it is a target of *miR-34a*.^{66,68} We tested the expression of *miR-34a* and another FOXP1-targeting miRNA, namely *miR-181a*,⁶⁹ in paired FL and tFL, but these miRNAs are clearly not downmodulated in most tFLs (supplemental Figure 1B-C). We also tested the expression of another known *miR-150* target, namely MYB, regulation of which by *miR-150* is critical in immature B cells.²⁵ MYB expression was not influenced by *miR-150* levels in our previous study in CLL,²² and we found no inverse correlation between MYB and *miR-150* levels in FL/tFL (data not shown). This indicates that FOXP1 is likely to be a more relevant *miR-150* target in FL/tFL B cells. We propose that MYC-induced repression of *miR-150* results in upregulation of FOXP1 in malignant B cells in tFL, contributing to transformation to DLBCL, as summarized in Figure 5. Altogether, our data suggest that MYC acts to activate oncogenic miRNAs (eg, *miR-17-92* family) and inhibits expression of tumor suppressor *miR-150* during tFL. Previous reports have also suggested a relevance of *miR-142-3p* and *miR-142-5p* in the FL/DLBCL biology, because their mutations have been described in DLBCL⁷⁰ and tFL.¹⁶ However, we detected no mutations in mature sequences of these miRNAs or *miR-150* in the 24 analyzed paired FL-tFL samples (supplemental Figure 19).

miR-150 expression could also determine the behavior of FL B cells in patients who do not undergo transformation. *miR-150* expression was reduced in FL samples with high Ki67 positivity (>30%) and in patients with high FLIPI score (3-5), and furthermore, patients with FL with lower *miR-150* levels had a shorter OS (median survival, 9.1 years vs not reached; $P = .008$; HR, 3.5; CI, 1.3-9.2). *miR-150* levels retained independent predictive power for OS (HR, 2.8; $P = .02$) in a multivariate analysis, which included several known prognostic markers (age, sex, FLIPI, hemoglobin levels, lactate dehydrogenase levels, number of lymph node areas involved, Ann Arbor clinical stage, presence of B symptoms, and *miR-150* levels). *miR-150* was also significantly lower in patients dying within 3 years from biopsy or diagnosis (FL transformation excluded) or those with early relapse after therapy. This finding suggests that measurement of *miR-150* levels could be useful in identifying patients with FL with unfavorable prognosis as defined by progression within 24 months after therapy⁴⁹ (supplemental Figure 17), and this should be studied prospectively in a clinical trial. The possibility to use FFPE samples to quantify *miR-150* levels suggests its use as a relatively simple prognostic biomarker based on analyzing FFPE material available from routine biopsy evaluation. The circulating levels of extracellular *miR-150* in serum or plasma might potentially also serve as a biomarker; however, previous findings in CLL suggest that the

serum/plasma *miR-150* levels reflect tumor burden and lymphocyte count rather than the biology of malignant B cells.^{71,72} Univariate analysis showed that high protein expression of FOXP1 as determined by IHC was associated with a significantly shorter OS in FL (median survival, 33 months vs not reached; $P = .03$; HR, 5.0; CI, 1.1-22.6). During preparation of this report, similar results demonstrating the association of higher FOXP1 levels with shorter failure-free survival in FL were reported.⁶⁷ The adverse prognostic implications of low-level *miR-150* expression and increased expression of FOXP1 support an important role in progression of tFL and FL. However, it is possible that *miR-150* could also influence FL biology by regulating translation of other genes besides FOXP1 that contribute to malignant B-cell biology and their interplay with genetic aberrations in individual FL cases.

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Authorship

Contribution: K.M. performed experiments, analyzed data, and wrote the paper; J.D., K.C., V.S., G.P., and S.S. performed experiments; J.O. analyzed data; L.Z. and K.L. performed immunostaining; L. Kruzova performed cytogenetics; A.M. performed immunoglobulin rearrangement analyses; P.B. and K.M.P. performed chromatin immunoprecipitation; M. Trbusek performed the experiments with L cells; R.P., V.P., Z.P., M.J., C.K., I.S.-K., A.-I.S., O.M., H.M., L. Kren, J.M., C.S.Z., M. Trneny, A.G.E., and A.J. provided samples and clinical data and interpreted data; M.M. designed the study, interpreted data, and wrote the paper; and all authors edited and approved the paper for submission.

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Footnotes

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REFERENCES

- Montoto S, Fitzgibbon J. Transformation of indolent B-cell lymphomas. *J Clin Oncol*. 2011;29(14):1827-1834.
- Al-Tourah AJ, Gill KK, Chhanabhai M, et al. Population-based analysis of incidence and outcome of transformed non-Hodgkin's lymphoma. *J Clin Oncol*. 2008;26(32):5165-5169.
- Giné E, Montoto S, Bosch F, et al. The Follicular Lymphoma International Prognostic Index (FLIPI) and the histological subtype are the most important factors to predict histological transformation in follicular lymphoma. *Ann Oncol*. 2006;17(10):1539-1545.
- Janikova A, Borticek Z, Campr V, et al. The incidence of biopsy-proven transformation in follicular lymphoma in the rituximab era. A retrospective analysis from the Czech Lymphoma Study Group (CLSG) database. *Ann Hematol*. 2018;97(4):669-678.
- Montoto S, Davies AJ, Matthews J, et al. Risk and clinical implications of transformation of follicular lymphoma to diffuse large B-cell lymphoma. *J Clin Oncol*. 2007;25(17):2426-2433.
- Okosun J, Bödör C, Wang J, et al. Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. *Nat Genet*. 2014;46(2):176-181.
- Pasqualucci L, Khiabani H, Fangazio M, et al. Genetics of follicular lymphoma transformation. *Cell Reports*. 2014;6(1):130-140.
- Lo Coco F, Gaidano G, Louie DC, Offit K, Chaganti RS, Dalla-Favera R. p53 mutations are associated with histologic transformation of follicular lymphoma. *Blood*. 1993;82(8):2289-2295.
- Yano T, Jaffe ES, Longo DL, Raffeld M. MYC rearrangements in histologically progressed follicular lymphomas. *Blood*. 1992;80(3):758-767.
- Lossos IS, Alizadeh AA, Diehn M, et al. Transformation of follicular lymphoma to diffuse large-cell lymphoma: alternative patterns with increased or decreased expression of c-myc and its regulated genes. *Proc Natl Acad Sci USA*. 2002;99(13):8886-8891.
- Alhejaily A, Day AG, Feilottter HE, Baetz T, Lebrun DP. Inactivation of the CDKN2A tumor-suppressor gene by deletion or methylation is common at diagnosis in follicular lymphoma and associated with poor clinical outcome. *Clin Cancer Res*. 2014;20(6):1676-1686.
- Kridel R, Sehn LH, Gascoyne RD. Can histologic transformation of follicular lymphoma be predicted and prevented? *Blood*. 2017;130(3):258-266.
- Fitzgibbon J, Iqbal S, Davies A, et al. Genome-wide detection of recurring sites of uniparental disomy in follicular and transformed follicular lymphoma. *Leukemia*. 2007;21(7):1514-1520.
- Elenitoba-Johnson KS, Gascoyne RD, Lim MS, Chhanabai M, Jaffe ES, Raffeld M. Homozygous deletions at chromosome 9p21 involving p16 and p15 are associated with histologic progression in follicle center lymphoma. *Blood*. 1998;91(12):4677-4685.
- Bouska A, McKeithan TW, Deffenbacher KE, et al. Genome-wide copy-number analyses reveal genomic abnormalities involved in transformation of follicular lymphoma. *Blood*. 2014;123(11):1681-1690.
- Bouska A, Zhang W, Gong Q, et al. Combined copy number and mutation analysis identifies oncogenic pathways associated with transformation of follicular lymphoma. *Leukemia*. 2017;31(1):83-91.
- Davies AJ, Rosenwald A, Wright G, et al. Transformation of follicular lymphoma to diffuse large B-cell lymphoma proceeds by distinct oncogenic mechanisms. *Br J Haematol*. 2007;136(2):286-293.
- Farinha P, Al-Tourah A, Gill K, Klasa R, Connors JM, Gascoyne RD. The architectural pattern of FOXP3-positive T cells in follicular lymphoma is an independent predictor of survival and histologic transformation. *Blood*. 2010;115(2):289-295.
- Farinha P, Kyle AH, Minchinton AI, Connors JM, Karsan A, Gascoyne RD. Vascularization predicts overall survival and risk of transformation in follicular lymphoma. *Haematologica*. 2010;95(12):2157-2160.
- Saifi M, Maran A, Raynaud P, et al. High ratio of interfollicular CD8/FOXP3-positive regulatory T cells is associated with a high FLIPI index and poor overall survival in follicular lymphoma. *Exp Ther Med*. 2010;1(6):933-938.
- Smeltzer JP, Jones JM, Ziesmer SC, et al. Pattern of CD14+ follicular dendritic cells and PD1+ T cells independently predicts time to transformation in follicular lymphoma. *Clin Cancer Res*. 2014;20(11):2862-2872.
- Mraz M, Chen L, Rassenti LZ, et al. miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1. *Blood*. 2014;124(1):84-95.
- Mraz M, Dolezalova D, Plevova K, et al. MicroRNA-650 expression is influenced by immunoglobulin gene rearrangement and affects the biology of chronic lymphocytic leukemia. *Blood*. 2012;119(9):2110-2113.
- Musilova K, Mraz M. MicroRNAs in B-cell lymphomas: how a complex biology gets more complex. *Leukemia*. 2015;29(5):1004-1017.
- Xiao C, Calado DP, Galler G, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb [published correction appears in *Cell*. 2016;165(4):1027]. *Cell*. 2007;131(1):146-159.
- He L, Thomson JM, Hemann MT, et al. A microRNA polycistron as a potential human oncogene. *Nature*. 2005;435(7043):828-833.
- Costinean S, Zanesi N, Pekarsky Y, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci USA*. 2006;103(18):7024-7029.
- Costinean S, Sandhu SK, Pedersen IM, et al. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of E(mu)-miR-155 transgenic mice. *Blood*. 2009;114(7):1374-1382.
- Gascoyne DM, Banham AH. The significance of FOXP1 in diffuse large B-cell lymphoma. *Leuk Lymphoma*. 2017;58(5):1037-1051.
- van Keimpema M, Grüneberg LJ, Mokry M, et al. FOXP1 directly represses transcription of proapoptotic genes and cooperates with NF- κ B to promote survival of human B cells. *Blood*. 2014;124(23):3431-3440.
- van Keimpema M, Grüneberg LJ, Schilder-Tol EJ, et al. The small FOXP1 isoform predominantly expressed in activated B cell-like diffuse large B-cell lymphoma and full-length FOXP1 exert similar oncogenic and transcriptional activity in human B cells. *Haematologica*. 2017;102(3):573-583.
- Flori M, Schmid CA, Sumrall ET, et al. The hematopoietic oncoprotein FOXP1 promotes tumor cell survival in diffuse large B-cell lymphoma by repressing S1PR2 signaling. *Blood*. 2016;127(11):1438-1448.
- Dekker JD, Park D, Shaffer AL III, et al. Subtype-specific addition of the activated B-cell subset of diffuse large B-cell lymphoma to FOXP1. *Proc Natl Acad Sci USA*. 2016;113(5):E577-E586.
- Barrans SL, Fenton JA, Banham A, Owen RG, Jack AS. Strong expression of FOXP1 identifies a distinct subset of diffuse large B-cell lymphoma (DLBCL) patients with poor outcome. *Blood*. 2004;104(9):2933-2935.
- Banham AH, Connors JM, Brown PJ, et al. Expression of the FOXP1 transcription factor is strongly associated with inferior survival in patients with diffuse large B-cell lymphoma. *Clin Cancer Res*. 2005;11(3):1065-1072.
- Brown P, Marafioti T, Kusec R, Banham AH. The FOXP1 transcription factor is expressed in the majority of follicular lymphomas but is rarely expressed in classical and lymphocyte predominant Hodgkin's lymphoma. *J Mol Histol*. 2005;36(4):249-256.
- Sagaert X, de Paep P, Libbrecht L, et al. Forkhead box protein P1 expression in mucosa-associated lymphoid tissue lymphomas predicts poor prognosis and transformation to diffuse large B-cell lymphoma. *J Clin Oncol*. 2006;24(16):2490-2497.
- Choi WW, Weisenburger DD, Greiner TC, et al. A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. *Clin Cancer Res*. 2009;15(17):5494-5502.
- Meyer PN, Fu K, Greiner TC, et al. Immunohistochemical methods for predicting cell of origin and survival in patients with diffuse large B-cell lymphoma treated with rituximab. *J Clin Oncol*. 2011;29(2):200-207.
- Visco C, Li Y, Xu-Monette ZY, et al. Comprehensive gene expression profiling and immunohistochemical studies support application of immunophenotypic algorithm for molecular subtype classification in diffuse large B-cell lymphoma: a report from the International DLBCL Rituximab-CHOP Consortium Program Study [published correction appears in *Leukemia*. 2014;28(4):980]. *Leukemia*. 2012;26(9):2103-2113.
- He M, Gao L, Zhang S, et al. Prognostic significance of miR-34a and its target proteins of FOXP1, p53, and BCL2 in gastric MALT

- lymphoma and DLBCL. *Gastric Cancer*. 2014; 17(3):431-441.
42. Nyman H, Jerkeman M, Karjalainen-Lindsberg ML, Banham AH, Enblad G, Leppä S. Bcl-2 but not FOXP1, is an adverse risk factor in immunochemotherapy-treated non-germinal center diffuse large B-cell lymphomas. *Eur J Haematol*. 2009;82(5):364-372.
43. Wong KK, Gascoyne DM, Brown PJ, et al. Reciprocal expression of the endocytic protein HIP1R and its repressor FOXP1 predicts outcome in R-CHOP-treated diffuse large B-cell lymphoma patients [published correction appears in *Leukemia*. 2014;28(2):470]. *Leukemia*. 2014;28(2):362-372.
44. Mraz M, Malinova K, Mayer J, Pospisilova S. MicroRNA isolation and stability in stored RNA samples. *Biochem Biophys Res Commun*. 2009;390(1):1-4.
45. Jiang X, Huang H, Li Z, et al. Blockade of miR-150 maturation by MLL-fusion/MYC/LIN-28 is required for MLL-associated leukemia. *Cancer Cell*. 2012;22(4):524-535.
46. Zemanova J, Hylse O, Collakova J, et al. Chk1 inhibition significantly potentiates activity of nucleoside analogs in TP53-mutated B-lymphoid cells. *Oncotarget*. 2016;7(38):62091-62106.
47. Schauer SL, Wang Z, Sonenshein GE, Rothstein TL. Maintenance of nuclear factor-kappa B/Rel and c-myc expression during CD40 ligand rescue of WEHI 231 early B cells from receptor-mediated apoptosis through modulation of I kappa B proteins. *J Immunol*. 1996;157(1):81-86.
48. Sagardoy A, Martinez-Ferrandis JI, Roa S, et al. Downregulation of FOXP1 is required during germinal center B-cell function. *Blood*. 2013;121(21):4311-4320.
49. Casulo C, Byrtek M, Dawson KL, et al. Early relapse of follicular lymphoma after rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone defines patients at high risk for death: an analysis from the National LymphoCare Study. *J Clin Oncol*. 2015;33(23):2516-2522.
50. Lawrie CH, Soneji S, Marafioti T, et al. MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. *Int J Cancer*. 2007;121(5):1156-1161.
51. Lawrie CH, Chi J, Taylor S, et al. Expression of microRNAs in diffuse large B cell lymphoma is associated with immunophenotype, survival and transformation from follicular lymphoma. *J Cell Mol Med*. 2009;13(7):1248-1260.
52. Zhong H, Xu L, Zhong JH, et al. Clinical and prognostic significance of miR-155 and miR-146a expression levels in formalin-fixed/paraffin-embedded tissue of patients with diffuse large B-cell lymphoma. *Exp Ther Med*. 2012;3(5):763-770.
53. Cui B, Chen L, Zhang S, et al. MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia. *Blood*. 2014;124(4):546-554.
54. Correia C, Schneider PA, Dai H, et al. BCL2 mutations are associated with increased risk of transformation and shortened survival in follicular lymphoma. *Blood*. 2015;125(4):658-667.
55. Thompson MA, Edmonds MD, Liang S, et al. miR-31 and miR-17-5p levels change during transformation of follicular lymphoma. *Hum Pathol*. 2016;50:118-126.
56. Di Lisio L, Gómez-López G, Sánchez-Beato M, et al. Mantle cell lymphoma: transcriptional regulation by microRNAs. *Leukemia*. 2010; 24(7):1335-1342.
57. Malumbres R, Sarosiek KA, Cubedo E, et al. Differentiation stage-specific expression of microRNAs in B lymphocytes and diffuse large B-cell lymphomas. *Blood*. 2009;113(16):3754-3764.
58. Zhao JJ, Lin J, Lwin T, et al. microRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. *Blood*. 2010;115(13):2630-2639.
59. Chang TC, Yu D, Lee YS, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet*. 2008;40(1):43-50.
60. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature*. 2005; 435(7043):839-843.
61. Kridel R, Mottok A, Farinha P, et al. Cell of origin of transformed follicular lymphoma. *Blood*. 2015;126(18):2118-2127.
62. Hu H, Wang B, Borde M, et al. Foxp1 is an essential transcriptional regulator of B cell development. *Nat Immunol*. 2006;7(8):819-826.
63. Brown PJ, Wong KK, Felce SL, et al. FOXP1 suppresses immune response signatures and MHC class II expression in activated B-cell-like diffuse large B-cell lymphomas. *Leukemia*. 2016;30(3):605-616.
64. van Keimpema M, Grüneberg LJ, Mokry M, et al. The forkhead transcription factor FOXP1 represses human plasma cell differentiation. *Blood*. 2015;126(18):2098-2109.
65. Walker MP, Stopford CM, Cederlund M, et al. FOXP1 potentiates Wnt/β-catenin signaling in diffuse large B cell lymphoma. *Sci Signal*. 2015;8(362):ra12.
66. Craig VJ, Cogliatti SB, Imig J, et al. Myc-mediated repression of microRNA-34a promotes high-grade transformation of B-cell lymphoma by dysregulation of FoxP1. *Blood*. 2011;117(23):6227-6236.
67. Mottok A, Jurinovic V, Farinha P, et al. FOXP1 expression is a prognostic biomarker in follicular lymphoma treated with rituximab and chemotherapy. *Blood*. 2018;131(2):226-235.
68. Rao DS, O'Connell RM, Chaudhuri AA, Garcia-Flores Y, Geiger TL, Baltimore D. MicroRNA-34a perturbs B lymphocyte development by repressing the forkhead box transcription factor Foxp1. *Immunity*. 2010;33(1):48-59.
69. Alencar AJ, Malumbres R, Kozloski GA, et al. MicroRNAs are independent predictors of outcome in diffuse large B-cell lymphoma patients treated with R-CHOP. *Clin Cancer Res*. 2011;17(12):4125-4135.
70. Kwanhian W, Lenze D, Alles J, et al. MicroRNA-142 is mutated in about 20% of diffuse large B-cell lymphoma. *Cancer Med*. 2012;1(2):141-155.
71. Stamatopoulos B, Van Damme M, Crompton E, et al. Opposite prognostic significance of cellular and serum circulating microRNA-150 in patients with chronic lymphocytic leukemia. *Mol Med*. 2015;21:123-133.
72. Pritchard CC, Kroh E, Wood B, et al. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer Prev Res (Phila)*. 2012;5(3):492-497.

LYMPHOID NEOPLASIA

Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis

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Key Points

- Microenvironmental interactions upregulate CD20 expression in CLL cells through the CXCR4/SDF-1 axis.
- Ibrutinib treatment causes downregulation of CD20 in CLL cells.

Agents targeting B-cell receptor (BCR) signaling-associated kinases such as Bruton tyrosine kinase (BTK) or phosphatidylinositol 3-kinase can induce mobilization of neoplastic B cells from the lymphoid tissues into the blood, which makes them potentially ideal to combine with anti-CD20 monoclonal antibodies (such as rituximab, obinutuzumab, or ofatumumab) for treatment of B-cell lymphomas and chronic lymphocytic leukemia (CLL). Here we show that interactions between leukemia cells and stromal cells (HS-5) upregulate CD20 on CLL cells and that administering ibrutinib downmodulates CD20 (MS4A1) expression *in vivo*. We observed that CLL cells that have recently exited the lymph node microenvironment and moved into the peripheral blood (CXCR4^{dim}CD5^{bright} subpopulation) have higher cell surface levels of CD20 than the cells circulating in the bloodstream for a longer time (CXCR4^{bright}CD5^{dim} cells). We found that CD20 is directly upregulated by CXCR4 ligand stromal cell-derived factor 1 (SDF-1 α , CXCL12) produced

by stromal cells, and BTK-inhibitor ibrutinib and CXCR4-inhibitor plerixafor block SDF-1 α -mediated CD20 upregulation. Ibrutinib also downmodulated Mcl1 levels in CLL cells *in vivo* and in coculture with stromal cells. Overall, our study provides a first detailed mechanistic explanation of CD20 expression regulation in the context of chemokine signaling and microenvironmental interactions, which may have important implications for microenvironment-targeting therapies. (*Blood*. 2016;128(12):1609-1613)

Introduction

The introduction of inhibitors of kinases involved in B-cell receptor (BCR) signaling, such as Bruton tyrosine kinase (BTK) or phosphatidylinositol 3-kinase, has been a major therapeutic advance in chronic lymphocytic leukemia (CLL).^{1,2} The small-molecule inhibitor of BTK kinase, ibrutinib, can disrupt CLL cells' capacity to interact with cells in the microenvironment by interfering with chemokine-receptor signaling, which is important for the chemotaxis of leukemia B cells to lymphoid tissues, and thus induce their massive and lasting mobilization in the peripheral blood.¹⁻⁶

The combined use of ibrutinib with anti-CD20 antibodies⁷⁻⁹ or other monoclonal antibodies (mAbs) has been suggested for the treatment of patients with CLL because they use different mechanisms for antileukemia activity. Additionally, we and others have previously shown that microenvironmental interactions protect CLL and lymphoma cells from rituximab-induced cytotoxicity¹⁰⁻¹² and chemotherapy-induced apoptosis.^{11,13} The ibrutinib-induced lymphocytosis suggests that a combinatorial therapy with mAbs might overcome adhesion-mediated antibody resistance and synergize with anti-CD20 mAbs.¹⁰⁻¹³

Here we examined whether ibrutinib has an effect on the CD20 expression, and this revealed that the CLL cells of patients treated with

ibrutinib have lower expression levels of CD20 than the CLL cells of the same patients prior to the therapy. Because ibrutinib interferes with leukemia-cell trafficking to the lymphoid microenvironment, we hypothesized that this downregulation might be because of the loss of stimulation by microenvironmental factors. Indeed, here we described that interactions of CLL cells with stromal cells induce the upregulation of CD20 expression through the CXCR4/SDF-1 axis. We also observed that ibrutinib inhibits SDF-1-induced CD20 expression and, in CLL patients, leads to CD20 downmodulation *in vivo*.

Study design

Peripheral blood samples were obtained from untreated CLL patients or patients treated with ibrutinib as a single agent (420 mg once daily). CLL cells were separated from the blood samples using negative selection by RosetteSep Human B Cell Enrichment Cocktail (StemCell Technologies) or Ficoll-Paque, followed by magnetic anti-CD3 MicroBeads separation (Miltenyi Biotec). The study was approved by the institutional review board, and samples were obtained with

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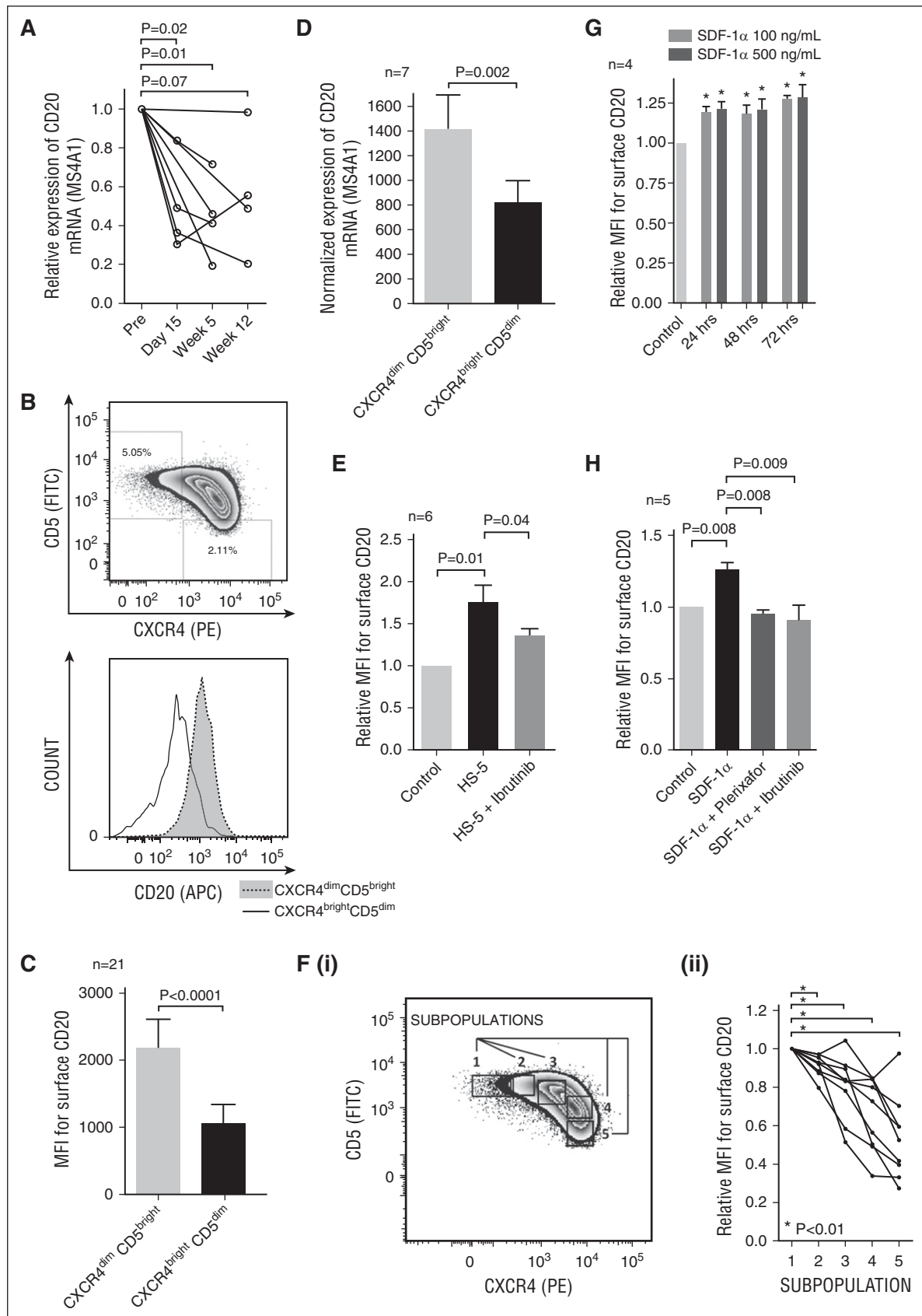


Figure 1. The effect of microenvironmental interactions on CD20 expression in CLL cells. (A) Relative CD20 mRNA (MS4A1) expression in samples obtained before and during ibrutinib treatment of CLL patients (N = 8 patients; for characterization of CLL samples, see supplemental Table 1, sample no. CLL102-109). Samples were acquired the day before the first administration of ibrutinib (Pre), and on day 15, and/or week 5 and/or week 12 after the first ibrutinib administration. (B) A representative example of the gating of CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} CLL cell populations analyzed using flow cytometry (top). The histogram of the surface CD20

written informed consent. The coculture experiments with immortalized HS-5 stromal cells were performed as previously described.¹¹ Briefly, CLL cells were seeded on plastic or a monolayer of HS-5 cells at a concentration of 2.5×10^6 cells per mL per well. The cells were incubated in RPMI with 10% fetal bovine serum (37°C, 5% CO₂) for the indicated time periods and harvested for flow cytometry, gene expression, or immunoblotting analyses (see supplemental Methods, available on the *Blood* Web site). Statistical analyses were performed using GraphPad Prism Software v 5.0.

Results and discussion

It has been suggested to therapeutically combine BCR-signaling inhibitors with anti-CD20 mAbs. Therefore, we investigated whether ibrutinib affects CD20 expression on CLL cells. We analyzed samples obtained from CLL patients treated with ibrutinib as a single agent (preibrutinib vs postibrutinib; patients' characteristics are summarized in supplemental Table 1) and observed significant CD20 downmodulation on the CLL cell surface and its messenger RNA (mRNA) levels (Figure 1A and supplemental Figures 1 and 2E-F). Ibrutinib-induced CD20 downmodulation has been also reported by others¹⁴ and suggests that CD20 expression might be regulated by a yet unknown mechanism in the context of microenvironmental interactions that are disrupted by ibrutinib. We therefore focused on investigating the effect of microenvironmental interactions on CD20 expression on malignant B cells.

We assessed CD20 expression on CLL cell subpopulations defined according to CXCR4 and CD5 levels. We and others have described that CLL cells that have recently exited the lymph node microenvironment and moved into the peripheral blood express lower levels of the chemokine receptor CXCR4 and higher levels of the activation marker CD5.^{15,16} The CXCR4^{dim}CD5^{bright} CLL cells have approximately twofold higher cell-surface and mRNA expression of CD20 than CXCR4^{bright}CD5^{dim} cells ($P < .01$; Figure 1B-D). This suggests that changes in surface CD20 levels within immune niches reflect changes in gene transcription. Ibrutinib treatment in vivo also induced a reduction of CXCR4^{dim}CD5^{bright} CLL cell subpopulation (supplemental Figure 2).

We next tested the effect of CLL cell coculture with the stromal cell line HS-5¹¹ on CD20 expression with and without ibrutinib treatment. This revealed that surface CD20 levels were significantly upregulated on CLL cells cocultured with the HS-5 cell line compared with control CLL cells cultured on plastic ($P < .05$; Figure 1E and supplemental

Figure 3), and ibrutinib inhibited the upregulation of CD20 ($P < .05$; Figure 1E).

We further analyzed the CXCR4/CD5 CLL subpopulations and observed that the CD20 expression gradually decreased with CLL cells' transition from CXCR4^{dim}CD5^{bright} to CXCR4^{bright}CD5^{dim} ($P < .01$; Figure 1F). This led us to hypothesize that the CXCR4/SDF-1 axis is directly involved in CD20 regulation. Indeed, CLL cells treated with SDF-1 α (CXCL12), a ligand for CXCR4 produced by stromal cells, significantly upregulated surface CD20 (Figure 1G). The CD20 upregulation induced by SDF-1 α or HS-5 stromal cells was inhibited by plerixafor (a CXCR4 inhibitor) ($P < .01$; Figure 1H and supplemental Figure 4). Similarly, ibrutinib treatment also inhibited the CD20 upregulation induced by SDF-1 α ($P < .01$; Figure 1H) or induced by CLL cell coculture with stromal cells ($P < .05$; Figure 1E). This can likely be explained by the previous finding that ibrutinib can directly prevent CXCR4 phosphorylation and alter the function of BTK downstream kinases.^{3,4,6} Altogether, these data suggest that the CD20 downmodulation induced by ibrutinib is at least partially because of the inhibition of CXCR4/SDF-1 α signaling that regulates its levels.

The addition of rituximab to ibrutinib largely mitigates ibrutinib-induced lymphocytosis.⁷ This implicates that rituximab is able to eliminate circulating CLL cells, at least to some extent, despite lower CD20 levels on ibrutinib-treated patients' CLL cells. This suggests that ibrutinib potentially has other mechanism(s) of action that facilitate rituximab efficacy when the 2 drugs are given in a combination. We therefore focused on the regulation of antiapoptotic molecules, namely Mcl1, because it has been shown that Mcl1 directly protects CLL cells from rituximab-induced apoptosis and complement dependent cytotoxicity,¹⁷ and Mcl1 is rapidly downmodulated by rituximab infusion in vivo.¹⁸ In Figure 2A, coculture of CLL cells with HS-5 cells leads to a strong Mcl1 protein upregulation in CLL cells. We also observed higher Mcl1 mRNA levels in the CXCR4^{dim}CD5^{bright} CLL subpopulation that recently exited the lymph node niche ($P < .05$; Figure 2B). Ibrutinib treatment resulted in significant ($P < .05$) downmodulation of Mcl1 protein levels in CLL cells (Figure 2C) and the CLL-derived cell line OSU-CLL cocultured with HS-5 (Figure 2D). Ibrutinib had no significant effect on cell viability in these experiments (supplemental Figure 5). Importantly, Mcl1 mRNA levels were also significantly downmodulated after ibrutinib treatment in vivo (Figure 2E), but other antiapoptotic molecules (such as Bcl2, Bcl-XL, Bcl-W, Bcl2A1, or XIAP) were not affected (supplemental Figure 6).

In summary, microenvironmental interactions upregulate CD20 expression in CLL B cells through the CXCR4/SDF-1 axis (summarized

Figure 1 (continued) expression on CLL cells gated in the top panel (bottom). (C) The mean fluorescence intensity (MFI) for the surface CD20 on CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} CLL cells (N = 21 pairs; for characterization of CLL samples, see supplemental Table 2). The statistical difference was tested by paired Student *t* test. (D) Normalized CD20 mRNA (*MS4A1*) expression in sorted CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} CLL cells (N = 7 pairs; purity >99% CD5⁺CD19⁺ cells; for characterization of CLL samples, see supplemental Table 2). The statistical difference was tested by paired Student *t* test. (E) Freshly obtained CLL cells (N = 6, purity >99% CD5⁺CD19⁺ cells; for characterization of CLL samples, see supplemental Table 2) were seeded (2.5×10^6 cells per mL) on plastic (control) or an HS-5 monolayer. CLL cells were pretreated with vehicle (labeled HS-5) or ibrutinib (HS-5 + ibrutinib) for 2 hours prior to being seeded on the HS-5 stromal cells. The CLL cells were treated with ibrutinib (1 μ M) prior to seeding on stromal cells to ensure full BTK inhibition before the contact of B cells with HS-5 cells. The control stands for CLL cells treated with vehicle and cultured on plastic with no contact with stromal cells. After 48 hours in cultivation, all cells in the wells were harvested and labeled with anti-CD20 antibody and Annexin-V, and CD20 MFI was analyzed on viable CD105-negative cells (ie, CLL cells) by flow cytometry. CD105 was used as a stromal cell marker. (F) A representative plot showing gating strategy of 5 subpopulations based on CXCR4 and CD5 expression (i). The relative surface CD20 expression in CLL subpopulations gated according to CXCR4/CD5 levels (ii; N = 9 CLL samples). The statistical difference was tested by paired Student *t* test. (G) Freshly obtained CLL cells (N = 4, purity >99% CD5⁺CD19⁺ cells; for characterization of CLL samples, see supplemental Table 2) were seeded on a plastic surface at a concentration of 2.5×10^6 cells per mL and treated with recombinant human SDF-1 α (100 or 500 ng/mL), or vehicle (control) and cultured for 24/48/72 hours. After the indicated cultivation period, the CLL cells were harvested and labeled with anti-CD20 antibody and Annexin-V, and CD20 MFI was analyzed on viable cells using flow cytometry. MFI on the control cells was set as 1. * $P \leq .05$. (H) Freshly obtained primary CLL cells (N = 5, purity >99% CD5⁺CD19⁺ cells; for characterization of CLL samples, see supplemental Table 2) were seeded on plastic (2.5×10^6 cells per mL) and treated with SDF-1 α (labeled SDF-1 α) or plerixafor and SDF-1 α (SDF-1 α + plerixafor) or ibrutinib and SDF-1 α (SDF-1 α + ibrutinib). Ibrutinib (1 μ M) or plerixafor (5 μ g/mL) was added to the cell culture 2 hours prior to SDF-1 α treatment (500 ng/mL) to ensure full BTK/CXCR4 inhibition before the contact of B cells with the SDF-1 α chemokine. The control stands for cells that were treated with vehicle and cultured on plastic with no inhibitor or SDF-1 α treatment. After 48 hours in cultivation, all cells in the wells were harvested and labeled with anti-CD20 antibody and Annexin-V, and CD20 MFI was analyzed on viable cells using flow cytometry.

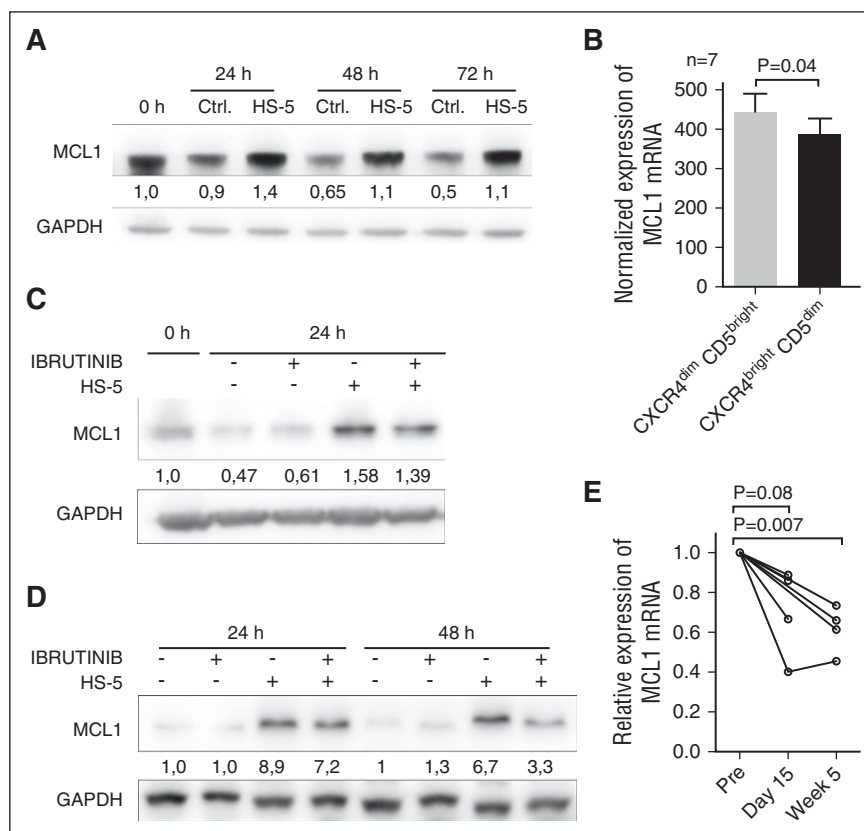


Figure 2. The effect of microenvironmental interactions and ibrutinib treatment on Mcl1 expression. (A) Representative immunoblot analysis of Mcl1 expression in CLL cells (purity >95% CD5⁺CD19⁺ cells) after culture on plastic (Ctrl.) or coculture with HS-5 cells (HS-5) for 24/48/72 hours. The purification of CLL cells after coculture with HS-5 cells was performed using magnetic anti-CD105 MicroBeads (purity >95% CD5⁺CD19⁺ cells). The blot images were quantified with UVitec Alliance 4.7 (UVitec Cambridge), and the Mcl1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the first sample was arbitrarily set at 1. (B) Normalized Mcl1 mRNA expression in sorted CXCR4^{dim}CD5^{bright} and CXCR4^{bright}CD5^{dim} CLL cells (N = 7 pairs, purity >99% CD5⁺CD19⁺ cells). (C) Representative immunoblot analysis of Mcl1 expression in CLL cells after culture on plastic or coculture with HS-5 (with or without ibrutinib treatment). The presence of ibrutinib target BTK in CLL was confirmed by immunoblotting (supplemental Figure 8). The CLL cells were pretreated with ibrutinib (10 μ M) or vehicle for 2 hours and then washed and seeded on plastic or confluent monolayer of HS-5 cells. The pretreatment of 2 hours with ibrutinib followed by washing was performed to minimize the off-target effects caused by the continuous ibrutinib presence. After the indicated cultivation period, all cells in the wells were harvested, and B-cell purification after coculture with HS-5 cells was performed using magnetic anti-CD105 MicroBeads (purity >95% CD5⁺CD19⁺ cells). The blot images were quantified with UVitec Alliance 4.7, and the Mcl1/GAPDH in the first sample was arbitrarily set at 1. Above each panel is a "+" or a "-" in the row labeled "ibrutinib" and/or "HS-5" to indicate the samples that were exposed to ibrutinib or cocultured with stromal cells. (D) Representative immunoblot analysis of Mcl1 in the OSU-CLL cell line after culture on plastic or coculture with HS-5 (with or without treatment with ibrutinib). The presence of ibrutinib target BTK in OSU-CLL cell line was confirmed by immunoblotting (supplemental Figure 8). The experiment was performed identically as described in panel C for CLL cells. The Mcl1/GAPDH in the control sample was arbitrarily set at 1. (E) Relative Mcl1 mRNA expression in samples obtained before (Pre) and during ibrutinib treatment of CLL patients (time of sampling indicated). The gene expression was analyzed in samples with enough RNA material available (N = 7 patients; for characterization of CLL samples, see supplemental Table 1, sample no. CLL101-103 and 106-109 [other samples did not have enough RNA available]).

in supplemental Figure 7). It is likely that inducing CD20 in the context of immune microenvironment is of physiological importance because CD20-knockout B cells have a reduced BCR signaling propensity, and CD20 physically couples with BCR in lipid rafts.¹⁹⁻²² The stroma-induced CD20 upregulation seems to be influenced by transcriptional changes rather than posttranscriptional regulation because microRNAs (miRs) involved in microenvironmental interactions (such as miR-29b, miR-181b, and miR-150)^{23,24} do not have evolutionary conserved binding sites in CD20 mRNA (data not shown). However, we cannot exclude the possibility that other miRs or microenvironmental factors can also affect CD20 levels. Additionally, our data demonstrate that the CD20 reduction is unlikely to contribute to stromal cell adhesion-mediated rituximab resistance described previously¹⁰⁻¹² because the interactions with stroma on the contrary upregulate the rituximab target, CD20. Our findings explain at least partially the mechanism of CD20 downmodulation with ibrutinib treatment and have potentially important implications for combinatorial studies of CD20-targeting antibodies and BCR inhibitors in patients with CLL.

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Authorship

Contribution: G.P. performed experiments, analyzed data, and wrote the manuscript; V.S., K.C., and R.C. performed experiments; M.B. and J.O. performed flow cytometry; J.M., M.S.D., M.D., S.P., T.J.K., M.T., and J.R.B. provided samples and interpreted data; M.M. designed the study, interpreted data, and wrote the manuscript; and all authors edited and approved the manuscript for submission.

References

- Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2013;369(1):32-42.
- Furman RR, Sharman JP, Coutre SE, et al. Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2014;370(11):997-1007.
- de Rooij MFM, Kuil A, Geest CR, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood*. 2012;119(11):2590-2594.
- Ponader S, Chen S-S, Buggy JJ, et al. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood*. 2012;119(5):1182-1189.
- Seda V, Mraz M. B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells. *Eur J Haematol*. 2015;94(3):193-205.
- Chen S-S, Chang BY, Chang S, et al. BTK inhibition results in impaired CXCR4 chemokine receptor surface expression, signaling and function in chronic lymphocytic leukemia. *Leukemia*. 2016;30(4):833-843.
- Burger JA, Keating MJ, Wierda WG, et al. Safety and activity of ibrutinib plus rituximab for patients with high-risk chronic lymphocytic leukaemia: a single-arm, phase 2 study. *Lancet Oncol*. 2014;15(10):1090-1099.
- Herman SEM, Niemann CU, Farooqui M, et al. Ibrutinib-induced lymphocytosis in patients with chronic lymphocytic leukemia: correlative analyses from a phase II study. *Leukemia*. 2014;28(11):2188-2196.
- O'Brien SM, Lamanna N, Kipps TJ, et al. A phase 2 study of idelalisib plus rituximab in treatment-naïve older patients with chronic lymphocytic leukemia. *Blood*. 2015;126(25):2686-2694.
- Buchner M, Brantner P, Stickel N, et al. The microenvironment differentially impairs passive and active immunotherapy in chronic lymphocytic leukaemia - CXCR4 antagonists as potential adjuvants for monoclonal antibodies. *Br J Haematol*. 2010;151(2):167-178.
- Mraz M, Zent CS, Church AK, et al. Bone marrow stromal cells protect lymphoma B-cells from rituximab-induced apoptosis and targeting integrin α -4 β -1 (VLA-4) with natalizumab can overcome this resistance. *Br J Haematol*. 2011;155(1):53-64.
- Marquez M-E, Hernández-Uzcátegui O, Cornejo A, Vargas P, Da Costa O. Bone marrow stromal mesenchymal cells induce down regulation of CD20 expression on B-CLL: implications for rituximab resistance in CLL. *Br J Haematol*. 2015;169(2):211-218.
- Lwin T, Hazlehurst LA, Li Z, et al. Bone marrow stromal cells prevent apoptosis of lymphoma cells by upregulation of anti-apoptotic proteins associated with activation of NF-kappaB (RelB/p52) in non-Hodgkin's lymphoma cells. *Leukemia*. 2007;21(7):1521-1531.
- Skarzynski M, Niemann CU, Lee YS, et al. Interactions between ibrutinib and anti-CD20 antibodies: competing effects on the outcome of combination therapy. *Clin Cancer Res*. 2016;22(1):86-95.
- Calissano C, Damle RN, Marsilio S, et al. Intracanal complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. *Mol Med*. 2011;17(11-12):1374-1382.
- Cui B, Chen L, Zhang S, et al. MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia. *Blood*. 2014;124(4):546-554.
- Hussain S-RA, Cheney CM, Johnson AJ, et al. Mcl-1 is a relevant therapeutic target in acute and chronic lymphoid malignancies: down-regulation enhances rituximab-mediated apoptosis and complement-dependent cytotoxicity. *Clin Cancer Res*. 2007;13(7):2144-2150.
- Byrd JC, Kitada S, Flinn IW, et al. The mechanism of tumor cell clearance by rituximab in vivo in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. *Blood*. 2002;99(3):1038-1043.
- Petrie RJ, Deans JP. Colocalization of the B cell receptor and CD20 followed by activation-dependent dissociation in distinct lipid rafts. *J Immunol*. 2002;169(6):2886-2891.
- Uchida J, Lee Y, Hasegawa M, et al. Mouse CD20 expression and function. *Int Immunol*. 2004;16(1):119-129.
- Polyak MJ, Li H, Shariat N, Deans JP. CD20 homo-oligomers physically associate with the B cell antigen receptor. Dissociation upon receptor engagement and recruitment of phosphoproteins and calmodulin-binding proteins. *J Biol Chem*. 2008;283(27):18545-18552.
- Franke A, Niederfellner GJ, Klein C, Bartscher H. Antibodies against CD20 or B-cell receptor induce similar transcription patterns in human lymphoma cell lines. *PLoS One*. 2011;6(2):e16596.
- Mraz M, Chen L, Rassenti LZ, et al. miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1. *Blood*. 2014;124(1):84-95.
- Musilova K, Mraz M. MicroRNAs in B-cell lymphomas: how a complex biology gets more complex. *Leukemia*. 2015;29(5):1004-1017.

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Komplexní mechanismy účinku inhibitorů „BCR signalizace“ a vzniku rezistence na tuto cílenou léčbu u chronické lymfocytární leukemie

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SOUHRN

Nedávné zavedení inhibitoru BTK ibrutinibu a inhibitoru PI3K idelalisibu představovalo revoluci v terapii malignit vznikajících z maturovaných B lymfocytů a u některých z nich se stalo primární léčebnou strategií. K všeobecnému překvapení však bylo zjištěno, že tyto inhibitory cílí na utlumení BCR dráhy fungují komplexnějším mechanismem, než se předpokládalo. Zároveň u pacientů léčených „BCR inhibitory“ vznikají bodové i chromozomální aberace a změny ve fyziologii maligních B buněk, které jim poskytují rezistenci k této léčbě. Nejdéle popsanými aberacemi je mutace PLCγ obcházející BCR komplex a mutace BTK bránící kovalentní vazbě ibrutinibu, ale stále jsou popisovány další mutace zodpovědné za rezistenci. Navíc relativní rezistence k léčbě BCR inhibitory může nastat i negenetickým adaptivním mechanismem spočívajícím v aktivaci alternativních drah a „kompenzaci“ inhibice kináz. Příkladem těchto kompenzačních mechanismů je aktivace PI3K-Akt a alternativní NFκB dráhy, či zvýšení hladiny BCL2, které dovolují přežítí buněk s inhibovanou BTK. Zde se tak nabízí potenciálně účinné terapeutické kombinace BTK/PI3K inhibitorů s dalšími cílenými inhibitory pro klinické testování. Alternativně lze využít léčiva, která by napodobovala klinický efekt BTK/PI3K inhibice například tím, že brání adhezi či migraci maligních B buněk (inhibice chemokinové dráhy či integrinů) či blokují odpověď na podpůrné signály od T lymfocytů v mikroprostředí jako je IL4 (inhibitory STAT signalizace).

KLÍČOVÁ SLOVA

CLL – BCR – inhibice – ibrutinib – idelalisib – rezistence – mikroprostředí

SUMMARY

Košťálová L., Šeda V., Mráz M.

Complex mechanisms of action of „BCR signalling“ inhibitors and development of resistance to this targeted therapy in chronic lymphocytic leukaemia

The emergence of the BTK inhibitor ibrutinib and PI3K inhibitor idelalisib represented a revolution in the therapy of B cell malignancies. In some of these malignancies, they became the primary therapeutic strategy. However „BCR inhibitors“ function by a more complex mechanism than anticipated. The evolution of point mutations, chromosomal aberrations and changes in the physiology of malignant B cells leads to resistance in some patients treated with „BCR inhibitors“. The most commonly described aberration is the mutation in PLCγ bypassing BCR signalosome or the mutation in BTK preventing the covalent binding of ibrutinib. However, additional mutations leading to resistance are still being described. Furthermore, relative resistance to „BCR inhibitors“ can be caused by non-genetic adaptive mechanisms leading to activation of alternative pathways and „compensation“ of kinase inhibition. For instance, PI3K-Akt and NFκB activation or BCL2 upregulation lead to B cell survival even after BTK inhibition. This suggests some potentially effective therapeutic combinations of BTK/PI3K inhibitors together with other targeted inhibitors for clinical trials. Alternatively, drugs mimicking the BTK/PI3K inhibition effect can be used to prevent adhesion and migration of malignant B cells (chemokine and integrin pathway inhibition) or to block the pro-proliferative signals from the microenvironment such as IL4 (STAT inhibitors).

KEY WORDS

CLL – BCR – inhibition – ibrutinib – idelalisib – resistance – microenvironment

ÚVOD

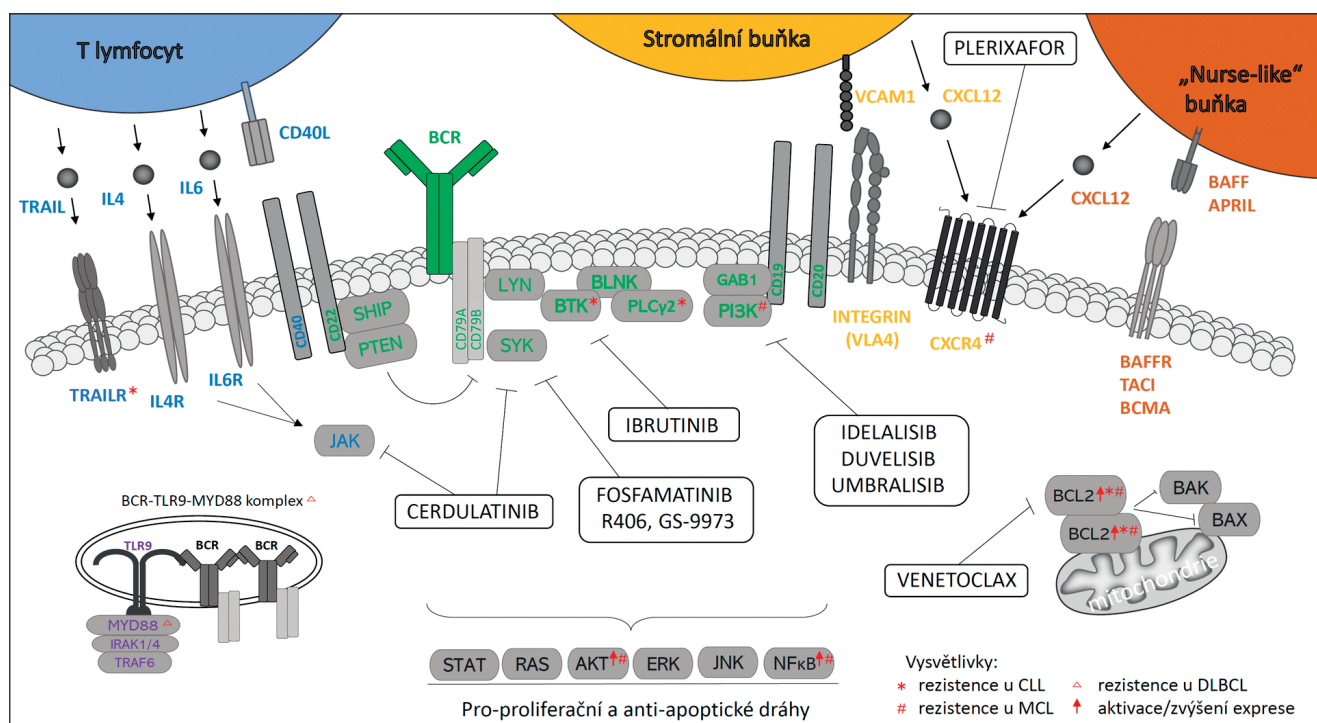
V posledních pěti letech byla do léčby B buněčných malignit vznikajících z maturovaných B lymfocytů zavedena léčiva cílicí na BCR-asociované kinázy. Prohlubováním poznatků o patogenezi těchto onemocnění se ukázalo, že signalizace z B-buněčného receptoru (BCR) úzce souvisí se vznikem a progresí onemocnění, jako je chronická lymfocytární leukemie (CLL), lymfom z plášťových buněk (MCL), difuzní velkobuněčný B lymfom (DLBCL), folikulární lymfom (FL) a Waldenströмова makroglobulinemie. Zavedení inhibitoru Brutonovy tyrosinkinázy (BTK), ibrutinibu a inhibitoru fosfatidylinositol 3-kinázy (PI3K) idelalisibu představovalo revoluci v terapii B buněčných malignit a u některých z nich se stalo standardní léčbou první linie, resp. relapsu choroby [1, 2]. Ke všeobecnému překvapení však bylo zjištěno, že „BCR inhibitory“ fungují komplexnějším mechanismem, než se iniciálně předpokládalo, který zahrnuje účinky na jiné dráhy než BCR. Zároveň také část pacientů na léčbu nereaguje nebo u nich dochází k vzniku rezistence. V následujícím textu se věnujeme popisu molekulárního mechanismu účinku „BCR inhibitorů“ se zaměřením na CLL a komplexním mechanismům

adaptace a rezistence maligních B buněk na tuto léčbu. Pro poskytnutí účinnější terapie je nutné bližší porozumění těmto kompenzačním mechanismům a návrh nových terapeutických kombinací tak, aby bylo možné u pacientů např. s CLL dosáhnout trvalé kontroly choroby, či případně její úplné eliminace pomocí režimů neobsahujících chemoterapii.

Komplexní provázanost BCR signalizace a interakcí v mikroprostředí u CLL

Pro vývoj, zrání a funkci B lymfocytů je důležitá správná funkce B buněčného receptoru (BCR), který je fyziologicky aktivován vazbou antigenu. Vazba antigenu generuje signál, který se kaskádou reakcí dostane až do jádra buňky, kde vyvolá produkci proteinů zodpovědných za přežívání, či případně podporuje klonální expanzi dané buňky. Do této reakce je zahrnut kromě transmembránového imunoglobulinu i systém proteinkináz, adaptorových molekul, ale též fosfatáz představujících negativní regulátory aktivace (obr. 1).

Transmembránový BCR na povrchu B lymfocytů je složen podobně jako jiné imunoglobuliny ze dvou těžkých a dvou lehkých imunoglobulinových řetězců. Imunoglobulinové komponenty BCR postrádají kiná-



Obr. 1. Schematické znázornění interakcí v mikroprostředí maligních B buněk a léčiv cílicích na tyto dráhy. Červené symboly (horní index) označují proteiny, které jsou u jednotlivých chorob v případě rezistence na BCR inhibitor ibrutinib mutovány nebo kompenzačně aktivovány (označeny ↑).
 *rezistence u CLL, #rezistence u MCL, Δrezistence u DLBCL, ↑aktivace/zvýšení exprese

zovou aktivitu a přenos signálu hlouběji do buňky je tak zajištěn jejich vazbou na dva heterodimery Igα a Igβ (CD79a/b), jejichž součástí jsou funkčně důležité tyroziny (ITAM). Při vazbě antigenu na BCR dochází k fosforylaci těchto tyrozinů pomocí kinázy LYN, což následně umožní aktivaci kinázy SYK (obr. 1). Za zmínku jistě stojí, že pro správnou aktivaci SYK je potřeba fosforylace jak Igα, tak i Igβ. Absence, případně zamezení fosforylace jedné z těchto molekul, vede k neschopnosti buněk navázat a fosforylovat SYK [3]. V další fázi signální dráhy je pomocí SYK fosforylována adaptorová molekula BLNK, která je důležitá pro spojení BCR s Brutonovou tyrozinokinázou a dalšími molekulami (viz obr. 1). Brutonova tyrozinokináza (BTK) je klíčovou molekulou signalizace a její defekty vedou k nesprávnému vývoji B lymfocytů a imunodeficitu (imunodeficit vázaný na chromozom X) [3]. Dalšími zúčastněnými kinázami jsou fosfatidylinositol 3-kináza (PI3K) a fosfolipáza C-γ (PLCγ), spouštějící signalizaci tzv. sekundárních posílů (inositol trifosfát, diacylglycerol a ionty Ca²⁺), které signál amplifikují a přenášejí ho z cytoplazmatické membrány k transkripčním faktorům v jádře. Společně s aktivací těchto signálních mechanismů dochází fyziologicky k aktivaci drah zodpovědných za utlumení právě probíhající signalizace. Kinázou zodpovědnou za tuto negativní regulaci je opět kináza LYN, která tak v biologii B lymfocytů zaujímá jedinečnou dvojí roli. Hlavním mechanismem jejího účinku je fosforylace molekul FCγRIIB a CD22 s následnou aktivací fosfatáz (např. SHIP1 či PTEN) inhibujících BCR dráhu (viz obr. 1).

Aktivita BCR dráhy se u jednotlivých CLL pacientů liší, obecně však platí, že schopnost snazší a silnější aktivace BCR signalizace je spojena s horší prognózou [4, 5]. Tyto poznatky přispěly ke snahám o identifikaci mutací způsobujících konstitutivní aktivaci BCR signalizace. Přestože jsou podobné aktivační mutace dobře popsány u B lymfomů (DLBCL a FL), a to s frekvencí až 30 %, u chronické lymfocytární leukemie se takovéto mutace nevyskytují s výjimkou rezistence na „BCR inhibitory“ [6, 7]. Historicky je však dobře známo, že důležitým prognostickým markerem u CLL je samotná struktura BCR, a to jak na úrovni využití specifických variabilních segmentů pro lehké a těžké řetězce imunoglobulinu, tak přítomnost tzv. somatických hypermutací v těžkém řetězci pro imunoglobulinu v oblasti determinující vazbu antigenu, tzv. CDR3 oblast [8, 9, 10]. U pacientů s těžkým řetězcem imunoglobulinu (IGHV), kde nedošlo k jeho somatickým hypermutacím, nacházíme typicky výraznější aktivaci BCR dráhy.

U části pacientů je v CLL buňkách exprimována také kináza ZAP-70, která je T-lymfocytární obdobou

SYK s funkcí v aktivaci signalizace od T buněčného receptoru. U CLL je její exprese častější v případě nemutovaného IGHV a je spojena s horší prognózou. Ukazuje se, že tato kináza obecně napomáhá v CLL buňkách silnější aktivaci BCR signalizace [11, 12], ale také podporuje adhezi CLL buněk ke stromálním buňkám a může modulovat i jejich odpověď na cytokiny [13]. Komplexní mechanismus působení ZAP-70 u CLL je stále předmětem zájmu, nicméně již bylo popsáno, že jeho fosforylovaná forma asociuje s CD79b, což vyvolává zvýšení aktivace SYK, a tím i posílení BCR signalizace [11].

Aktivace BCR signalizace reprezentuje důležitý pro-proliferální a anti-apoptický signál, ale sama o sobě není u většiny CLL buněk dostatečná k spuštění proliferace. CLL buňky zůstávají (podobně jako normální B lymfocyty) závislé na kostimulačních a podpůrných signálech poskytovaných mikroprostředím imunitních orgánů (především lymfatických uzlin), kde oproti periferní krvi CLL buňky prokazatelně aktivně proliferují [14, 15]. Mezi základní složky mikroprostředí jsou řazeny především Th lymfocyty [16], dendritické, „nurse-like“ buňky [17, 18] a stromální buňky mezenchymálního původu (viz obr. 1). Tyto buňky produkují solubilní či membránově vázané proteiny, které u zdravých B lymfocytů indukují proliferaci a procesy spjaté s reakcí na antigen, kdežto u maligních B buněk podporují především přežívání a expanzi. Esenciální je pro CLL buňky interakce s Th lymfocyty, které poskytují maligním B lymfocytům stimulaci prostřednictvím produkce IL4 a CD40 ligandu. Sekrece IL4 vede u CLL buněk jednak k nárůstu produkce anti-apoptického proteinu BCL2, jednak ke zvýšení produkce BCR a s tím spjaté vnitrobuněčné signalizace [19]. Vazba B lymfocytu na CD40 ligand silně podporuje proliferaci, což lze napodobit *in vitro* přidáním CD40L k CLL buňkám. Analogické procesy probíhají také u příbuzných malignit, jako je DLBCL, FL či MCL [6].

Další důležitou komponentou mikroprostředí jsou stromální buňky, které jsou zdrojem několika chemokínů a také produkují základní komponenty extracelulární matrix. Adhezi CLL buněk k extracelulární matrix a buňkám zajišťují integriny a tato adheze je zásadním způsobem potencována BCR signalizací [3]. Terapeutická inhibice BCR signalizace tak přispívá k vzniku lymfocytózy a zmenšení lymfatických uzlin u pacientů právě inhibicí schopnosti CLL buněk adherovat. Pro CLL je pravděpodobně nejvýznamnějším integrinem VLA4 (CD49d) zprostředkovávající interakci se stromálními buňkami vazbou na VCAM1 a k extracelulární matrix vazbou na fibronectin (viz obr. 1). VLA4 je důležitým prognostickým markerem a jeho

vyšší hladiny jsou asociované s horší prognózou CLL [20]. Tento integrin hraje také klíčovou úlohu v tzv. adhezí zprostředkované rezistenci na chemo-imunoterapii [21]. Mezi molekuly produkované stromálními buňkami a dalším podpůrným typem buněk tzv. „nurse-like cells“ patří také cytokiny BAFF a APRIL (viz obr. 1). Navázáním těchto ligandů z rodiny tumor nekrotizujícího faktoru (TNF) dochází k aktivaci NFκB dráhy a ochraně před spontánní i indukovanou apoptózou. Ibrutinib a idelalisib minimálně částečně inhibují signální dráhy adheze, CD40L, IL4, BAFF, APRIL a tyto jsou zároveň možným terapeutickým cílem nezávisle nebo společně s inhibicí BCR signalizace.

Velmi zajímavou vlastností CLL buněk je jejich opakující se re-cirkulace mezi periferní krví a lymfatickými orgány [22]. V krevním řečišti dochází u CLL buněk ke zvýšení exprese membránového receptoru CXCR4. Ten následně CLL buňky „navádí“ ke svému ligandu, kterým je SDF1 produkovaný myeloidními či stromálními buňkami v kostní dřeni. Vazba SDF1 (též označený CXCL12) na CXCR4 indukuje mobilizaci vápenatých iontů, aktivuje kinázy PI3K, MAPK a protein STAT3 a napomáhá reorganizaci cytoskeletu, což je základní předpoklad pro migraci [23]. Po vstupu buňky do mikroprostředí dochází v důsledku interakcí ke snížení CXCR4, což umožní buňce opustit uzlinu/kostní dřeň. Takováto CLL buňka pak vykazuje charakteristický imunofenotyp CXCR4dim/CD5bright, což je možné využít pro rozlišení buněk, které právě vycestovaly z mikroprostředí [22]. Dalším receptorem, který pomáhá B lymfocytům vcestovat do mikroprostředí je CXCR5 a CXCR7 [24, 25]. Bylo ukázáno, že BTK se účastní nejen signalizace vedoucí z BCR, ale také signalizace z chemokinových receptorů. Použití ibrutinibu, jakožto látky inhibující BTK, tak interferuje nejen s aktivitou BCR, ale také s migrací CLL buněk a jejich retencí v uzlinách.

Principy terapeutického účinku „BCR inhibitorů“ a rezistence

Propojení signálních drah vedoucích z BCR, cytokinových receptorů a adheze je klíčové pro pochopení mechanismu účinku klinicky podávaných „inhibitorů BCR signalizace“. Přestože léčba ibrutinibem vede ve většině případů CLL iniciálně k dobré klinické odpovědi, tak postupně dochází u některých pacientů k relapsu a nežádka i k Richterově transformaci. Selhání terapie bylo později dáno do souvislosti se vznikem mutací v BTK, čímž se výrazně sníží afinita ibrutinibu. Nejčastěji se jedná o substituci C481S, ale detekovány byly i varianty C481F, C481R, C481Y nebo T474I a T474S (26). Vlivem toho dochází k „prostu“ signálů z BCR a přežití maligních B buněk [27]. Mutace C481S sama o sobě stačí

k získání relativní rezistence, ale pravděpodobně ne úplné rezistence. Vznik úplné rezistence je dáván do souvislosti s mutací v genu pro fosfolipázu C-γ (PLCγ2; především R665W, S707Y a L845F) [28]. Mutace PLCγ v pozici 665 byly detekovány u ibrutinib rezistentních pacientů samostatně nebo společně s mutacemi v BTK [29]. Funkčním důsledkem této mutace je konformační změna PLCγ, a s tím související aktivace B lymfocytů, která je již nezávislá na BTK [27].

Nejen bodové mutace, ale i chromozomové změny přispívají u CLL pacientů k rezistenci. Delece krátkého raménka chromozomu 8 s lokusem kódujícím receptor pro TRAIL způsobuje rezistenci k TRAIL indukované apoptóze [30]. Naopak získ genetického materiálu na krátkém raménku chromozomu 2 (v lokusu 2p16.1-15) vede ke zvýšené expresi XPO1, který reguluje transport nádorových supresorů p53 a FOXO1 mezi buněčným jádrem a cytoplazmou, což pravděpodobně ovlivňuje přežití maligních B buněk [31,32].

Zajímavé mechanismy rezistence byly popsány i u DLBCL, kde je patogeneze ABC subtypu charakterizována chronickou aktivací BCR signalizace díky mutacím v CD79 [33] a mutacemi v MYD88, který v signální dráze Toll-like receptoru (TLR) reguluje NFκB transkripční faktor. Bylo popsáno, že v případě DLBCL tyto genetické změny specificky regulují odpověď na léčbu BCR-inhibitory. Například buňky ABC-DLBCL nesoucí mutaci v MYD88, ale nikoliv v CD79, jsou rezistentní k ibrutinibu. Kdežto případy s mutací pouze v CD79 nebo s mutací jak v CD79, tak v MYD88 na léčbu reagují. Mechanismus, díky kterému jsou rezistentní buňky s mutací pouze v MYD88, není zatím zcela znám, ale může to souviset s nově objevenou funkcí MYD88 v multi-proteinovém komplexu BCR společně s TLR9-MYD88 [34, 35]. MYD88^{L265P} mutace se nachází i u Waldenströmovy makroglobulinémie (~ 90 % pacientů), ale u těchto pacientů je ibrutinib při léčbě efektivní [36]. Naopak mutace v chemokinovém receptoru CXCR4 vedou k rezistenci na ibrutinib u ~30 % pacientů s Waldenströmovou makroglobulinémií. Zde dochází k narušení internalizace CXCR4 receptoru, nepřetržité aktivaci ERK a AKT kinázy a vysoké životaschopnosti buňky. Signalizaci přes mutovaný CXCR4 receptor je možné inhibovat plerixaforem (CXCR4 inhibitor), který senzitivizuje buňky k ibrutinibu [37].

Kromě mutací v genomu buněk se u CLL a B buněčných malignit uplatňují negenetické mechanismy adaptace či rezistence, spočívající v zapojení alternativních signálních drah a interakcích v mikroprostředí (viz obr. 1). Bylo popsáno, že u MCL buněk rezistentních k ibrutinibu bez mutací v BTK dochází k využití alternativních mechanismů, mezi které patří aktivace

PI3K-AKT molekul a alternativní NFκB signální dráhy. Buněčné linie MCL využívající klasickou NFκB signální dráhu jsou senzitivní k léčbě ibrutinibem, rezistenci naopak vykazují buňky využívající alternativní NFκB dráhu [38]. Obecně je překvapivé, že CLL buňky na inhibici BTK reagují jen velmi pomalou apoptózou projevující se mimo jiné měsíce přetrvávající lymfocytózou u pacientů. Toto by naznačovalo, že buňky využívají kompenzatorní mechanismy podporující jejich přežití, jako je aktivace PI3K/Akt dráhy, a předpokládá se tedy, že kombinace inhibitorů BTK a PI3K-AKT by mohla vykazovat synergický terapeutický efekt.

Terapeutické strategie překonání rezistence na „BCR inhibitory“

Cílená inhibice proteinkináz je efektivní léčebnou strategií, ale podobně jako u chemoimunoterapie část pacientů na léčbu nereaguje nebo u nich postupem času dojde ke vzniku rezistence. Jak bylo popsáno výše, ke vzniku rezistence k ibrutinibu vede nejčastěji mutace C481S v BTK či mutace R665W v PLCγ. Přestože inhibitor BTK nové generace, acalabrutinib, vykazuje vyšší aktivitu a užší specifitu než ibrutinib, jsou pacienti s mutací C481S k této léčbě podobně rezistentní. Důvodem je stejný mechanismus účinku acalabrutinibu, který se podobně jako ibrutinib váže na BTK v místě C481S. Řešením může být vývoj nekovalentních inhibitorů nezávislých na C481S, které dokážou překonat rezistenci a inhibovat BCR signalizaci. Vysoce selektivní nekovalentní inhibitory BMS-986142 a GDC-0853 jsou již v raných fázích klinického testování [39, 40]. Mutace R665W v PLCγ vede ke zvýšené aktivaci této molekuly proteinem Rac2, a zdá se, že aktivace mutované PLCγ stále do určité míry vyžaduje aktivitu kináz LYN a SYK. Jednou z možných strategií překonání rezistence způsobené touto mutací je proto kombinace ibrutinibu s inhibitorem Rac2 (EHT 1864) [28] či inhibice proximálních kináz LYN/SYK inhibi-

tory R406 nebo GS-9973 (inhibice SYK) či dasatinibem (inhibice LYN) [29].

Jak již bylo naznačeno dříve, negenetické mechanismy rezistence nejčastěji zahrnují aberantní aktivaci dalších molekul BCR signální dráhy např. PI3K/AKT. Molekula PI3K se jako součást signálních drah účastní mnoha buněčných procesů a její inhibice je v kombinaci s ibrutinibem potenciálním terapeutickým cílem. V případě idelalisibu a dalších PI3K inhibitorů jako umbralisib je tato léčebná kombinace již v relativně pokročilé fázi klinického testování u MCL a CLL. Navíc bylo zjištěno, že další inhibitory PI3K duvelisib a PR6530 inhibují fosforylaci AKT a přežití maligních B buněk i u pacientů s BTK mutací C481S či kompenzatorní aktivací Akt [41, 42]. Naproti tomu ani idelalisib či duvelisib nedokážou inhibovat signalizaci přes mutovanou PLCγ, a není tak asi zcela vhodné jejich použití u pacientů v případě rezistence na ibrutinib způsobené specificky touto mutací [29]. Zdá se tedy, že je racionální primární kombinace BTK a PI3K inhibitoru nebo aplikace PI3K inhibitoru při vzniku rezistence na ibrutinib díky BTK mutaci. Podobně racionální strategií je pravděpodobně přidání inhibitorů molekul AKT, MEK1/2 nebo MAPK k ibrutinibu (shrnutí v tabulce 1).

Kromě kombinace dvou různých inhibitorů je další možností vývoj jednoho léčiva inhibujícího dva rozdílné cíle. V současnosti tak vznikají molekuly inhibující současně BTK a PI3K [43], případně SYK a JAK-STAT (cerdulatinib). JAK-STAT signální dráha zprostředkovává signalizaci spouštěnou interleukiny IL4 a IL6, které přes JAK kinázy aktivují proteiny STAT3/6 (viz obr. 1). Ty regulují produkci anti-apoptických proteinů MCL1 a BCL_{XL} a buňky po navýšení jejich množství snáze unikají apoptóze [44]. Cerdulatinib byl popsán jako potentní inhibitor blokující proliferaci primárních CLL buněk a oproti ibrutinibu je schopný snáze indukovat apoptózu blokací signálů pro přežití vyvolaných IL4, anti-IgM (aktivuje BCR) a CD40L [45]. Význam JAK-STAT

Tab. 1. Shrnutí možných strategií překonání rezistence/adaptace při léčbě „BCR inhibitory“

Příčina rezistence/adaptace	Možné strategie překonání rezistence
Mutace v BTK	nekovalentní inhibitory BTK (GDC-0853, BMS-986142)
	inhibitory LYN (dasatinib) a SYK (R406, GS-9973)
Mutace v PLCγ	inhibitor Rac2 (EHT 1864), inhibitory LYN a SYK
Aktivace AKT/PI3K dráhy	kombinace ibrutinibu a PI3K inhibitoru (idelalisib, duvelisib, umbralisib)
Aktivace JAK-STAT signalizace	cerdulatinib, ruxolitinib
Zvýšená exprese BCL2	venetoclax
Chemokinová signalizace přes CXCR4	plerixafor

signální dráhy pro přežívání buněk naznačuje také klinická studie kombinující ibrutinib s inhibítor JAK ruxolitinibem [46].

Jedním ze slibných přístupů je také inhibice anti-apoptických molekul z rodiny BCL2, jejichž součástí jsou pro-apoptické proteiny BAX, BAK, BAD, BIM a PUMA a anti-apoptické proteiny BCL2, BCL_{XL}, BCL_L, MCL1 a BFL1. Bylo popsáno, že u CLL a MCL pacientů léčených ibrutinibem dochází k mírnému zvýšení exprese BCL2 a naopak poklesu MCL1 [22]. Není zcela jasné, jakým mechanismem k tomu dochází a zda se jedná o selekci buněk rezistentnějších k buněčné smrti či o adaptační mechanismus. Důsledkem snížení exprese MCL1 je zvýšení „závislosti“ CLL buněk na expresi BCL2, neboť tyto dvě antiapoptické molekuly hrají u této choroby klíčovou roli. Venetoclax je malou molekulou inhibující antiapoptický protein BCL2, jehož úkolem je sekvestrace proteinů BAX a BAK (viz obr. 1). Uvolnění BAX a BAK proteiny způsobují apoptózu buněk permeabilizací mitochondriální membrány. Venetoclax vykazuje skvělý klinický efekt při podání samostatně či v kombinaci s monoklonálními protilátkami proti CD20 a uvedená pozorování naznačují, že jeho kombinace s ibrutinibem by mohla být z biologického pohledu přirozeně synergická. Je třeba připomenout, že kombinace BTK inhibice s rituximabem nepřináší klinický benefit, neboť inhibice BTK vede k snížení hladin CD20 [22].

Jak již bylo zmíněno, migrace CLL buněk do mikroprostředí je důležitá pro přežívání nádorových buněk. Cílená léčba se tedy zaměřuje i na chemokinové signální dráhy, které řídí tento proces. Antagonisté CXCR4 původně vyvinutí pro léčbu HIV, slouží CXCR4 viru jako koreceptor při vstupu do T lymfocytů. Nicméně záhy se ukázalo, že antagonist CXCR4 plerixafor nebo analog T140 významně narušují migraci a adhezi CLL buněk k stromálním buňkám [23] a u CLL pacientů vedou k vypavení CLL buněk z lymfatických tkání do krve, kde jsou přístupnější konvenčním léčivům (monoklonální protilátka, chemoterapie).

V neposlední řadě se kromě signálních drah zaměřuje pozornost i na inhibici ostatních buněčných procesů asociovaných s BCR. Jednou z možných strategií překonání rezistence je inhibice autofagie či chaperonu Hsp90, který stabilizuje proteiny, brání ubikvitinaci a proteosomální degradaci [47]. Inhibice Hsp90 vede k degradaci BTK a IκB kinázy a inhibuje MAPK a alternativní NFκB signální dráhu [48]. Navíc Hsp90 inhibitor AUY922 dokáže obejít rezistenci k ibrutinibu u MCL. Podobně selinexor, inhibitor jaderného přenašeče Exportinu-1 (XPO1), snižuje aktivaci AKT, ERK a potlačuje expresi genu pro BTK, což vykazuje efektivitu i u rezistentních buněk s BTK mutací C481S

[49, 50]. Výzkum těchto inhibitorů cílící na kompenzační mechanismy buněk je nicméně v raných stádiích klinického testování a bude nutné bližší porozumění alternativním mechanismům rezistence maligních B buněk.

ZÁVĚR

Cílená terapeutická inhibice BCR signální dráhy je klíčová pro léčbu pacientů s B buněčnými malignitami pocházejícími z maturovaných B lymfocytů. Prvními zavedenými a relativně velmi úspěšnými „BCR inhibitory“ jsou léčiva cílící na kinázy BTK a PI3K. Pro část pacientů však není léčba BCR inhibitory účinná a/nebo u nich dochází ke vzniku rezistence vznikem bodových i chromozomových aberací a změnami ve fyziologii maligních B buněk. Nejdéle popsanou aberací způsobující rezistenci na ibrutinib je mutace PLCγ obcházející BCR komplex či mutace BTK bránící kovalentní vazbě léčiva. Zároveň je třeba říci, že velmi málo rozumíme mechanismům rezistence na PI3K inhibitory. Je zajímavé, že relativní rezistence k léčbě inhibitory může nastat i negenetickým adaptivním mechanismem díky aktivaci alternativních drah signalizace a „kompenzací“ inhibice kináz (shrnutí v tabulce 1). Příkladem těchto kompenzačních mechanismů je aktivace PI3K-AKT a alternativní NFκB dráhy, které obchází inhibovanou BTK a působí anti-apopticky. Zde se nabízí terapeutický potenciál kombinace inhibice PI3K a BTK, což dobře ilustruje existence téměř desítky probíhajících klinických studií cílených na souběžnou inhibici BTK ibrutinibem a PI3K idelalisibem a několika dalších studií využívajících jiné PI3K inhibitory.

Vzhledem ke zvyšujícímu se počtu pacientů se získanou rezistencí u CLL či primární rezistencí u B lymfomů jsou v současnosti vyvíjeny nové léčebné strategie. Zejména se jedná o testování dalších inhibitorů molekul BCR signalizace, jako jsou LYN, SYK, PI3K (jiné než delta podjednotky), AKT, inhibitorů chemokिनových receptorů a anti-apoptických proteinů. Možným přístupem je také použití multikinázových inhibitorů (např. SYK + STAT). V budoucnu lze, doufejme, očekávat, že kombinace BTK inhibitoru s jedním až dvěma dalšími inhibitory povede ke kombinacím, které budou schopny alespoň u některých CLL pacientů dosáhnout hluboké molekulární odpovědi bez nutnosti aplikace chemoterapie.

LITERATURA

1. Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med* 2013;369(1):32–42.

2. Furman RR, Sharman JP, Coutre SE, et al. Idelalisib and rituximab in relapsed chronic lymphocytic leukemia. *N Engl J Med* 2014;370(11):997–1007.
3. Seda V, Mraz M. B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells. *Eur J Haematol* 2015;94(3):193–205.
4. D'Avola A, Drennan S, Tracy I, et al. Surface IgM expression and function are associated with clinical behavior, genetic abnormalities, and DNA methylation in CLL. *Blood* 2016;128(6):816–826.
5. Mraz M, Chen L, Rassenti LZ, et al. miR-150 influences B-cell receptor signaling in chronic lymphocytic leukemia by regulating expression of GAB1 and FOXP1. *Blood* 2014;124(1):84–95.
6. Devan J, Janikova A, Mraz M. New concepts in follicular lymphoma biology: From BCL2 to epigenetic regulators and non-coding RNAs. *Semin Oncol* 2018;45(5-6):291–302.
7. Mraz M, Trbusek M, Dolezalova D, et al. Identifikace patogeneticky významných mutací u chronické lymfocytární leukemie pomocí „sekvenování nové generace“. *Transfúze Hematologie dnes* 2012;18(2):72–75.
8. Stamatopoulos B, Smith T, Crompot E, et al. The light chain IgLV3-21 defines a new poor prognostic subgroup in chronic lymphocytic leukemia: results of a multicenter study. *Clin Cancer Res* 2018;24(20):5048–5057.
9. Stamatopoulos K, Agathangelidis A, Rosenquist R, Ghia P. Antigen receptor stereotypy in chronic lymphocytic leukemia. *Leukemia* 2017;31(2):282–291.
10. Mráz M, Pavlová Š, Malčíková J, Malinová K, Mayer J, Pospíšilová, Š. Molekulární patogeneze chronické lymfocytární leukemie. *Transfúze Hematol dnes* 2010;16(1):16–20.
11. Chen L, Apgar J, Huynh L, et al. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. *Blood* 2005;105(5):2036–2041.
12. Rassenti LZ, Huynh L, Toy TL, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med* 2004;351(9):893–901.
13. Lafarge ST, Li H, Pauls SD, et al. ZAP70 expression directly promotes chronic lymphocytic leukaemia cell adhesion to bone marrow stromal cells. *Br J Haematol* 2015;168(1):139–142.
14. Pavlasova G, Borsky M, Svobodova V, et al. Rituximab primarily targets an intra-clonal BCR signaling proficient CLL subpopulation characterized by high CD20 levels. *Leukemia* 2018;32(9):2028–2031.
15. Herishanu Y, Pérez-Galán P, Liu D, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood* 2011;117(2):563–574.
16. Kneitz C, Goller M, Wilhelm M, et al. Inhibition of T cell/B cell interaction by B-CLL cells. *Leukemia* 1999;13(1):98–104.
17. Ten Hacken E, Burger JA. Microenvironment interactions and B-cell receptor signaling in chronic lymphocytic leukemia: implications for disease pathogenesis and treatment. *Biochim Biophys Acta* 2016;1863(3):401–413.
18. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood* 2000;96(8):2655–2663.
19. Aguilar-Hernandez MM, Blunt MD, Dobson R, et al. IL-4 enhances expression and function of surface IgM in CLL cells. *Blood* 2016;127(24):3015–3025.
20. Shanafelt TD, Geyer SM, Bone ND, et al. CD49d expression is an independent predictor of overall survival in patients with chronic lymphocytic leukaemia: a prognostic parameter with therapeutic potential. *Br J Haematol* 2008;140(5):537–546.
21. Mraz M, Zent CS, Church AK, et al. Bone marrow stromal cells protect lymphoma B-cells from rituximab-induced apoptosis and targeting integrin α - β -1 (VLA-4) with natalizumab can overcome this resistance. *Br J Haematol* 2011;155(1):53–64.
22. Pavlasova G, Borsky M, Seda V, et al. Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis. *Blood* 2016;128(12):1609–1613.
23. Burger M, Hartmann T, Krome M, et al. Small peptide inhibitors of the CXCR4 chemokine receptor (CD184) antagonize the activation, migration, and antiapoptotic responses of CXCL12 in chronic lymphocytic leukemia B cells. *Blood* 2005;106(5):1824–1830.
24. Bürkle A, Niedermeier M, Schmitt-Gräff A, Wierda WG, Keating MJ, Burger JA. Overexpression of the CXCR5 chemokine receptor, and its ligand, CXCL13 in B-cell chronic lymphocytic leukemia. *Blood* 2007;110(9):3316–3325.
25. Till KJ, Lin K, Zuzel M, Cawley JC. The chemokine receptor CCR7 and α 4 integrin are important for migration of chronic lymphocytic leukemia cells into lymph nodes. *Blood* 2002;99(8):2977–2984.
26. Maddocks KJ, Ruppert AS, Lozanski G, et al. Etiology of ibrutinib therapy discontinuation and outcomes in patients with chronic lymphocytic leukemia. *JAMA Oncol* 2015;1(1):80–87.
27. Woyach JA, Furman RR, Liu TM, et al. Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. *N Engl J Med* 2014;370(24):2286–2294.
28. Walliser C, Hermkes E, Schade A, et al. The Phospholipase $\text{C}\gamma$ 2 mutants R665W and L845F identified in ibrutinib-resistant chronic lymphocytic leukemia patients are hypersensitive to the Rho GTPase Rac2 protein. *J Biol Chem* 2016;291(42):22136–22148.
29. Liu TM, Woyach JA, Zhong Y, et al. Hypermorphic mutation of phospholipase C, γ 2 acquired in ibrutinib-resistant CLL confers BTK independency upon B-cell receptor activation. *Blood* 2015;126(1):61–68.
30. Rubio-Moscardo F, Blesa D, Mestre C, et al. Characterization of 8p21.3 chromosomal deletions in B-cell lymphoma: TRAIL-R1 and TRAIL-R2 as candidate dosage-dependent tumor suppressor genes. *Blood* 2005;106(9):3214–3222.
31. Cosson A, Chapiro E, Bougacha N, et al. Gain in the short arm of chromosome 2 (2p+) induces gene overexpression and drug resistance in chronic lymphocytic leukemia: analysis of the central role of XPO1. *Leukemia* 2017;31(7):1625–1629.
32. Burger JA, Landau DA, Taylor-Weiner A, et al. Clonal evolution in patients with chronic lymphocytic leukaemia developing resistance to BTK inhibition. *Nat Commun* 2016;7:11589.

33. Davis RE, Ngo VN, Lenz G, et al. Chronic active B-cell-receptor signaling in diffuse large B-cell lymphoma. *Nature* 2010;463(7277):88–92.
34. Wilson WH, Gerecitano JF, Goy A, et al. The Brutons tyrosine kinase (BTK) inhibitor, ibrutinib (PCI-32765), has preferential activity in the ABC subtype of relapsed/refractory de novo diffuse large B-cell lymphoma (DLBCL): interim results of a multicenter, open-label, phase 2 study. *Blood* 2012;120 (21): 686 LP-686.
35. Phelan JD, Young RM, Webster DE, et al. A multiprotein supercomplex controlling oncogenic signalling in lymphoma. *Nature* 2018;560(7718):387–391.
36. Yang G, Zhou Y, Liu X, et al. A mutation in MYD88 (L265P) supports the survival of lymphoplasmacytic cells by activation of Bruton tyrosine kinase in Waldenström macroglobulinemia. *Blood* 2013;122(7):1222–1232.
37. Cao Y, Hunter ZR, Liu X, et al. The WHIM-like CXCR4(S338X) somatic mutation activates AKT and ERK, and promotes resistance to ibrutinib and other agents used in the treatment of Waldenström's macroglobulinemia. *Leukemia* 2015;29(1):169–176.
38. Rahal R, Frick M, Romero R, et al. Pharmacological and genomic profiling identifies NF- κ B-targeted treatment strategies for mantle cell lymphoma. *Nat Med* 2014;20(1):87–92.
39. Byrd JC, Smith S, Wagner-Johnston N, et al. First-in-human phase 1 study of the BTK inhibitor GDC-0853 in relapsed or refractory B-cell NHL and CLL. *Oncotarget* 2018;9(16):13023–13035.
40. Lee SK, Xing J, Catlett IM, et al. Safety, pharmacokinetics, and pharmacodynamics of BMS-986142, a novel reversible BTK inhibitor, in healthy participants. *Eur J Clin Pharmacol* 2017;73(6):689–698.
41. Dong S, Guinn D, Dubovsky JA, et al. IPI-145 antagonizes intrinsic and extrinsic survival signals in chronic lymphocytic leukemia cells. *Blood* 2014;124(24):3583–3586.
42. Vakkalanka S, Nyayapathy S, Viswanadha S. Addition of RP6530, a dual PI3K delta/gamma inhibitor, overcomes ibrutinib resistance in DLBCL cells in vitro. *Blood* 2014;124(21):4497.
43. Pujala B, Agarwal AK, Middya S, et al. Discovery of pyrazolopyrimidine derivatives as novel dual inhibitors of BTK and PI3K δ . *ACS Med Chem Lett* 2016;7(12):1161–1166.
44. Steele AJ, Prentice AG, Cwynarski K, et al. The JAK3-selective inhibitor PF-956980 reverses the resistance to cytotoxic agents induced by interleukin-4 treatment of chronic lymphocytic leukemia cells: potential for reversal of cytoprotection by the microenvironment. *Blood* 2010;116(22):4569–4577.
45. Guo A, Lu P, Coffey G, Conley P, Pandey A, Wang YL. Dual SYK/JAK inhibition overcomes ibrutinib resistance in chronic lymphocytic leukemia: Cerdulatinib, but not ibrutinib, induces apoptosis of tumor cells protected by the microenvironment. *Oncotarget* 2017;8(8):12953–12967.
46. Spaner DE, McCaw L, Wang G, Tsui H, Shi Y. Persistent janus kinase-signaling in chronic lymphocytic leukemia patients on ibrutinib: Results of a phase I trial. *Cancer Med* 2019;8(4):21540–21550.
47. Zuehlke A, Johnson JL. Hsp90 and co-chaperones twist the functions of diverse client proteins. *Biopolymers* 2010;93(3):211–217.
48. Jacobson C, Kopp N, Laver JV, et al. HSP90 inhibition overcomes ibrutinib resistance in mantle cell lymphoma. *Blood* 2016;128(21):2517–2526.
49. Zhong Y, El-Gamal D, Dubovsky JA, et al. Selinexor suppresses downstream effectors of B-cell activation, proliferation and migration in chronic lymphocytic leukemia cells. *Leukemia* 2014;28(5):1158–1163.
50. Hing ZA, Mantel R, Beckwith KA, et al. Selinexor is effective in acquired resistance to ibrutinib and synergizes with ibrutinib in chronic lymphocytic leukemia. *Blood* 2015;125(20):3128–3132.

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Supplementary material 11

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Title of the Invention	Inhibitors for Treatment of Hematological Malignancies		
Attorney Docket Number	J507-127 US	Small Entity Status Claimed ☒	
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	7	Suggested Figure for Publication (if any)	

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	J507-127 US
		Application Number	
Title of Invention	Inhibitors for Treatment of Hematological Malignancies		

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	☒ Customer Number	☐ US Patent Practitioner	☐ Limited Recognition (37 CFR 11.9)
Customer Number	21706		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status			Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	J507-127 US
		Application Number	
Title of Invention	Inhibitors for Treatment of Hematological Malignancies		

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)ⁱ the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

		Remove	
Application Number	Country ⁱ	Filing Date (YYYY-MM-DD)	Access Code ⁱ (if applicable)
Additional Foreign Priority Data may be generated within this form by selecting the Add button.			

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013. NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.
--

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	J507-127 US
		Application Number	
Title of Invention	Inhibitors for Treatment of Hematological Malignancies		

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	J507-127 US
		Application Number	
Title of Invention	Inhibitors for Treatment of Hematological Malignancies		

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

[Clear](#)

☒ Assignee
 ☐ Legal Representative under 35 U.S.C. 117
 ☐ Joint Inventor

☐ Person to whom the inventor is obligated to assign.
 ☐ Person who shows sufficient proprietary interest

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name

Mailing Address Information For Applicant:

Address 1

Address 2

City State/Province

Country Postal Code

Phone Number Fax Number

Email Address

Additional Applicant Data may be generated within this form by selecting the Add button.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	J507-127 US
		Application Number	
Title of Invention	Inhibitors for Treatment of Hematological Malignancies		

Applicant 2			
If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section. Clear			
☒ Assignee		☐ Legal Representative under 35 U.S.C. 117	
☐ Person to whom the inventor is obligated to assign.		☐ Person who shows sufficient proprietary interest	
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:			
Name of the Deceased or Legally Incapacitated Inventor:			
If the Applicant is an Organization check here. ☒			
Organization Name	Fakultni Nemocnice Brno		
Mailing Address Information For Applicant:			
Address 1	Jihlavská 20		
Address 2			
City	Brno	State/Province	
Country	CZ	Postal Code	62500
Phone Number		Fax Number	
Email Address			
Additional Applicant Data may be generated within this form by selecting the Add button.			

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1
Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.
If the Assignee or Non-Applicant Assignee is an Organization check here. ☒

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	J507-127 US
		Application Number	
Title of Invention	Inhibitors for Treatment of Hematological Malignancies		

Organization Name	Masarykova Univerzita
-------------------	-----------------------

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1		Zerotinovo namesti 617/9	
Address 2			
City	Brno	State/Province	
Country ⁱ	CZ	Postal Code	60177
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

--

Assignee 2

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

--

If the Assignee or Non-Applicant Assignee is an Organization check here.



Organization Name	Fakultni Nemocnice Brno
-------------------	-------------------------

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1		Jihlavská 20	
Address 2			
City	Brno	State/Province	
Country ⁱ	CZ	Postal Code	62500
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	J507-127 US
		Application Number	
Title of Invention	Inhibitors for Treatment of Hematological Malignancies		

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/NADER ABADIR/			Date (YYYY-MM-DD)	2020-07-06
First Name	NADER	Last Name	ABADER	Registration Number	52,537

Additional Signature may be generated within this form by selecting the Add button.

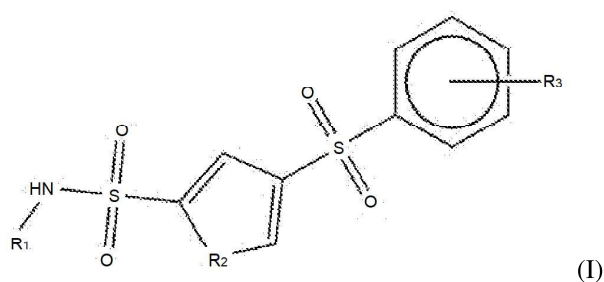
This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

CLAIMS

What is claimed is:

1. A method of treatment of a hematological malignancy, said method comprising the step of administering at least one GAB1 inhibitor to a subject in need of such treatment.
2. The method of claim 1, wherein the hematological malignancy is a myeloid neoplasm.
3. The method of claim 2, wherein the myeloid neoplasm is Myelodysplastic syndrome.
4. The method of claim 2, wherein the myeloid neoplasm is myeloid leukemia.
5. The method of claim 4, wherein the myeloid leukemia is Acute myeloid leukemia.
6. The method of claim 4, wherein the myeloid leukemia is Chronic myeloid leukemia.
7. The method of claim 4, wherein the myeloid leukemia is Monocytic leukemia.
8. The method of claim 4, wherein the myeloid leukemia is Promyelocytic leukemia.
9. The method of claim 1, wherein the hematological malignancy is a lymphoid neoplasm.
10. The method of claim 9, wherein the lymphoid neoplasm is Multiple myeloma.
11. The method of claim 9, wherein the lymphoid neoplasm is a lymphocytic leukemia.
12. The method of claim 11, wherein the lymphocytic leukemia is Chronic lymphocytic leukemia.
13. The method of claim 11, wherein the lymphocytic leukemia is Acute lymphocytic leukemia.
14. The method of claim 11, wherein the lymphocytic leukemia is Small lymphocytic leukemia.
15. The method of claim 9, wherein the lymphoid neoplasm is a lymphoma.
16. The method of claim 15, wherein the lymphoma is a relapsed or refractory lymphoma.

17. The method of claim 15, wherein the lymphoma is non-Hodgkin's lymphoma.
18. The method of claim 15, wherein the lymphoma is Mantle cell lymphoma.
19. The method of claim 15, wherein the lymphoma is Burkitt's lymphoma.
20. The method of claim 15, wherein the lymphoma is Diffuse large B cell lymphoma.
21. The method of claim 15, wherein the lymphoma is Follicular lymphoma.
22. The method of claim 15, wherein the lymphoma is Marginal zone lymphoma.
23. The method of claim 15, wherein the lymphoma is Hodgkin's lymphoma.
24. The method of claim 15, wherein the lymphoma is T cell lymphoma.
25. The method of claim 15, wherein the lymphoma is NK cell lymphoma.
26. The method of claim 15, wherein the lymphoma is Primary thyroid lymphoma.
27. The method of claim 15, wherein the lymphoma is Waldenstrom's macroglobulinemia.
28. The method of claim 1, wherein the GAB1 inhibitor is a compound of formula I:

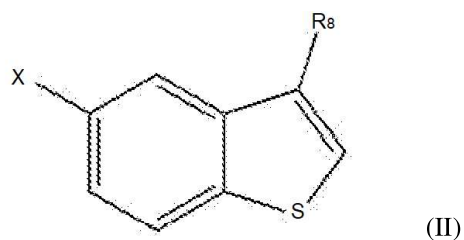


wherein

R2 is an S;

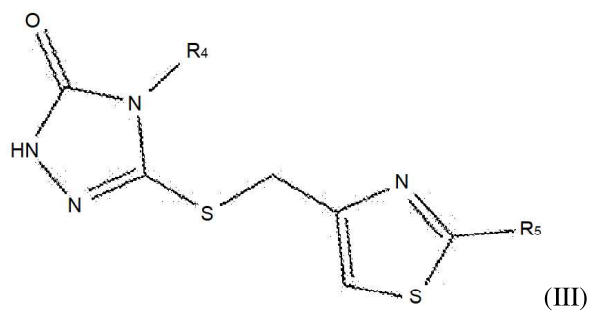
R3 is H, an alkyl, or a halogen; and

R1 is an aromatic heterocycle or formula II



wherein R8 is an alkyl bonded to the NH group in formula I, and X is a halogen.

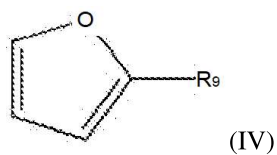
29. The method of claim 1, wherein the GAB1 inhibitor is a compound of formula III:



wherein

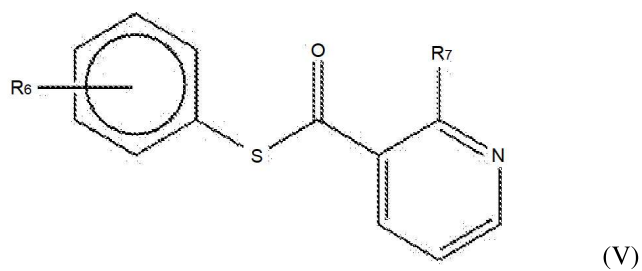
R5 is a benzene, or a (substituted) aryl group, or a (substituted) heteroaryl group; and

R4 is a moiety of formula IV:



wherein R9 is an alkyl bonded to the N group of formula III.

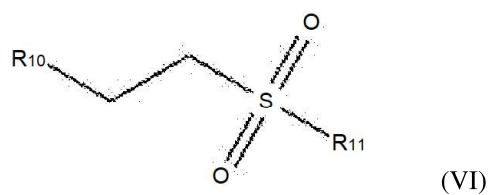
30. The method of claim 1, wherein the GAB1 inhibitor is a compound of formula V:



wherein

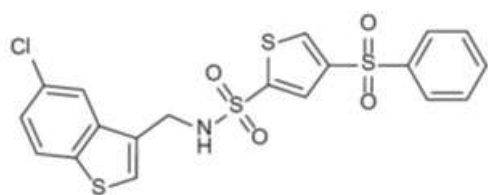
R6 is an H, an alkyl, or a halogen.

R7 is a moiety of formula VI:

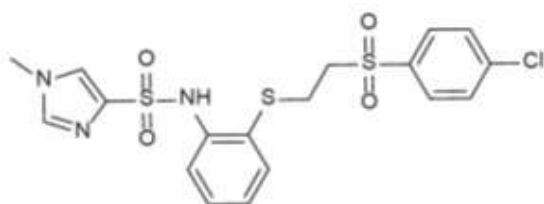


wherein R10 is an S or an alkyl bonded to the N-containing aromatic group of formula V; and R11 is an H or an alkyl.

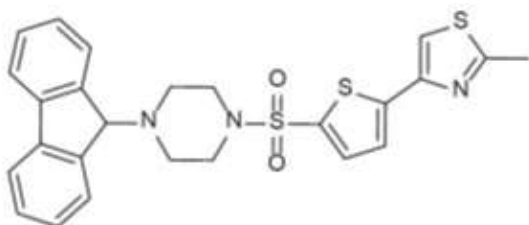
31. The method of claim 1, wherein the GAB1 inhibitor is a compound selected from the group consisting of:



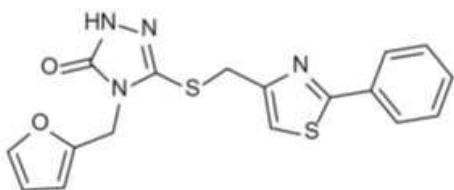
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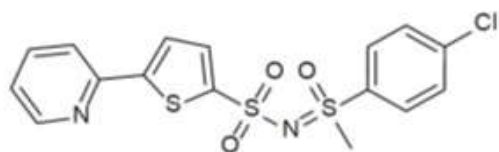
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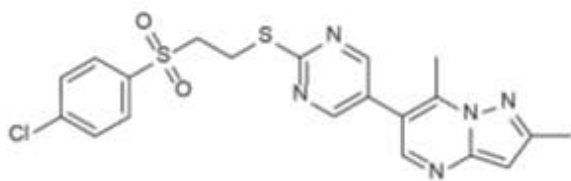
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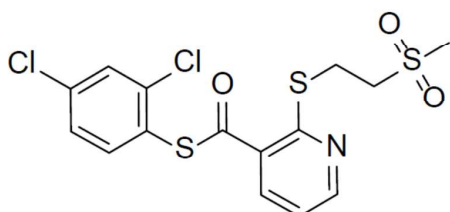
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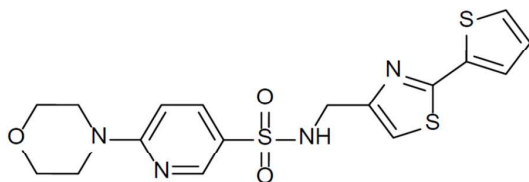
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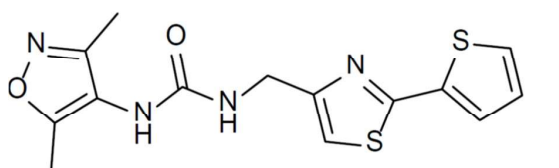
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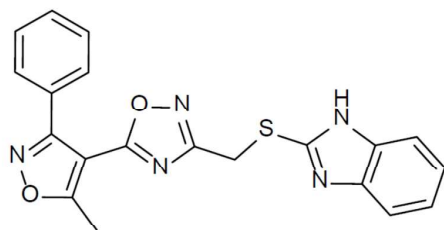
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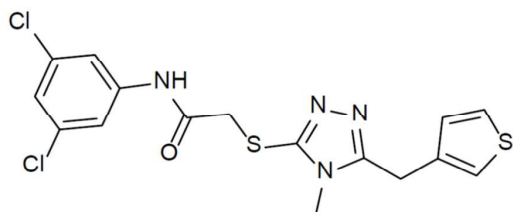
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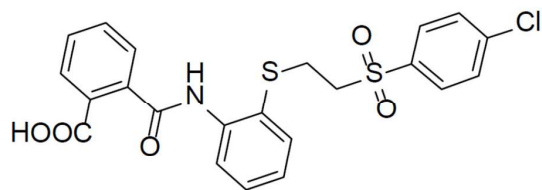
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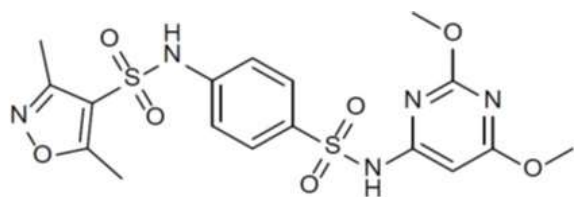
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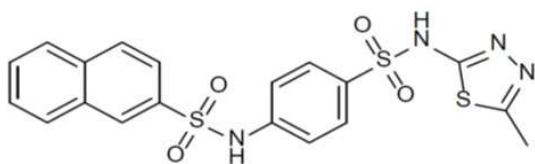
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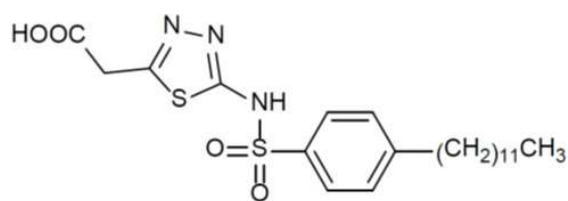
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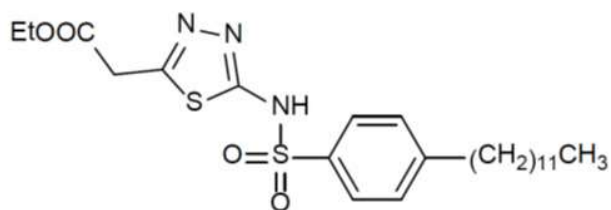
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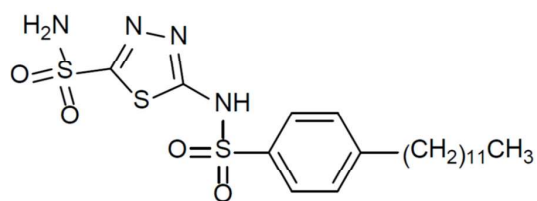
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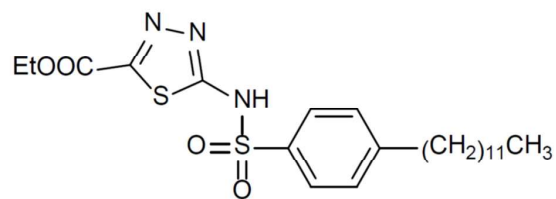
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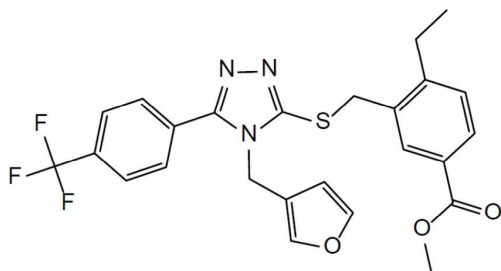
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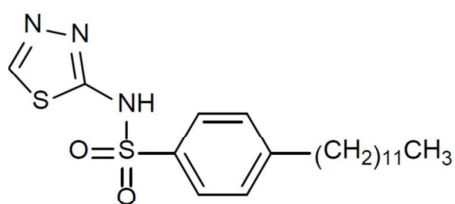
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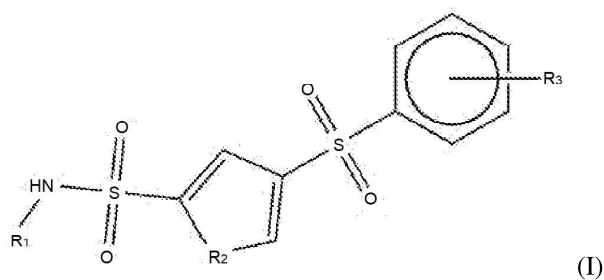


inhGAB1-020

32. A method of treatment of at least one hematological malignancy, said method comprising a step of co-administering at least one GAB1 inhibitor and at least one BTK inhibitor.
33. The method of claim 32, wherein the step of co-administering at least one GAB1 inhibitor and at least one BTK inhibitor is a step of sequentially administering at least one GAB1 inhibitor and at least one BTK inhibitor.
34. The method of claim 32, wherein the step of co-administering at least one GAB1 inhibitor and at least one BTK inhibitor is a step of simultaneously administering at least one GAB1 inhibitor and at least one BTK inhibitor.
35. The method of claim 32, wherein the hematological malignancy is a myeloid neoplasm.
36. The method of claim 35, wherein the myeloid neoplasm is Myelodysplastic syndrome.
37. The method of claim 35, wherein the myeloid neoplasm is myeloid leukemia.
38. The method of claim 37, wherein the myeloid leukemia is Acute myeloid leukemia.
39. The method of claim 37, wherein the myeloid leukemia is Chronic myeloid leukemia.
40. The method of claim 37, wherein the myeloid leukemia is Monocytic leukemia.
41. The method of claim 37, wherein the myeloid leukemia is Promyelocytic leukemia.
42. The method of claim 32, wherein the hematological malignancy is a lymphoid neoplasm.
43. The method of claim 42, wherein the lymphoid neoplasm is Multiple myeloma.
44. The method of claim 42, wherein the lymphoid neoplasm is a lymphocytic leukemia.

45. The method of claim 44, wherein the lymphocytic leukemia is Chronic lymphocytic leukemia.
46. The method of claim 44, wherein the lymphocytic leukemia is Acute lymphocytic leukemia.
47. The method of claim 44, wherein the lymphocytic leukemia is Small lymphocytic leukemia.
48. The method of claim 42, wherein the lymphoid neoplasm is a lymphoma.
49. The method of claim 48, wherein the lymphoma is a relapsed or refractory lymphoma.
50. The method of claim 48, wherein the lymphoma is non-Hodgkin's lymphoma.
51. The method of claim 48, wherein the lymphoma is Mantle cell lymphoma.
52. The method of claim 48, wherein the lymphoma is Burkitt's lymphoma.
53. The method of claim 48, wherein the lymphoma is Diffuse large B cell lymphoma.
54. The method of claim 48, wherein the lymphoma is Follicular lymphoma.
55. The method of claim 48, wherein the lymphoma is Marginal zone lymphoma.
56. The method of claim 48, wherein the lymphoma is Hodgkin's lymphoma.
57. The method of claim 48, wherein the lymphoma is T cell lymphoma.
58. The method of claim 48, wherein the lymphoma is NK cell lymphoma.
59. The method of claim 48, wherein the lymphoma is Primary thyroid lymphoma.
60. The method of claim 48, wherein the lymphoma is Waldenstrom's macroglobulinemia.

61. The method of claim 32, wherein the GAB1 inhibitor is a compound of formula I:

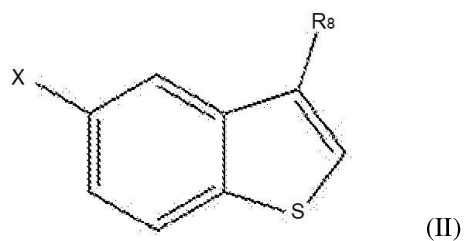


wherein

R2 is an S;

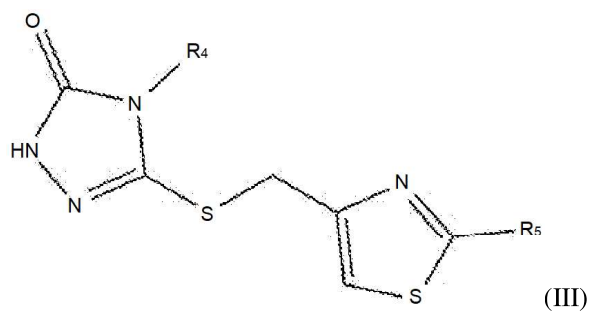
R3 is H, an alkyl, or a halogen; and

R1 is an aromatic heterocycle or formula II



wherein R8 is an alkyl bonded to the NH group in formula I, and X is a halogen.

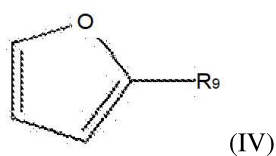
62. The method of claim 32, wherein the GAB1 inhibitor is a compound of formula III:



wherein

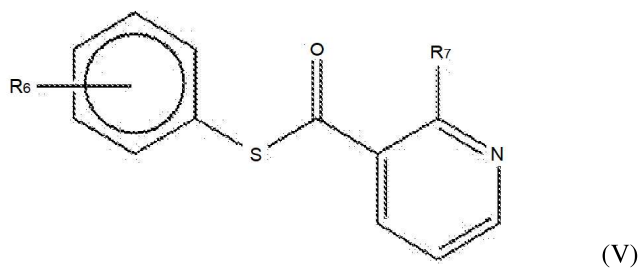
R5 is a benzene, or a (substituted) aryl group, or a (substituted) heteroaryl group; and

R4 is a moiety of formula IV:



wherein R9 is an alkyl bonded to the N group.

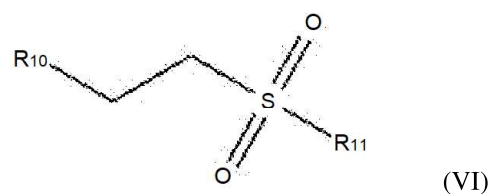
63. The method of claim 32, wherein the GAB1 inhibitor is a compound of formula V:



wherein

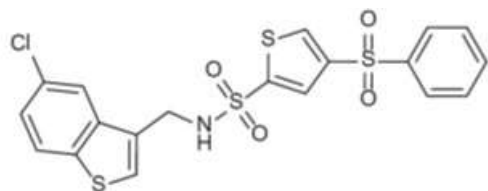
R6 is an H, an alkyl, or a halogen.

R7 is a moiety of formula VI:

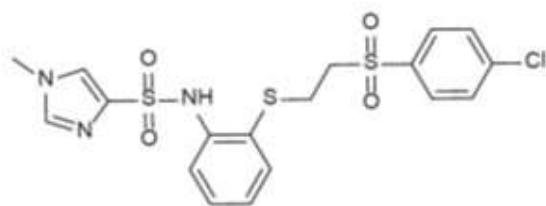


wherein R10 is an S or an alkyl bonded to the N-containing aromatic group of formula V; and R11 is an H or an alkyl.

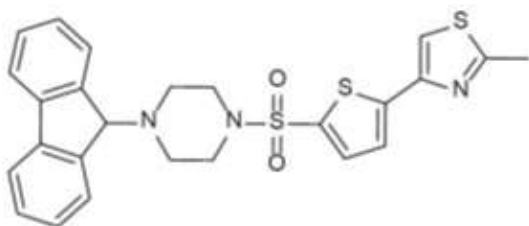
64. The method of claim 32, wherein the GAB1 inhibitor is a compound selected from the group consisting of:



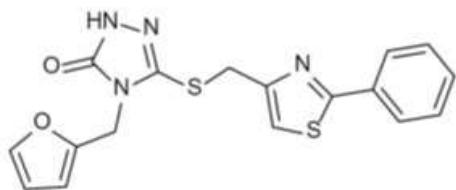
inhGAB1-001



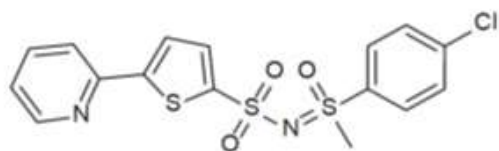
inhGAB1-002



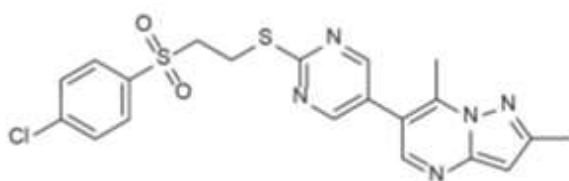
inhGAB1-003



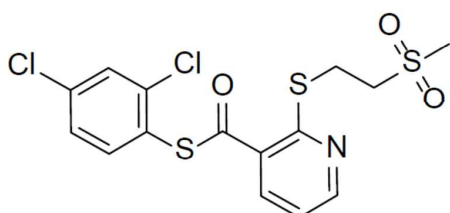
inhGAB1-004



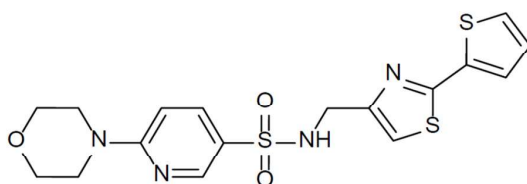
inhGAB1-005



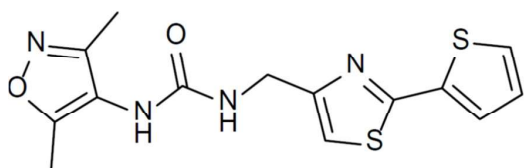
inhGAB1-006



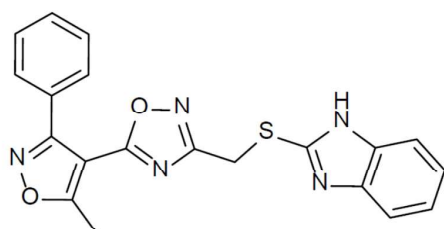
inhGAB1-007



inhGAB1-008

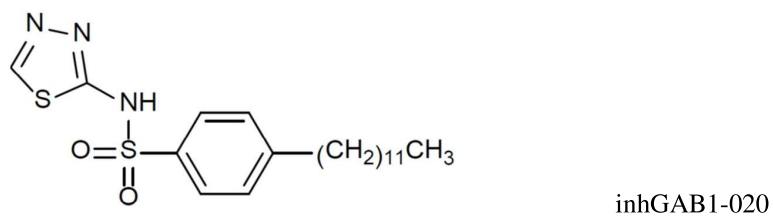
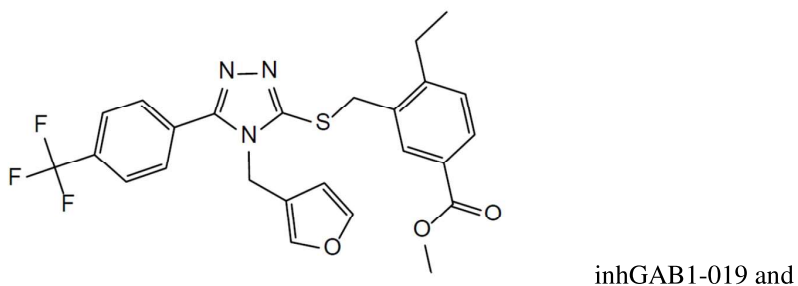
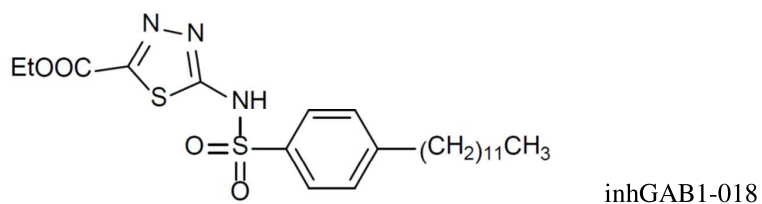


inhGAB1-009



inhGAB1-010

Clc1ccc(cc1)S(=O)(=O)CCSc2ccccc2NC(=O)c3ccccc3C(=O)OCc1c(C)c2nc(C)c(C)oc2s1(=O)=O.Nc1ccc(S(=O)(=O)Nc2cc(OC)c3ncnc3c2OC)cc1Cc1nc(NC(=O)c2ccc(NC(=O)c3ccc4ccccc43)cc2)s1CCCCCCCCCCCCCCCC(=O)Nc1ncnc(CCC(=O)O)s1CCCCCCCCCCCCCCCCc1ccc(cc1)S(=O)(=O)NC2=CN=C(CC(=O)OCC)S2NC(=O)C1=NC(=NS1)NC(=O)S(=O)(=O)c2ccc(cc2)CCCCCCCCCCCC



65. The method according to claim 32, wherein the BTK inhibitor is selected from the group consisting of ibrutinib, acalabrutinib, zanubrutinib, GDC-0834; RN-486; CGI-560; CGI-1764; HM-71224; CC-292; ONO-4059; CNX-774; and LFM-A13.