Cellular Signalling 26 (2014) 198–207

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

# Increased activity of mitogen activated protein kinase pathway in flotillin-2 knockout mouse model

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ARTICLE INFO

*Article history:*
Received 10 October 2013
Accepted 4 November 2013
Available online 9 November 2013

*Keywords:*
Cell signaling
Gene transcription
MAP kinases
p53
Signal transduction
Extracellularly regulated kinase

ABSTRACT

Flotillins are highly conserved and widely spread proteins that function in receptor tyrosine kinase signaling and membrane trafficking processes. Flotillin-1 and flotillin-2 have been shown to form both homo- and hetero-oligomers, and their cellular localization changes during signaling. Increased expression of flotillins has been detected in several types of cancer and shown to correlate with poor survival. Consistently, flotillin-2 knockout mice show a reduced formation of metastases in a breast cancer animal model. Our recent data have shown that flotillin-1 depletion results in diminished activation of the epidermal growth factor receptor and impairs its downstream signaling towards the mitogen activated protein kinases and the respective transcriptional response. Here we show that genetic ablation of flotillin-2 in a mouse model or its knockdown in cultured cells increases extracellular signal regulated kinase (ERK) activation. Furthermore, the downstream transcriptional targets of ERK and p53 are upregulated at both mRNA and protein levels. These data suggest that opposite effects are obtained upon ablation of one of the two flotillins, with flotillin-2 knockout/knockdown enhancing and flotillin-1 knockdown inhibiting ERK signaling. Due to their overexpression in cancers, flotillins may be considered as cancer therapy targets. However, our findings suggest that care needs to be taken when interfering with flotillin function, as undesired effects such as deregulation of growth-associated genes may emerge in certain cell types.



## 1. Introduction

The mitogen activated protein (MAP) kinase signaling pathway, well known for its crucial role in carcinogenesis, is the canonical downstream cascade of many growth factor receptors. Stimulation of receptor tyrosine kinases results in activation of kinases such as RAF1 (rapidly accelerating fibrosarcoma), a Ser/Thr kinase that in turn phosphorylates and activates MEK (MAP kinase kinase, a dual specificity Tyr/Thr kinase) which is responsible for activating the MAP kinases such as extracellular signal regulated kinases 1 and 2 (ERK1/2). Phosphatases are important regulators of intracellular signaling events during many biological processes. Dual-specificity phosphatases (Dusps) are phosphatases that can dephosphorylate both tyrosine and serine/threonine residues of their substrates. For example, Dusp1 was initially discovered to negatively regulate the activities of MAP kinases by dephosphorylating the TXY motif in their kinase domains [1].

MAP kinases have a large repertoire of substrates that become phosphorylated upon activation of the MAP kinase pathway. Some of these substrates reside in the cytoplasm, and their ERK mediated phosphorylation can influence the cytoskeleton or cell matrix adhesion. Alternatively, ERK can translocate into the nucleus and activate transcription factors which then induce the transcription of certain genes that are important e.g. for the cell cycle progression or function as transcription factors themselves.

Among the transcription factors that are induced by ERK activation are Fos and Egr1 (early growth response protein 1) which are also involved in growth regulation. Fos forms a dimer with other transcriptional regulators of the JUN, ATF and MAF families, giving rise to the activator protein 1 (AP-1) complex that can regulate transcription of growth genes. The most common AP-1 complex is a dimer composed of Fos and c-Jun [2]. Egr1 is a member of the zinc finger transcription factor family. It has been shown to be involved in the regulation of the transcriptional response induced not only by growth factors and mitogens, but also by stress, and thus to control both cell proliferation and differentiation [3,4]. Egr1 has been suggested to function as a tumor suppressor protein, and its expression is reduced or undetectable in many tumor cell lines and in tumors, implicating that its downregulation may enhance tumorigenesis, with the exception of prostate

*Abbreviations:* AP-1, activator protein 1; Dusp, dual-specificity phosphatase; Egr1, early growth response protein 1; ERK, extracellular signal regulated kinase; F2-KO mice, flotillin-2 knockout mice; Flot-1, flotillin-1; Flot-2, flotillin-2; HB-EGF, Heparin Binding-EGF; KO, knockout; MAPK, mitogen activated protein kinase; MEF, mouse embryonic fibroblasts; PUMA, p53 regulated modulator of apoptosis; RAF, rapidly accelerating fibrosarcoma; SRE, serum responsive element; WT, wildtype.

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http://dx.doi.org/10.1016/j.cellsig.2013.11.001

cancer in which Egr1 is oncogenic [5]. Egr1 directly controls the transcription of growth regulatory genes such as p53 and transforming growth factor β1 (TGFβ1) [6]. In line with this, Egr1 dependent enhancement of p53 expression has been shown to be important for the induction of apoptosis in melanoma cells [7].

Flotillin-1 (flot-1) and flotillin-2 (flot-2) are highly conserved and ubiquitously expressed proteins [8,9] that have recently been shown to be connected with growth factor signaling, MAP kinase regulation [10,11] and cancer [12–15]. The exact molecular function of flotillins has remained somewhat enigmatic, as they do not possess any clearly identifiable functional domains. Flot-1 and flot-2 show a high tendency to form both homo- and hetero-oligomers [16,17]. Both proteins can be Tyr phosphorylated by the Src family kinases, implicating a function in signaling [18]. In fact, flotillins are strongly interdependent of each other in their expression, and upon flot-2 downregulation, flot-1 expression is severely reduced [19]. In most cell types, however, flot-1 depletion results in a considerably milder or even no loss of flot-2 protein [10], suggesting that flot-1 is more dependent on flot-2 than vice versa.

Our recent findings show that flot-1 is required for the proper activation of the EGF receptor/MAP kinase signaling pathway [10]. Within this cascade, flot-1 plays a dual role in that it mediates the clustering and activation of EGF receptor at the plasma membrane but also functions as a MAP kinase scaffolding protein that regulates MAP kinase signaling during later stages of the pathway.

In this study, we have generated genetically ablated mice for the *flot-2* gene (F2-KO mice) and studied the transcriptional alterations associated with the loss of flotillins in several organs. Surprisingly, we found that the MAP kinase signaling cascade was hyperactivated in F2-KO mice, and several target genes (direct and successive) of ERK were upregulated. Since the products of these genes are associated with the regulation of cell growth and survival, and flotillins themselves are transcriptional targets of ERK signaling [20], our data reveal an interesting feedback loop within the MAP kinase cascade that is observed in both the knockout mouse model and upon RNA interference mediated depletion of flot-2 expression.

## 2. Material and methods

### 2.1. Flotillin-2 knockout mice

F2-KO mice were generated by deleting exon one which contains the ATG and parts of the promoter (Genoway, Lyon, France). The targeting vector containing a long and a short homology arm, two loxP sites flanking exon one, an FRT-flanked neomycin cassette and a Diphteria toxin A selection marker was electroporated in 129/SvPas embryonic stem cells. Verified ES cell clones were injected into C57BL/6J blastocysts. Chimeric animals were mated with C57BL/6J deleter mice constitutively expressing the Flp recombinase to remove the neomycin cassette. The resulting heterozygous floxed mouse line was mated with C57BL/6J deleter mice constitutively expressing the Cre recombinase to generate heterozygous, constitutive F2-KO mice. The presence of the knockout allele was confirmed by Southern blot. Heterozygous F2-KO mice were backcrossed with C57BL/6J mice three more times before the start of the first experiments.

For the microarray experiments, animals were housed under standard conditions (12 h dark–light cycle and free access to food and water). Animal experiments and husbandry were carried out in accordance with guidelines from the Federation of European Laboratory Animal Science Associations. The studies were approved by local authorities under the number V54-19c 20/15 c GI 20/2. Heterozygous animals were crossed to receive wild type and knockout littermates. PCR-based genotyping was performed with tail-clippings. Male animals were sacrificed at the age of four months by cervical dislocation under deep Isoflurane anesthesia. The organs and tissues were removed quickly, freeze-clamped in liquid nitrogen and stored at −80 °C for further analysis.

### 2.2. Cell culture

Primary murine embryonic F2-KO or wildtype fibroblasts (MEF) were isolated at embryonic day 13.5 from heterozygous matings. MEFs were genotyped and PCR-sexed according to [21]. Only wildtype and F2-KO MEF littermates of the same sex were used for direct comparisons. HeLa cells (human cervix carcinoma) and MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Karlsruhe, Germany), high glucose, supplemented with 10% fetal calf serum (FCS, Gibco), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Sigma-Aldrich, Taufkirchen, Germany) at 8% $CO_2$ and 37 °C. Cell culture medium for MEFs was additionally supplemented with 1% nonessential amino acids and 1 mM sodium pyruvate. In some experiments, HeLa cells were cultured in serum-free medium overnight.

### 2.3. Knockdown of flotillin-2

HeLa cells grown on 12 well plates were transfected with siRNA oligonucleotide duplexes targeting flot-2 (StealthTM siRNA; Invitrogen, Karlsruhe, Germany; final concentration 85 nM) using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. As a control, an oligo which does not target any human sequence, with a medium GC content (48%; StealthTM RNAi Negative Control Duplexes Med GC) was used (Invitrogen, Karlsruhe, Germany). The cells were used for the assays three days post-transfection. The knockdown was verified by Western blotting of equal protein amounts. Stable lentivirus mediated flot-2 knockdown cells and respective controls have been described before [16].

### 2.4. RNA isolation and qPCR

Lung, liver, colon and kidney tissues were ground under liquid nitrogen, and 20–30 mg of tissue powder was suspended in 800 μl of peqGold Trifast (Peqlab, Erlangen, Germany), homogenized with a tissue lyzer (Retsch, Haan, Germany), and RNA was isolated according to the manufacturer's protocol. DNA was eliminated by on-column DNase digestion and cleanup with RNeasy mini columns (Qiagen, Hilden, Germany). RNA purity and quality were checked photometrically and with RNA gels.

HeLa cell total RNA was isolated using peqGold Trifast reagent. For qPCRs, 3 μg of RNA was reverse transcribed as described [10]. Real time PCRs (Mx3005PTM QPCR System, Stratagene, Amsterdam, Netherlands) were performed in triplicates with 0.2 μl cDNA in 25 μl reaction mixtures using QPCR SensiMixTM SYBR (Bioline, Luckenwalde, Germany). The annealing temperature was 60 °C for all PCR reactions. Primers were designed with PerlPrimer to be specific for the cDNA and to give an amplicon of 100–188 nucleotides. PCR products were quantified with the ΔCt-method. The geometric mean of the reference genes Rpl13a and GAPDH was used for normalization of cell culture data and the geometric mean of HPRT, B2M, and Rpl13a for the mouse data.

### 2.5. Microarray analysis

For the microarray experiments, 1.5 μg of total RNA of seven male wildtype or 12 male F2-KO animals were pooled. Biotin-labeled cRNA was produced using a linear amplification kit (Ambion, Austin, TX, United States) with 400 ng of quality-checked total RNA. Chip hybridizations, washing, Cy3-streptavidin staining, and scanning were performed on the Illumina BeadStation 500 (Illumina, San Diego, CA, United States) platform using reagents and protocols of the manufacturer. cRNA samples were hybridized onto Illumina mouse-6v2 BeadChips.

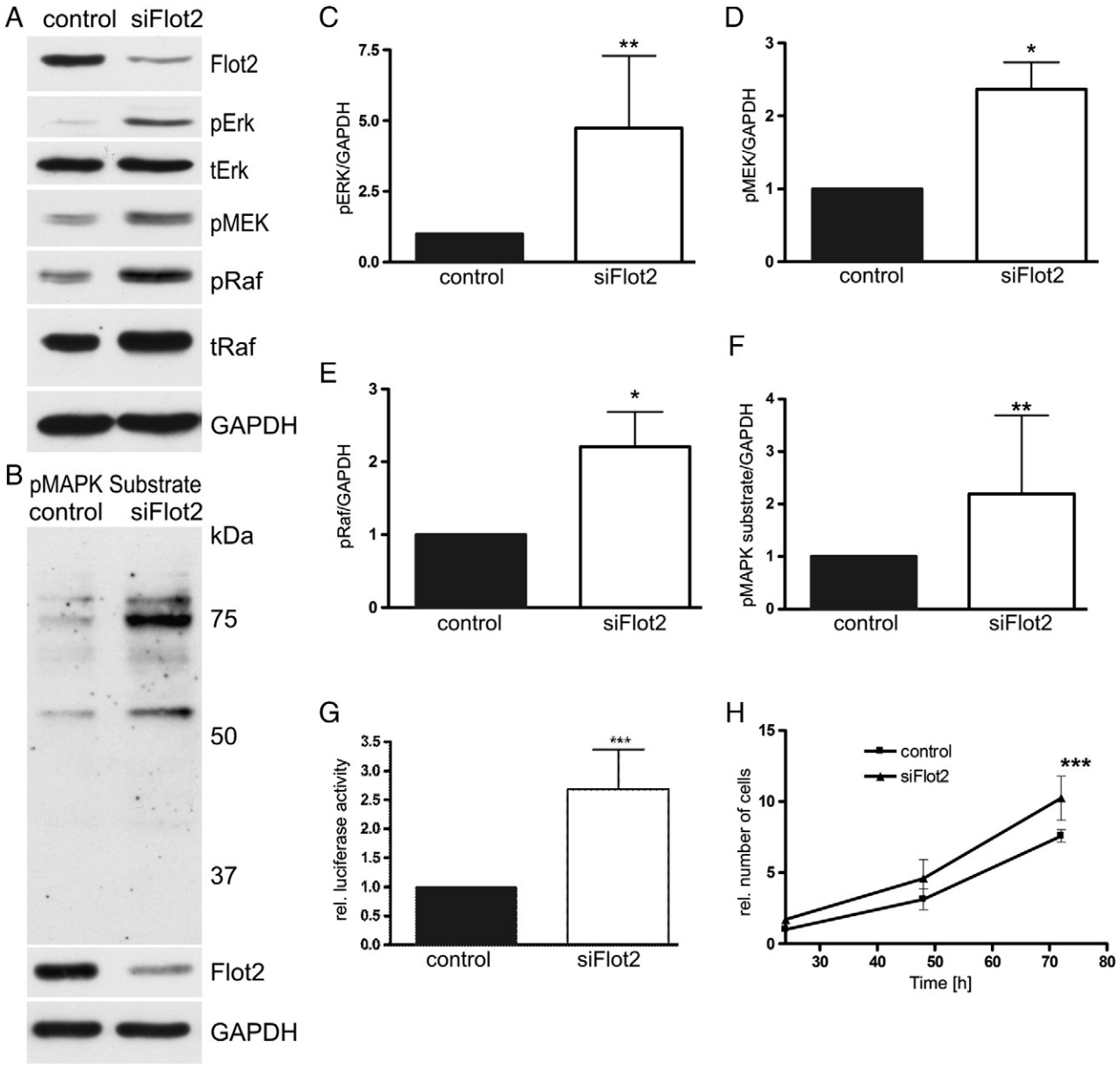


**Fig. 1.** Increased activation of MAP kinase pathway upon flot-2 knockdown in HeLa cells. Flot-2 was knocked down in HeLa cells using siRNAs. A: Activation of CRAF, MEK and ERK was measured with phospho-specific antibodies and depicted as pERK/pMEK/pRAF. B: ERK activity was determined with an antibody that specifically detects phosphorylated MAPK substrates. C–F: The blots in A & B were quantified and the signals normalized to GAPDH. G: A reporter gene construct containing Serum Responsive Elements and the luciferase enzyme was used to measure transcriptional response after MAPK activation. H: The proliferation rate of flot-2 knockdown HeLa cells was analyzed in an MTT assay 24, 48 and 72 h after seeding an identical number of cells in 96-well plates. The data show a significant increase in the activation of the MAP kinase signaling pathway, the transcriptional response coupled with ERK activation and cell proliferation.

Basic expression data analysis was carried out with BeadStudio 3.0 software. Raw data were background-subtracted and normalized using the "rank invariant" algorithm. Normalized data were then filtered for significant expression on the basis of negative control beads. Selection for differentially expressed genes was performed on the basis of arbitrary thresholds for fold changes [22]. This study was designed to detect the potential involvement of specific genes, and we thus used a ratio cut-off of 1.5-fold change.

### 2.6. Reporter gene assays

Five copies of the serum responsive element (SRE) were PCR amplified using the PathDetect® pSRE-Luc Cis-Reporter Plasmid (Stratagene, Waldbronn, Germany) as a template and subcloned into the NheI and XhoI sites of the pGL3-promoter plasmid (Promega, Mannheim, Germany), yielding the construct SRE-pGLprom. For p53 reporter gene assays, an oligonucleotide containing two copies of the consensus p53 responsive element [23] was cloned into the NheI and XhoI sites of pGL3-promoter, yielding the construct p53RE–pGL3prom. Correctness of the constructs was verified by sequencing.

Cells were transfected on 24 well plates with 15 ng of pRL-TK renilla luciferase expression plasmid and 150 ng of reporter gene construct or empty pGL3-promoter using Lipofectamine 2000 (Invitrogen). In the case of SRE-pGL3prom, cells were lysed in passive lysis buffer (Promega) after 48 h. In the case of p53RE–pGL3prom transfected cells, cells were starved overnight and UV-treated in PBS for 15 min on a NU-72 UV-table (Benda, Wiesloch, Germany). After UV-treatment, PBS was replaced by a serum-free medium and the cells were returned to the incubator. 6 h after UV-treatment, cells were lysed in passive lysis buffer. Determination of firefly and renilla luciferase activity was performed with a Tecan infinite M200 plate reader using 20 µl of lysate and 85 µl of beetle or renilla juice (PJK, Kleinblittersdorf, Germany) as

reagents. Relative luciferase activity was calculated by dividing firefly luciferase activity by renilla luciferase activity.

### 2.7. MTT-assay

Cells were seeded onto 96-well plates (5000 cells/well). After 24, 48, and 72 h, 20 µl MTT-solution (Sigma-Aldrich, 5 mg/ml in PBS) was added to the medium at 37 °C for 40 min. Cells were lysed in 5% formic acid and 95% isopropanol for 10 min. Extinction was measured at 550 nm versus 690 nm in a microplate reader.

### 2.8. Antibodies

Rabbit polyclonal antibody against Dusp1 and mouse polyclonal antibody against pErk were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit monoclonal Egr1 antibody and antibodies against pMEK, pMAPK-substrate and pCRAF (Ser338) were from Cell Signaling (NEB, Frankfurt, Germany). A mouse monoclonal antibody against GAPDH was obtained from Biozol (Eching, Germany). For detection of flot-1 and flot-2 in Western blots, polyclonal rabbit antibodies from Sigma (Taufkirchen, Germany) were used. Secondary antibodies goat anti-mouse and goat anti-rabbit coupled to horseradish peroxidase (HRP) were obtained from Southern Biotechnologies (Birmingham, AL, USA) and Zymed (Invitrogen, Karlsruhe, Germany), respectively.

### 2.9. Western blotting

Tissues were ground in liquid nitrogen, and 20–30 mg of tissue powder was suspended in 500 µl lysis buffer (50 mM Tris pH 7.4, 0.15 M NaCl, 2 mM EDTA, 1% NP-40) containing protease inhibitor

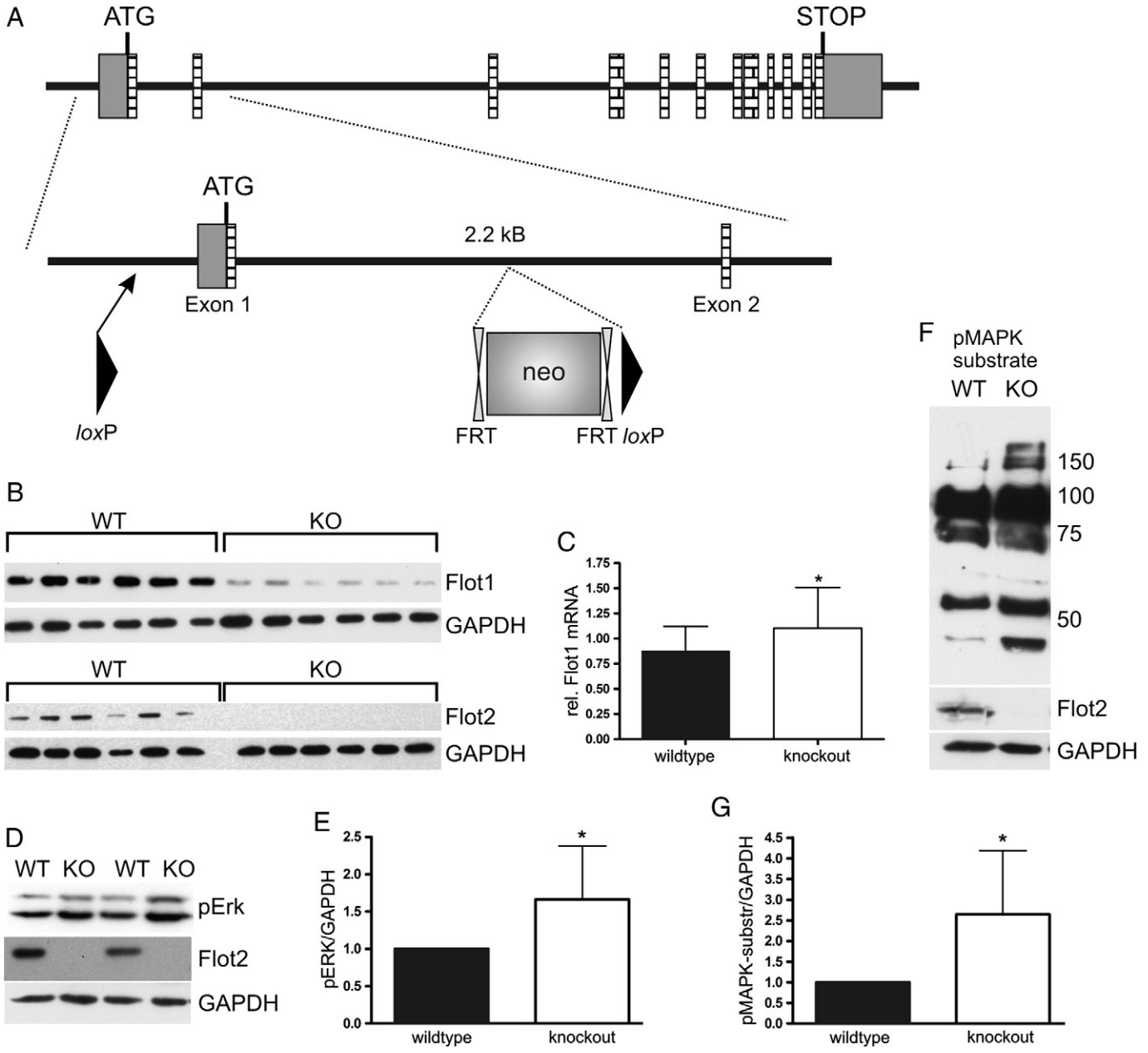


**Fig. 2.** Increased ERK activity in flot-2 knockout mice. A: Schematic representation of the mouse knockout strategy for the genetic ablation of the flot-2 gene. B: Western blot of wildtype and knockout mouse lung lysates shows that flot-2 is not expressed and the amount of flot-1 is severely reduced in the knockout tissue. C: Flot-1 mRNA is increased in flot-2 knockout mice. D–G: In mouse embryonic fibroblasts, flot-2 knockout cells show a significantly higher basal ERK activity, as assessed by an increased ERK phosphorylation (D, E), as well as increased phosphorylation of MAPK-substrates (F, G) as compared to the wildtype MEFs.

cocktail (Sigma-Aldrich, Taufkirchen, Germany). Homogenization was achieved with a tissue lyzer (Retsch, Haan, Germany), for 2 × 2 min at 25 Hz. Cellular debris were removed by centrifugation at 20,800 *g*, 15 min, 4 °C. Hela or MEF cells were washed with PBS and lysed in 50 mM Tris pH 7.4, 0.15 M NaCl, 2 mM EDTA, 1% NP-40 supplemented with protease inhibitor cocktail on ice for 30 min. Protein content was assessed according to Bradford using the BioRad Protein Assay reagent (BioRad, Munich, Germany). Equal protein amounts were analyzed by 10% SDS-polyacrylamide gel electrophoresis and Western blot.

### 2.10. Statistical analysis

Unless otherwise stated, all experiments were performed at least three times. Data are expressed as mean ± SD. Statistical comparisons between groups were made using Student's t-tests, 1-way or 2-way ANOVA, as appropriate (GraphPad Prism 5, San Diego, CA, USA). Western blot bands were quantified by scanning densitometry using Quantity One Software (Bio-Rad) and normalized against GAPDH. Values of $p < 0.05$ were considered significant (*) while values of $p < 0.01$ were considered very significant (**) and $p < 0.001$ extremely significant (***).

## 3. Results

### 3.1. Flotillin-2 knockdown results in increased MAP kinase signaling

We have earlier shown that flot-1 is important for the regulation of MAP kinase signaling, and flot-1 knockdown results in reduced phosphorylation of e.g. EGFR and ERK [10]. Flot-1 knockdown HeLa cells still abundantly express flot-2, whereas upon flot-2 knockdown, flot-1 protein is destabilized and degraded, resulting in severely reduced expression of both flotillins in many cell lines. Since flot-1 protein is also missing in flot-2 knockdown cells, one would expect to observe a similar effect on signaling by both flot-1 and flot-2 depletions. However, siRNA mediated knockdown of flot-2 in HeLa cells showed that the basal phosphorylation of ERK2, MEK1/2 and CRAF was increased (Fig. 1A). The increased ERK2 phosphorylation was accompanied by increased ERK activity, as was evidenced by increased phosphorylation of MAPK substrates (Fig. 1B). Quantification of the data showed that these differences were statistically significant (Fig. 1C–F), whereas total ERK amount was not changed (data not shown). Furthermore, in flot-2 knockdown cells, transcriptional activation of a luciferase reporter under the control of five successive SREs, used as a measurement of MAPK activation, was highly significantly increased (Fig. 1G). Very similar results were obtained using a second siRNA against flot-2 (data not shown). In addition, flot-2 knockdown cells displayed a significantly higher proliferation rate than control cells (Fig. 1H).

### 3.2. Flotillin-2 knockout mouse model

To corroborate these findings in a more physiological model system, we generated a flot-2 knockout (F2-KO) mouse using the strategy depicted in Fig. 2A. The mouse *flot-2* gene was ablated by inserting loxP sites in the first intron 3′ of the translation start codon and in the *flot-2* promoter sequence (see Section 2.1 for details). This strategy results in a complete impairment of flot-2 expression, as shown for lung tissue in Fig. 2B. No expression of flot-2 was detected in any of the tissues analyzed (data not shown). In accordance with the RNA interference data, flot-1 protein was also barely detectable in the lung (Fig. 2B), whereas flot-1 mRNA was even increased in the F2-KO lung (Fig. 2C). This is consistent with the transcriptional control of flot-1 by the ERK signaling cascade [20]. The F2-KO mice are viable and fertile and do not display any obvious phenotype.

### 3.3. MAP kinase signaling is increased in the flotillin-2 knockout mouse model

Our data showed that flot-2 knockdown in HeLa cells resulted in increased ERK activation (Fig. 1). To demonstrate that this also takes place in our knockout mice, we analyzed the amount of phosphorylated ERK

**Table 1**
Microarray results. List of genes that are >1.5 fold upregulated in F2-KO mice in at least 2 organs. Egr1 and Fos are regulated in all 4 organs analyzed.

| Gene | | Accession no. | Liver | Lung | Kidney | Colon |
|---|---|---|---|---|---|---|
| Atf3 | Activating transcription factor 3 | NM_007498.2 | **2.16** | 1.47 | **1.69** | 1.35 |
| Atf4 | Activating transcription factor 4 | NM_009716.2 | **1.50** | **1.70** | **1.60** | 1.41 |
| Axud1 | AXIN1 up-regulated 1 | NM_153287.3 | 1.49 | **1.67** | **1.60** | 1.11 |
| Capn2 | Calpain 2 | NM_009794.2 | 1.09 | **1.57** | **1.54** | **1.55** |
| Ccl9 | Chemokine (C-C motif) ligand 9 | NM_011338.2 | **1.62** | **1.70** | 0.92 | 1.09 |
| Cdkn1a | Cyclin-dependent kinase inhibitor 1A (P21) | NM_007669.2 | **1.70** | **1.72** | **1.52** | 1.22 |
| Cenpl | Centromere protein L | NM_027429.2 | **1.64** | 0.93 | 1.10 | **1.56** |
| Cxcl1 | Chemokine (C-X-C motif) ligand 1 | NM_019494.1 | **6.67** | 1.30 | **1.65** | 1.18 |
| Cyr61 | Cysteine rich protein 61 | NM_010516.1 | n.e. | **1.77** | **1.75** | n.e. |
| Dnajb1 | DnaJ (Hsp40) homolog, subfamily B, member 1 | NM_018808.1 | **2.32** | 1.32 | 1.43 | **1.51** |
| Dusp1 | Dual specificity phosphatase 1 | NM_013642.2 | **2.05** | **1.75** | 1.42 | **1.63** |
| Ear11 | Eosinophil-associated ribonuclease A family member 11 | NM_053113.2 | n.e. | **2.15** | n.e. | **1.90** |
| Egr1 | Early growth response 1 | NM_007913.5 | **1.93** | **3.24** | **10.90** | **4.23** |
| Fcer1g | Fc receptor, IgE, high affinity I, gamma polypeptide | NM_010185.4 | **1.82** | **2.36** | **1.53** | 1.45 |
| Fos | FBJ osteosarcoma oncogene | NM_010234.2 | **10.75** | **2.01** | **6.21** | **2.00** |
| Gadd45g | Growth arrest and DNA-damage-inducible 45 gamma | NM_183358.2 | **2.15** | 0.94 | **1.53** | 1.23 |
| Gdpd3 | Glycerophosphodiester phosphodiesterase dom. cont. 3 | NM_024228.2 | 1.08 | **1.50** | 1.05 | **1.54** |
| Hspa1a | Heat shock protein 1A | NM_010479.2 | n.e. | **2.57** | **2.33** | **2.22** |
| Il1b | Interleukin 1 beta | NM_008361.3 | **2.30** | **2.42** | 1.16 | 1.33 |
| Junb | Jun-B oncogene | NM_008416.1 | **2.58** | **1.99** | **2.12** | 1.30 |
| Paip1 | Polyadenylate binding protein-interacting protein 1 | NM_145457.3 | 1.34 | **1.94** | **1.56** | 1.42 |
| Pvrl2 | Poliovirus receptor-related 2 | NM_008990.2 | 1.15 | **1.51** | **1.55** | 1.12 |
| S100a8 | S100 calcium binding protein A8 (calgranulin A) | NM_013650.2 | **1.96** | **2.69** | 1.35 | 1.43 |
| S100a9 | S100 calcium binding protein A9 (calgranulin B) | NM_009114.1 | **1.89** | **2.38** | 1.18 | n.e. |
| Saa3 | Serum amyloid A 3 | NM_011315.3 | **2.21** | **2.87** | n.e. | n.e. |
| Scand1 | SCAN domain-containing 1 | NM_020255.2 | **1.52** | **1.51** | 1.01 | 0.99 |
| Shmt1 | Serine hydroxymethyltransferase 1, mitochondrial | NM_009171.2 | **1.79** | n.e. | **1.52** | 1.02 |
| Tnfrsf12a | Tumor necrosis factor receptor superf., member 12a | NM_013749.1 | **2.35** | 1.00 | 1.19 | **1.50** |
| Tnrc6a | Trinucleotide repeat containing 6a | NM_144925.3 | **2.48** | 1.30 | **1.61** | 0.85 |
| Uap1 | UDP-N-acetylglucosamine pyrophosphorylase 1 | NM_133806.4 | 1.29 | **1.63** | **1.96** | **1.76** |
| Zfp36 | Zinc finger protein 36 | NM_011756.4 | **2.43** | 1.43 | **2.18** | 1.30 |

All values above 1.5 have been marked in bold as they are above the cutoff.

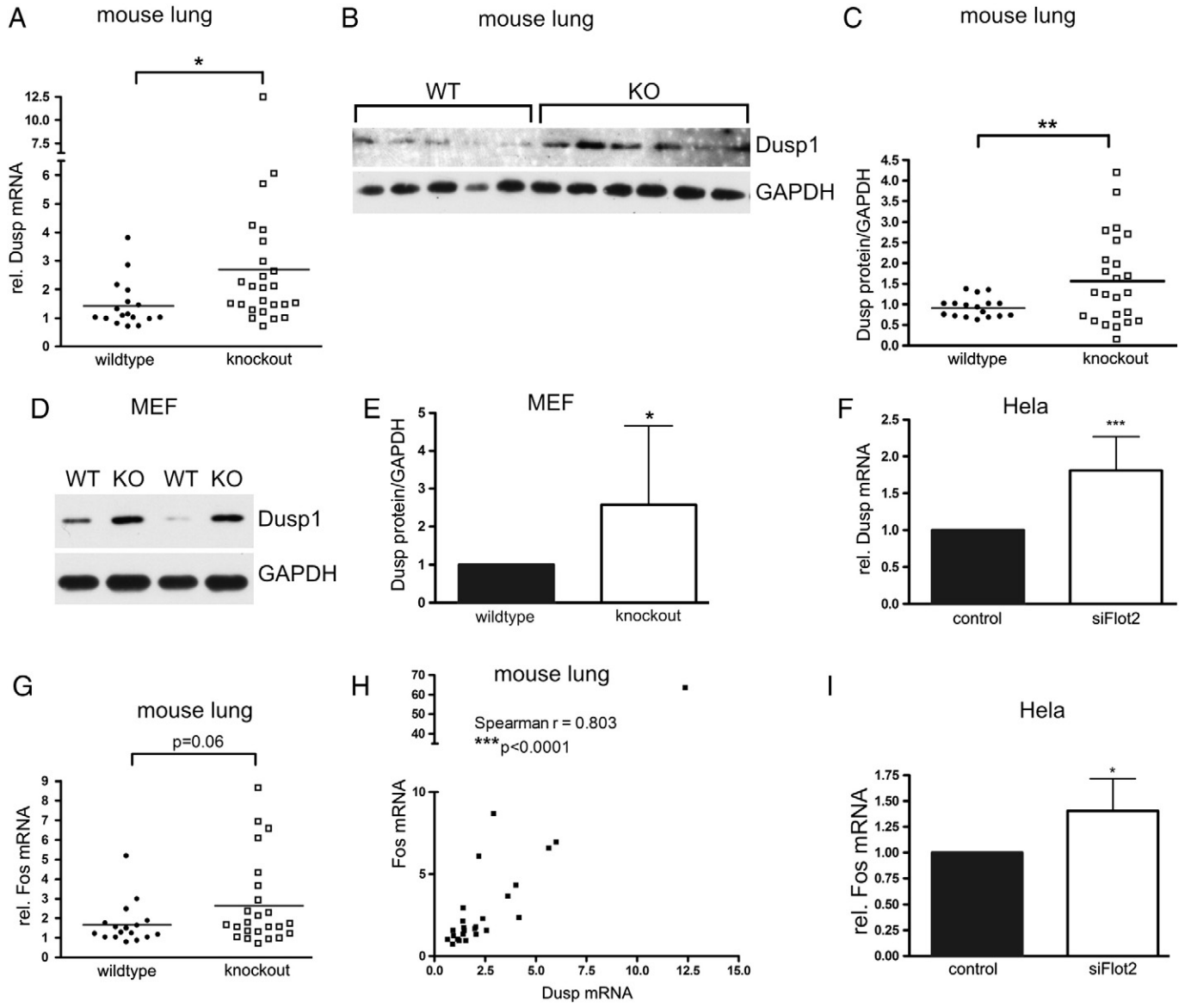


**Fig. 3.** Dusp1 and Fos are increased upon flot-2 knockout. Dusp1 mRNA (A) and protein (B, quantification in C) are significantly increased in the flot-2 knockout mice lung tissue and in the mouse embryonic fibroblasts (D, quantification in E). Dusp1 mRNA is also significantly higher in stable flot-2 knockdown HeLa cells (F). Fos mRNA shows a tendency to be increased in flot-2 knockout mouse lungs (G), and displays a significant correlation with the expression of Dusp1 (H). Fos mRNA was significantly higher in stable flot-2 knockdown HeLa cells (I).

and the phosphorylation of MAPK substrates in WT and F2-KO MEFs. Two independent pairs of MEFs (1 male WT/KO pair, 1 female WT/KO pair) were used for the experiments. Consistently, the knockout MEFs displayed a significantly increased ERK activity (Fig. 2D, quantification in Fig. 2E) which was accompanied by an increased phosphorylation of MAPK-substrates (Fig. 2F, quantification in Fig. 2G).

Gene expression in the organs (liver, lung, kidney, colon) of the F2-KO mice was analyzed by means of Illumina genome chips using pooled RNA samples of 7 WT or 12 KO mice. Table 1 shows the genes that were upregulated in the F2-KO at least 1.5 fold in at least 2 of the 4 organs analyzed. Intriguingly, many of these genes are involved in the regulation of cell growth, signaling, or transcription. For further analysis, we chose Dusp1, a dual-specificity phosphatase, and the transcription factors Fos and Egr1 (early growth regulated 1), all of which are involved in the MAP kinase signaling pathway.

The results of the Illumina analysis were verified in the lung of the mice using quantitative real time PCR (qPCR) and Western Blot. For this, we used the individual mice that were used for the Illumina analysis plus additional animals. Fig. 3 shows that the Dusp1 mRNA (Fig. 3A) and protein were significantly increased in the F2-KO lung (Fig. 3B–C) and in isolated MEFs (Fig. 3D–E). An increase of Dusp1 mRNA was also detected in flot-2 knockdown HeLa cells (Fig. 3F). Fos mRNA also showed an increase in both the F2-KO mouse lung (Fig. 3G) and flot-2 knockdown HeLa (Fig. 3I), although the results in mouse lung remained just below the significance level ($p = 0.06$). However, there was a highly significant correlation between the increased Dusp1 and Fos mRNA in the lung of the F2-KO mice (Fig. 3H), indicating that mice with a high Dusp1 expression also have an increased expression of Fos and vice versa. Similarly, Egr1 exhibited an increased mRNA (Fig. 4A) and protein (Fig. 4C–D) expression in the F2-KO lung. However, the knockout mice showed large individual variations in Egr1 expression level, and the results remained just below significance level ($p = 0.07$). However, Egr1 expression levels highly significantly correlated with increased Fos expression (Fig. 4B). This demonstrates that although the degree of increased expression varies between individual mice, the same mice exhibit an increased

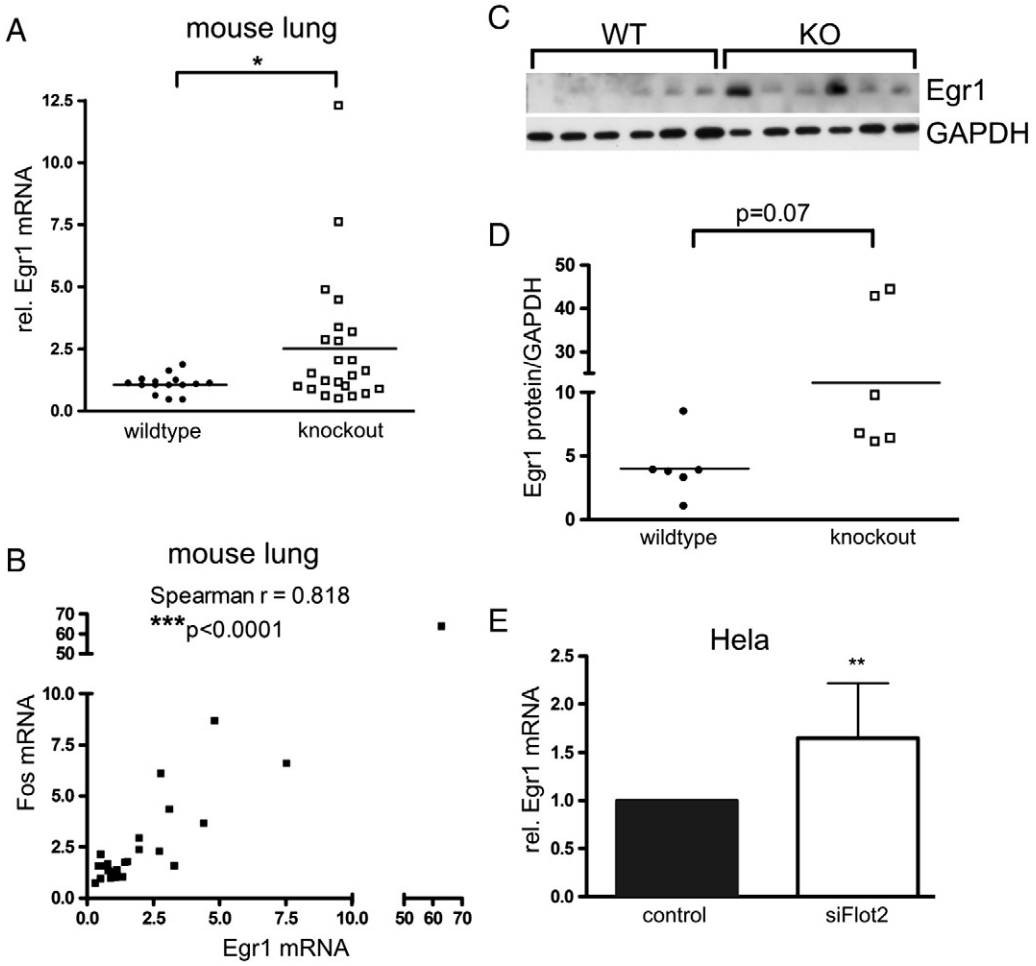


**Fig. 4.** Egr1 is increased in flot-2 knockout mice and upon flot-2 knockdown. Egr1 mRNA (A) and protein (C and D) are increased in the lung tissue of flot-2 knockout mice and in stable flot-2 knockdown HeLa cells (E). A significant correlation between increased Egr1 and Fos expression is observed in flot-2 knockout mouse lung (B).

expression of all three ERK target genes (Fos, Egr1 and Dusp1) analyzed. Again, flot-2 knockdown HeLa cells (Fig. 4E) recapitulated these findings.

The analysis of the mRNA of Egr1 target genes *p53* (Fig. 5A) and *fibronectin-1* (not shown) revealed a significant increase in the F2-KO mice lungs, implicating that Egr1 overexpression resulted in the activation of its downstream transcriptional targets. Since p53 itself controls the cell cycle and transcription of several important growth regulatory genes, we measured the mRNA levels of Dusp5, Heparin Binding-EGF (HB-EGF) and PUMA (p53 regulated modulator of apoptosis), all of which showed an increase in the F2-KO lung (Fig. 5B–D). Consistently, p21 which is regulated by both Egr1 and p53 exhibited increased mRNA levels in the knockout mouse lungs (Fig. 5E). An increased p53 activity was also detected after UV exposure of flot-2 knockdown HeLa cells transfected with a p53 reporter gene construct (Fig. 5F).

## 4. Discussion

Our data show that upon genetic ablation of the *flot-2* gene in the mouse, several important regulators of transcription of growth associated genes are upregulated in various organs, especially in the lung. These genes include transcription factors such as ATF3, ATF4, JunB, Fos and Egr1, which are positive regulators of cell proliferation and survival. On the other hand, the dual specificity phosphatases Dusp1 and Dusp5, which are MAPK phosphatases and may thus negatively regulate MAP kinase signaling, also exhibited a higher expression in the F2-KO mouse. Since flotillins have been shown to be involved in MAPK signaling [10], we here focused on three genes, i.e., *Egr1*, *Fos* and *Dusp1*, which all are targets and regulators of the said pathways and were increased in either all four (*Egr1* and *Fos*) or three (*Dusp1*) of the four organs tested.

Our findings in cultured cells in which flot-2 was knocked down and in MEFs of the knockout mice revealed an increased activation of ERK and a concomitant upregulation of Egr1, Fos and Dusp1, which was verified in the F2-KO mouse tissues. In line with this, the Egr1 downstream target genes p53 and fibronectin-1, and the p53 regulated genes Puma, Dusp5, HB-EGF, and p21 [24–27] which is also regulated by Egr1 directly [28], were also increased in the F2-KO lung. As the transcriptional regulation of these genes is connected as a network of hierarchy levels (Fig. 5G), the primary reason for the upregulation of these genes is likely to be the derailed MAP kinase signaling, manifesting as an increased ERK activity.

Although Dusp5, p21 and PUMA are likely to display a negative effect on ERK activation (see below), HB-EGF, which is a growth factor, may well act by providing a positive feedback loop and further increase the upstream signaling. The transcription factors Egr1 and Fos both belong to the immediate-early gene family and are well known pro-proliferative or pro-survival effectors. Egr1 has also been firmly established to exhibit tumor suppressive properties [5,6]. On the other hand, in prostate cancer, Egr1 may function in the opposite way and facilitate carcinogenesis and metastasis formation [29,30]. Interestingly, flot-1 expression was found to be downregulated in MEFs isolated from Egr1 knockout mice [5], which is in accordance with our data that

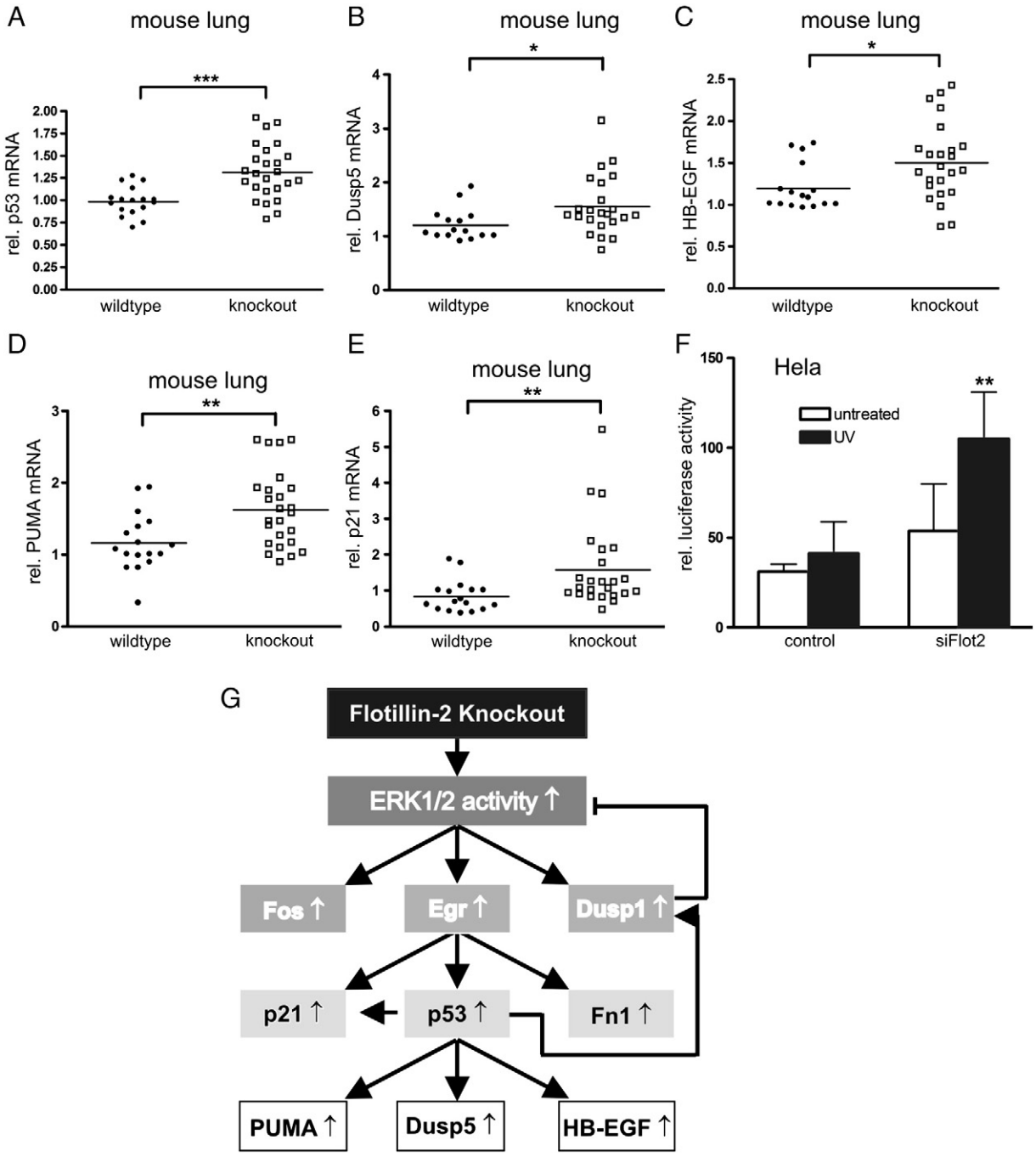


**Fig. 5.** Increased mRNA and activity of p53 and its target genes in the lung of flot-2 knockout mice. (A):p53, a downstream transcriptional target of Egr1, is significantly more expressed in flot-2 knockout mouse lung. The p53 target genes Dusp5 (B), HB-EGF (C), PUMA (D) and p21 (E) display an increased mRNA amount in flot-2 knockout mouse lungs. Please note that p21 is also directly regulated by Egr1. (F) Stable flotillin-2 knockdown cells were transfected with p53 reporter gene constructs and treated with UV light. Flotillin-2 knockdown cells showed a stronger increase in p53 activity upon UV treatment. (G) Flot-2 knockout in a mouse or knockdown in cultured cells results in increased activation of the MAP kinase pathway that culminates in increased ERK activity and increased transcription of its direct downstream targets Fos, Egr1 and Dusp1. The transcription factor Egr1 enhances the transcription of its target genes p53, p21 (which is also regulated by p53) and fibronectin-1 (Fn1). Consistently, the downstream targets of p53 (PUMA, Dusp5 and HB-EGF) are upregulated due to increased p53 amount. Dusp1 may display a negative feedback regulation by means of dephosphorylation of ERK1/2.

flotillins are target genes of Egr1 [20], and many transcriptional targets of Egr1 are also upregulated upon flot-2 ablation. This again points to a transcriptional regulatory loop within the MAP kinase cascade that also involves flotillins.

Dusp1 preferentially dephosphorylates p38 and Jnk, while ERK1/2 are poor substrates. Thus, Dusp1$^{-/-}$ cells show a dysregulation of JNK and p38 but no abnormalities in ERK activation [31]. All nuclear Dusps (Dusp1, 2, 4 and 5) are immediate-early genes that are rapidly induced by the same stimuli that activate MAPK, but they exhibit varying half-lives. However, phosphorylation of Dusp1 by ERK doubles its half-life [1]. The expression of all nuclear Dusps is regulated by p53 [27,32–34] whose upregulation in the F2-KO may contribute to the increased amount of Dusp1 and -5. Interestingly, the regulation appears to be somewhat specific for Dusp1 and -5, as other members of the Dusp family were only mildly increased, remaining clearly below a 1.5 fold cut-off, and were not systematically regulated in the studied organs.

Upregulation of Dusps is at first glance somewhat counter intuitive, considering that especially Dusp5 is a negative regulator of ERK

signaling during interleukin 2 signaling [35]. On the other hand, Dusp5 overexpression has been shown to enhance a migratory phenotype and anchorage independent growth in human breast cancer cells [36]. Importantly, it has been suggested that upon prolonged stimulation and even oncogenic transformation, Dusps exert negative feedback loops to ERK, which are required to prevent overt ERK signaling [37]. Dusps were originally suggested to exert their function by means of dephosphorylating and thus regulating the activity of their substrates. However, it has been shown that Dusp5 can modulate the function of ERK by sequestering it in the nucleus, a process that does not require Dusp5 phosphatase activity [38]. Furthermore, the Dusp5 sequestered, inactive ERK is still phosphorylated and activated by MEK, suggesting that Dusp5 may be more important as a nuclear anchor of ERK than as ERK phosphatase. In line with this, genetic ablation of specific Dusps does not always result in an increased phosphorylation of their in vitro substrates. For example, hyperphosphorylation of ERK is not observed upon depletion of Dusp2, although it has been shown to function as an ERK and p38 phosphatase [39], and Dusp10 knockout does not result in increased p38 phosphorylation [40]. These observations make it likely that the differential MAP kinase activation upon genetic ablation of specific Dusps also rely on MAP kinase crosstalk [41] and make it more difficult to judge the true molecular cause of the resulting phenotypes.

The p53 target gene PUMA [24], on the other hand, is a positive modulator of apoptosis in that it interacts with Bcl2, thereby liberating proapoptotic factors which can then enhance the intrinsic apoptosis pathway through mitochondria [42]. Interestingly, our recent data show that flotillin knockdown neuroblastoma and HeLa cells are more prone to undergo apoptosis upon various apoptotic stimuli (our unpublished data). Although the molecular mechanisms still need to be clarified in detail, it is plausible that upregulation of PUMA may also contribute to the enhanced apoptosis sensitivity of flotillin knockdowns.

We have shown that flot-1 not only exerts an effect on the activation of EGF receptor at the plasma membrane, but also is involved in further downstream signaling steps [10]. Flot-1 is capable of binding several of the MAPK signaling proteins (e.g. ERK, MEK and CRAF) and thus appears to function as a *bona fide* scaffolding protein during MAPK signaling [10]. As most flot-2 knockdown cells and F2-KO MEFs do not express flot-1 either, one would expect to see a similar phenotype as with flot-1 knockdown. Thus, the observed hyperphosphorylation and increased activity of ERK upon flot-2 depletion was surprising. Flotillins exhibit a strong tendency to form oligomeric complexes, and both homo-oligomers containing either of the flotillins and hetero-oligomers containing both flotillins are observed in most cells [16,43,44]. However, the functional relevance of each oligomer type is not clear. Thus, in the light of our data it is possible that flot-1 is a positive regulator of MAPK signaling and its absence results in a decreased signaling, whereas flot-2 may represent a negative regulator whose ablation impairs the control mechanisms that are required for the downregulation of signaling.

Recent clinical findings have suggested that flotillin expression is increased in several types of cancer, such as breast cancer [13,14,45], hepatocellular carcinoma [46], esophageal squamous cell carcinoma [15], gastric cancer [47], nasopharyngeal carcinoma [48] and melanoma [49]. The expression levels of either flotillin-1 or flotillin-2 were concordantly correlated with poor prognosis and reduced patient survival. Hence, flotillins were proposed as prognostic biomarkers [45–47] or even therapeutic cancer targets [13,14]. However, a mechanistic study to elucidate the functional role of flotillins in carcinogenesis is still lacking. In the breast cancer cell line SKBR3, flotillins were shown to stabilize ErbB2, a member of the EGFR family, which is frequently overexpressed in breast cancer [14]. In contrast, stable depletion of flotillin-1 in MCF7 breast cancer cells resulted in an increased expression of EGFR without altering ErbB2 levels (N. Kurrle & RT, in preparation). So far, except for the gene ablation, no direct method for interfering with flotillin function in humans or animals has been described. The F2-KO mice (this study and in [12]) and the previously published flot-1 knockout mice [50] do not show any obvious developmental defects, are fertile and exhibit a normal life span. However, our male KO mice exhibit a significantly decreased body weight as compared to their WT littermates (AB & RT, in preparation). Thus, the increased growth signaling paradoxically results in a smaller size which may derive from increased energy metabolism. It is tempting to speculate that interfering with flotillin expression may provide an alternative for inhibiting cancer cell proliferation, as flotillins do not appear to be essential for the survival of an organism. On the other hand, when F2-KO mice (generated independently of our model) were bred with an established breast cancer model, the resulting offspring did not show an impaired growth of primary tumors, whereas metastasis formation was reduced [12]. Thus, the effects of functional ablation of flotillins may not always be predictable.

## 5. Conclusions

Our study here shows that unexpected effects, such as increased ERK activity, which is observed in many cancers and provides a pro-proliferative stimulus, and upregulation of growth genes may result from a permanent flotillin ablation. In the light of these data, a permanent inhibition of flotillins, especially flot-2, may result in an unwanted compensatory upregulation of cellular signaling pathways. Thus, care needs to be taken when designing possible strategies for targeting flotillins in cancer, in order not to end up “from one ditch to another”.

## Acknowledgments

Petra Janson is acknowledged for her excellent technical support. This study has been financially supported by the Deutsche Forschungsgemeinschaft personal grant to RT (grant number Ti291/6-2). The funding body had no influence on the design of the study, data collection and interpretation or to the decision when and where to publish the data.

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