Telomere function and evolution in health and disease

06-11 May- 2024
Rome, Italy

EMBO Workshop

Workshop

Telomere function and evolution in health and disease

06 – 11 May 2024 | Roma, Italy

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Conference venue

Hotel Mercure Roma West
Viale dei Eroi di Cefalonia, 301

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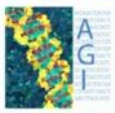

Special thanks are extended to Prof. Antonella Sgura (Università Roma Tre) for her invaluable assistance and support throughout the organization of the workshop.

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16:00-18:00	*Registration and Cocktails*	
18:00-18:30	Opening of the meeting - Introduction	Claus M. Azzalin Simon Boulton Miguel Godinho Ferreira Grazia Daniela Raffa Maria Teresa Teixeira
Keynote Lecture I		
	Chair: Ylli Doksani	
18:30-19:30	What can ALS tell us about our telomeres?	Jack Griffith
19:30-21:00	*Dinner*	

07 May 2022

08:00-09:00	*Breakfast*	

Session 1a: Telomere protection and dysfunction - I

Chair: Francisca Lottersberger

09:00-09:30	Characterizing the role of individual shelterin components in telomerase-mediated telomere elongation	Eros Lazzerini-Denchi
09:30-09:45	Identification of key regulators of DDR signaling and DNA repair at deprotected telomeres	Jacqueline Jacobs
09:45-10:00	Insights into compaction of telomeric chromatin: Structured yet dynamic	Aghil Soman
10:00-10:15	Unraveling the genetic and epigenetic impacts of large-scale chromosomal rearrangements in *Arabidopsis thaliana*	Miloslava Fojtová
10:15-10:45	The hidden functions and power of telomeric repeats in plants	Petra Prochazkova Schrumpfova
10:45-11:15	*Coffee break*	

Session 1b: Telomere protection and dysfunction - II

Chair: Martin Kupiec

11:15-11:45	Formation of telomeric circles reveals a general mechanism of eccDNA formation at tandem repeats	Ylli Doksani
11:45-12:00	smFRET Investigation of Structural Dynamics During T-Loop Formation	Arianna Lacen
12:00-12:15	Regulation of the shelterin complex by phosphorylation of TRF2	Libor Macurek
12:15-12:30	Interplay between lamin B1 and the shelterin protein TRF2 to maintain genome stability	Julien Picotto
12:30-12:45	Restriction on Ku Inward Translocation Caps Telomere Ends	Stéphane Marcand
12:45-13:00	Identification of the first poly (ADP-ribose) polymerase in yeasts and its involvement in telomere maintenance	Regina Sepšiová
13:00-14:00	*Lunch*	

Session 1c: Telomere protection and dysfunction - III

Chair: Ctirad Hofr

14:00-14:30	Processing of oxidative damage at telomeres regulates cellular senescence	Patricia Opresko
14:30-14:45	Regulation of Taz1 functions in telomere replication in fission yeast	Stephane Coulon
14:45-15:00	Cytoplasmic exposure facilitates resolution of non-sister telomere entanglements	Rishi Nageshan
15:00-15:15	Epigenetic inheritance of a telomere capping defect triggers longevity in *C. elegans*	Ben McCarthy

15:15-15:30	Telomeric DNA damage response activation mediates Aβ42 oligomers toxicity in Alzheimer's disease.	Sara Sepe
15:30-16:00	*Coffee break*	

Session 1d: Telomere protection and dysfunction - IV

Chair: Marita Cohn

16:00-16:30	Functions of the *Saccharomyces cerevisiae* CST complex in double-strand break repair	Zhou Xu
16:30-16:45	Chromosome end protection by RAP1-mediated inhibition of DNA-PK	Max Douglas
16:45-17:00	DNA-PK controls Apollo's access to leading-end telomeres	Ceylan Sonmez
17:00-17:20	**Microtalks I**	
17:30-19:30	**Poster Session - I** (odd number posters)	
19:30-21:00	*Dinner*	

08 May 2024

08:00-09:00	*Breakfast*	

Session 2: Telomerase biogenesis and function

Chair: Dirk Hockeymeyer

09:00-09:30	Analysis of telomerase assembly using RNP-MaP	Jens Schmidt
09:30-09:45	Single-molecule imaging of telomerase provides mechanistic insights into cancer-associated POT1 mutations	Pascal Chartrand
09:45-10:00	The DBHS family of RNA/DNA binding proteins regulate telomerase recruitment to the telomere	Alexander Sobinoff
10:00-10:15	Nuclear F-actin and DNA replication stress regulate the recruitment of human telomerase to telomeres	Tracy Bryan
10:15-10:30	Depletion of the *Leishmania* spp. TERT and TER telomerase components triggered telomere shortening on different chromosomes	Maria Isabel Nogueira Cano
10:30-10:45	*S. pombe* telomerase RNA: secondary structure and flexible-scaffold function	David Zappulla
10:45-11:15	*Coffee break*	

Session 3: Regulation and consequences of telomere transcription

Chair: Sara Selig

11:15-11:45	TERRA Dynamics, R-loops and the Induction of Telomere Fragility	Joachim Lingner
11:45-12:00	TERRA Nanopore Direct RNA Sequencing and Its Upregulation with Human Aging	Hsueh-Ping Catherine Chu
12:00-12:30	Understanding the role of TERRA in the regulation of human telomerase	Emilio Cusanelli
12:30-12:45	Transcription stress at telomeres leads to cytosolic DNA release and paracrine senescence	Kalliopi Stratigi
12:45-13:00	Mechanisms of telomere protection in the *Drosophila* embryo	Livia Scatolini
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19:30-21:00	*Dinner at Villa dei Cesari*	

09 May 2024

08:00-09:00	*Breakfast*	

Session 4: The cellular response to dysfunctional telomeres

Chair: Susan Smith

09:00-09:30	Autophagy and innate immunity in telomere-mediated tumor suppression	Joe Nassour
09:30-09:45	cGAS-STING activation causes premature aging and cancer in zebrafish.	Giulia Allavena
09:45-10:00	The interplay between telomere attrition and EV biology	Yie Liu
10:00-10:15	Micronucleus Derived from Chromosome Fusion Is Not a Potent Inducer of cGAS-STING Pathway	Makoto Hayashi
10:15-10:30	RNA-seq analysis of telomerase-deficient *S. cerevisiae* reveals upregulation of distinct cellular pathways during senescence and survivorship	Arpana Upadhyay
10:30-11:00	CRISPR-based screens to illuminate the genetic landscape in response to telomere erosion and senomodulation	Lea Harrington
11:00-11:30	*Coffee break*	

Session 5a: Alternative mechanisms of telomere maintenance - I

	Chair: Junho Lee	
11:30-12:00	The fatty connection of telomeres	Anabelle Decottignies
12:30-12:45	ZBTB48 causes persistent chromatin bridges in ALT-positive cells	Dennis Kappei
12:45-13:00	Ultrabright telomeric foci and telomere clustering in the alternative lengthening of telomeres.	Sarantis Gagos
13:00-14:00	*Lunch*	

Session 5b: Alternative mechanisms of telomere maintenance - II

	Chair: Hilda Pickett	
14:00-14:30	Elucidating the Role of Heterochromatin Marks in the Alternative Lengthening of Telomeres (ALT) Mechanism	Nausica Arnoult
14:30-14:45	Replication Fork Proteome Profiling by BLOCK-ID Identifies the Chromatin Reader TRIM24 in ALT	Kyle Miller
14:45-15:00	Multi-omics analyses unveiled distinctive features of alternative lengthening of telomeres in mouse embryonic stem cells	Sanghyun Sung
15:00-15:30	Widely interspaced Zinc finger (WIZ) induced H3K9 trimethylation is necessary for ALT activity	Agnel Sfeir
15:30-16:00	*Coffee break*	

Session 5c: Alternative mechanisms of telomere maintenance - III

	Chair: Antonella Sgura	
16:00-16:15	Silencing of SLFN11 confers replication stress tolerance in ATRX deficient ALT cancers	Sandra Segura-Bayona
16:15-16:30	Heterogeneity of the activity of the alternative lengthening of telomeres pathway in neuroblastoma tissue	Emma Koeleman
16:30-16:50	**Microtalks - II**	
17:30-19:30	**Poster Session - II** (even number posters)	
19:30-21:00	*Dinner*	

<u>10 May 2024</u>

08:00-09:00	*Breakfast*	

Session 6a: Consequences of telomere dysfunction-cancer and telomere biology disorders - I

Chair: Alison Bertuch

09:00-09:30	Genetic Basis, Manifestations and Clinical Implications of Human Telomere-Mediated Disease	Mary Armanios
09:30-09:45	Engineered telomerase RNA to increase telomere length in human cells	Neha Nagpal
09:45-10:00	Consequences of telomere dysfunction in different cell types for kidney fibrosis development	Maria A. Blasco
10:00-10:15	POT1a humanized mouse models	Paula Martinez
10:15-10:30	Deep scanning mutagenesis of Shelterin for high-throughput characterization of clinical variants of unknown significance	Annika Martin
10:30-10:50	*Coffee break*	

Session 6b: Consequences of telomere dysfunction-cancer and telomere biology disorders - II

Chair: Fabrizio d'Adda di Fagagna

10:50-11:20	Towards systemic therapies for telomere biology disorders	Suneet Agarwal
11:20-11:50	Apollo deficiency in humans and mice	Patrick Revy
11:50-12:05	Digital telomere measurement distinguishes health and disease	Santiago Sanchez
12:05-12:20	Connecting the Dots - From Bench to Life, Not Just Bedside	Katie Stevens
12:20-14:00	*Meet the Speakers (Lunch with assigned tables)*	

Session 7: Regulation of telomere length and structure: evolution and ecology

Chair: Scott Cohen

14:00-14:30	NanoTelSeq enables accurate analysis of single telomeres and their subtelomeric methylation states in healthy individuals and telomere biology disease patients	Yehuda (Dudy) Tzfati
14:30-14:45	Telomere length inheritance through the budding yeast sexual cycle	Vasilisa Sidarava
14:45-15:00	Individual cell fate and population dynamics revealed by a mathematical model linking telomere length and replicative senescence	Marie Doumic
15:00-15:30	Chromatin identity crisis: meiotic telomere functions reveal centromere-telomere interactions and interchangeability	Julia Promisel Cooper

15:30-15:45	*Coffee break*	
Keynote Lecture II		
	Chair: Joachim Lingner	
15:45-16:15	My fieldtrip: from telomeres to telomerase and back.	Mundy Wellinger
16:15-16:45	Meeting Discussion and Concluding Remarks	Claus Maria Azzalin Simon Boulton Miguel Godinho Ferreira Grazia Daniela Raffa Maria Teresa Teixeira
17:30-19:00	*Cocktail*	
20:00-onwards	*Gala Dinner and After party*	

11 May 2024

08:00-09:00	*Breakfast*
09:00-11:30	*Departure*

SESSION I: ODD NUMBER POSTERS 07th May 2024

17:00-17:20 Microtalks I (in bold)

17:30-19:30 Posters session I

poster #	Presenter	Abstract Title
1	Acocella Giulia	The nucleoside analogue 6-thio-2'-deoxyguanosine (6-thio-dG) sensitize breast cancer cells to ionizing radiation exposure
3	Andolfato Marta	Characterization of the long noncoding RNA TERRA in a vertebrate
5	Bar-nes Kamil	Impaired glucose homeostasis in the short-telomere Telomouse.
7	Bazzo Catto Luiz	Clinical and molecular features associated with immunodeficiency in telomere biology disorders.
9	Bertuch Alison	Investigating proteins that associate with TIN2 via the region mutated in dyskeratosis congenita and related telomere biology disorders
11	Bruno Flavia	Identification of factors involved in the excision of telomeric i-loops as eccDNA
13	Buck Brandon	Fragmentation patterns in human telomeric cell-free DNA
15	Cano Maria Isabel Nogueira	Depletion of the Leishmania spp. TERT and TER telomerase components triggered telomere shortening on different chromosomes
17	Casajús Pelegay Ester	Structural studies of TRF1-Tankyrase interaction
19	**Červenák Filip**	**Evolution of yeast telomeres is shaped by the flexibility of telomere-protecting systems**
21	**Clatterbuck Soper Sarah**	**PML promotes organization of nuclear f-actin to facilitate ALT**
23	Cohn Marita	Subtelomeric sequences stabilize the shortened telomeres in N. castellii ALT cells.
25	De Laurentiis Evelina	Understanding the Full Lifespan and Dynamics of the Telomerase RNP in Saccharomyces cerevisiae.
27	de Tocqueville Sophie	ZCCHC8 mutations associated with telomere biology disorders affect both hTR biogenesis and telomere stability
29	Derumier Amélie	The fatty connection of telomeres
31	Drissi Rachid	THIO-induced Telomere Dysfunction Leads to Cytoskeleton Remodeling
33	Fatma Khushnood	Telomere Targeting using Small Molecule Mediated G-Quadruplex Stabilization in Cancer Cells
35	**Gaela Venus Marie**	**Orphan nuclear receptors promote human cell immortalization by activating ALT**
37	Germe Thomas	A structural role for Topoisomerase II in resolving telomere entanglements in late anaphase

39	Giraud-Panis Marie-Josèphe	Biochemical studies reveal a possible correlation between the strength of the Apollo-TRF2 complex and longevity
41	Gonzalez-Rodriguez Nayim	Structural studies of Candida glabrata Cdc13 by cryoEM
43	Grupelli Glenda Paola	Investigating the role of TERRA R-loops in telomerase regulation
45	Hajikazemi Mona	Telomerase-mediated targeting of telomeric G4s via the 6-thio-dG as a potential treatment strategy for Melanoma
47	Hillairet Alexia	HOT1 acts as a DYNLL1-mediated dimer to influence telomeric chromatin organization.
49	Holzmann Klaus	Measurement of telomere variant repeats by monochromatic quantitative PCR
51	Hourvitz Noa	HHS patient cells with homozygous RTEL1 mutations exhibit accelerated telomere shortening and disrupted nuclear morphology associated with cytoplasmic localization of nuclear factors
53	Idilli Aurora	Landscape of genetic vulnerabilities in ALT cancers
55	Johnson Noah	The biophysical environment of telomeres at the nuclear envelope
57	Kochman Rima	RPA copes with replication stress at telomeres: missense mutations in RPA2 gene cause telomere biology disorders
59	Kyriacou Eftychia	Live tracking of TERRA in cancer cells
61	Larini Luca	Exploring the role of telomeric transcripts in the cytoplasm of ALT cancer cells
63	Lee Hui-Ting	The Potential Regulatory Role of Telomeric Overhang Structure in Telomere Looping
65	**Li Bibo**	**Telomere end processing in Trypanosoma brucei**
67	Lidonnici Jacopo	Prognostic role of TERRA levels in peripheral blood mononuclear cells from elderly colorectal cancer patients: an exploratory study
69	Ling Justin	Consequences of ribonucleotides in telomeres
71	Sidarava Vasilisa	Telomere length inheritance through the budding yeast sexual cycle
73	Marzullo Marta	Role of the E2-variant ubiquitin conjugating enzyme AKTIP in chromatin conformation, telomere stability and aging.
75	Mattarocci Stefano	MRX Inhibition by Rap1 at Telomeres
77	Micheli Emanuela	The riddle of TERT inside the mitochondria
79	Müller Jörg	A model of the dynamics of individual telomere lengths
81	Novo Clara	Telomere end protection in mouse embryonic stem cells
83	Orioli Alex	Mechanistic differences between disease-related mutations of human Telomerase RNA

85	Pandolfo Francesca	The function of SLX4IP at ALT telomeres
87	Park Kyumin	Proteomic Landscape of Type 2 ALT Maintenance in Mouse Embryonic Stem Cells
89	PERALTA Juan Manuel	Oxidative stress and telomere length during replicative senescence in Saccharomyces cerevisiae
91	**PONCET Delphine**	**The TeloDIAG: Insights into Glioma Pathogenesis and Diagnosis Through Telomeric Analysis**
93	Quintana Verdaguer Maria	The yeast Hog1 MAPK controls telomere homeostasis during stress adaptation
95	Richter Felix	A high-throughput screening assay for telomere length and dynamics in S. cerevisiae based on luminescence
97	**Rose Anna**	**Hypoxia induces Alternative Lengthening of Telomeres pathway activity in ATRX-deficient cells**
99	Rotondo Julianna	Telomere length homeostasis: S. cerevisiae to humans
101	**Salgado Sara**	**The nucleic acids-binding protein SUB1 supports the viability of ALT cancer cells**
103	Sanford Samantha	6-thio-2'-deoxyguanosine drives telomere shortening by impairing telomerase translocation after nucleotide addition
105	Silimperi Kayli	*S. cerevisiae* telomerase RNA flexibly scaffolds the essential Est1-arm Conserved Region (ECR), bound by Est1 and Pop proteins
107	Smoom Riham	Separation of telomere protection from length regulation by different point mutations at position 492 of mRtel1
109	Taylor Erin	Investigating the functional consequences of telomeric heterochromatin formation in ALT vs non-ALT cells
111	Trastus Lucrezia	From telomere dysfunction to bone marrow failure: investigating hematopoietic vulnerability in mice upon Trf2 knockout
113	**Tseng Chi-Kang**	**Development of RNA-based therapy for telomere biology disorders**
115	Udroiu Ion	Terc gene duplication in Murinae: evolutionary evidence and functional implication
117	Uttarilli Anusha	Relative Telomere Length measurement of Colorectal cancer, adenoma and healthy controls from Austria - a CORSA study
119	Vaurs Mélina	TRF1-dependent repression of human telomere fragility requires fork reversal and telomerase activity
121	**Vodicka Pavel**	**Genomic instability, microenvironment and telomere homeostasis in solid malignancies.**
123	Zanella Elia	DNA damage at tandem repeats generates i-loops that can be excised as eccDNA
125	Zhu Jiyue	Humanization of the mTert gene reset mouse telomeres to human length

SESSION II: EVEN NUMBER POSTERS

09th May 2024
16:30-16:50 Microtalks II (in bold)
17:30-19:30 Posters session

poster #	Presenter	Abstract Title
2	Alayoubi Hosnia	The different functions of RTEL1 variants in telomere maintenance
4	Azeroglu Benura	Characterizing mechanisms and factors in the generation of telomeric ssDNA in ALT-positive cells
6	Baudoin Nicolaas	A single-cell sequencing-based approach for telomere length analysis reveals telomere length dynamics in the human breast during aging and tumor evolution.
8	BERARDI PRISCA	Processing and initiation of genome instability at the shortest telomere in cells in the absence of telomerase
10	Bosso Giuseppe	A novel role for BRAF in telomere biology
12	Brzacova Zuzana	Telomere reprogramming to test the compatibility of different telomeric repeats with telomere-binding proteins in Saccharomyces cerevisiae
14	Cannistrà Federica	Genome and telomere integrity depend on type I ESCRT subunits
16	Carlund Olivia	Telomerase activity and proliferative capacity in activated T-lymphocytes in telomere biology disorders
18	Castelli Sara	Dissecting a mechanism of telomere end protection against NHEJ
20	Choi Hon Fung	CRISPR/Cas9 Approach to Unravel the Molecular Basis of Telomeric DNA Damage and the Disparity in DNA Damage Responses between Genomic and Telomeric Regions
22	**Cohen Scott**	**Structural characterization of small-molecule inhibitors of human telomerase**
24	**Coloma Javier**	**Characterization of the oligomeric state of the C. glabrata CST complex.**
26	De Rosa Mariarosaria	OGG1 and MUTYH repair activities promote telomeric 8-oxoguanine induced cellular senescence
28	Deimler Nathaniel	hTERC Template Mutation Impairs Telomerase Processivity Resulting in DKC
30	**Domínguez de Barros Angélica Teresa**	**Study of telomere length dynamics and oxidative stress-mediated biomarkers in Psittacidae species with different longevity.**
32	Fajkus Jiří	Evolutionary Divergence in Hymenoptera and Lepidoptera Telomerase RNAs: A Shift from the Norm in Animal TR Biogenesis
34	**Flores Ignacio**	**Telomerase activation under the p21-promoter induces cardiomyocyte proliferation and heart regeneration**
36	Gaiatto Alice	Telomere maintenance and structural variants: the impact of ALT on complex genomic rearrangements
38	Giladi Liran	Does RECQL5 play a role in protecting telomere integrity?

ABSTRACTS

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What can ALS tell us about our telomeres?

Jack Griffith
Lineberger Cancer Center, University of North Carolina at Chapel Hill

While it was assumed that telomeres do not encode their own proteins, we showed recently (Al Turki and Griffith, PNAS, 2023) that telomeric G-rich RNA transcripts can be translated via the RAN mechanism first discovered in the ALS/FTD disease by Ranum and colleagues to generate repeating glycine-leucine (GL) and valine-arginine (VR) dipeptide proteins. VR is present at higher levels in cells from an osteosarcoma cancer line and from a patient with a telomere biology disease as contrasted to a primary fibroblast line. Both VR and GL activate innate immune pathways and induction of dysfunctional telomeres leads to higher levels and deposits of VR in the nuclei. Recently we observed high expression of VR in cultured cells in mitosis and in 16-day mouse embryonic neural progenitor precursor cells that have a high mitotic index. Our hypothesis is that VR expression serves to help depress translation during mitosis.

My fieldtrip: from telomeres to telomerase and back.

Raymund Wellinger , Many Team-members
Université de Sherbrooke

I started my wanderings in the telomere field with a keen interest in exploring how this part of the chromosome was maintained. Early on, the work focused on how the replication of the bulk DNA of the chromosomes would affect the replication of telomeric DNA (or vice-versa). Experiments targeting the dynamics of replication fork movements on telomeres did reveal unexpected replication intermediates that suggested that DNA ends with telomeric DNA, at least for some time, are not that different from DNA-breaks at other loci. Reinforcing this idea, our work unveiled that some well-known DNA-break binders also functioned on telomeres (yKU, MRX). While working on the changing ends, we got interested in how telomerase would fit-in with a focus on the RNA component, which seemed so different in yeasts. Phylogenetics yielded a secondary structure prediction and after RNA-tagging, the dynamics of single molecules in live cells could be recorded, showing various modes of telomere-telomerase encounters. Also, adaptations of those RNA tools eventually yielded new essential constituents of the yeast telomerase RNP. Most recently, we developed a refined model for how and when the yeast telomerase RNP is assembled. That then allowed us to loop back to our roots and discover that reading old papers (properly!!) can be rewarding: it turns out that not all telomeres are maintained equally. We worked out the molecular mechanism of how in yeast at least one telomere, TEL03L, manages to remain much longer than the others, which could have important repercussions for human telomeres and biology.

Towards systemic therapies for telomere biology disorders

Suneet Agarwal
Division of Hematology/Oncology and Stem Cell Program, Boston Children's Hospital; Pediatric Oncology, Dana-Farber Cancer Institute; Department of Pediatrics, Harvard Medical School; Boston, MA, USA

Telomere biology disorders (TBDs) comprise a range of genetic diseases unified by impaired maintenance of telomere integrity. Bone marrow failure, liver disease and pulmonary fibrosis are frequent life-threatening sequelae amongst myriad pathologies, indicating that TBDs are degenerative disorders affecting the whole body. In the past two decades, genetic discoveries and improved awareness have increased the diagnosis of TBDs across age ranges, but with clinical impacts primarily on the management of bone marrow failure and family risk assessment and counseling. A major unmet need now is to develop safe approaches to restore telomere maintenance systemically – throughout the body – based on the hypothesis that tissue dysfunction can be ameliorated and/or prevented by targeting the underlying molecular defect. This talk will focus on how genetic discoveries in TBDs are being translated to the clinic, exemplifying the critical role basic science plays in understanding human diseases, and vice versa. The impact of telomere length measurement in a bone marrow transplant clinical trial will be reported, demonstrating important advances but also the limitations of fixing one part of the body in a systemic disorder. Our efforts to address this by identifying and validating targets potentially amenable to small molecule modulation, such as PAPD5 and nucleotide metabolism, will be described. Prospects and challenges for clinical translation of small molecule telomere therapeutics will be discussed.

Genetic Basis, Manifestations and Clinical Implications of Human Telomere-Mediated Disease

Mary Armanios
Departments of Oncology and Genetic Medicine, Telomere Center at Johns Hopkins, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins School of Medicine
Baltimore, Maryland USA

The short telomere syndromes are the most common of premature aging syndromes and their most common manifestation is adult-onset pulmonary fibrosis. One-third of individuals with familial pulmonary fibrosis carry a germline mutant telomerase or other telomere maintenance gene. These genetic insights have led to the discovery of syndromic patterns that inform the diagnostic and treatment approaches for this large group of patients including in the lung transplant setting. There is also growing appreciation for an emerging group of long telomere-mediated genetic disorders that manifest as an increased risk for benign and malignant neoplasia. This risk is associated and is driven by an exquisitely high risk of clonal hematopoiesis that is sustained by long telomere length with aging. Here, I will discuss how these disorders inform fundamental human telomere biology as well as approaches at the bedside for patient care.

Elucidating the Role of Heterochromatin Marks in the Alternative Lengthening of Telomeres (ALT) Mechanism

Bruce Proctor III [1, 2], Erin Taylor [1, 2], Nausica Arnoult [1]
[1] *University of Colorado, Boulder*
[2] *Contributed equally to the work*

Despite ALT telomeres exhibiting a less condensed chromatin structure with reduced nucleosome density, they are paradoxically enriched with H3K9me3— a constitutive heterochromatin mark. The depletion of H3K9me3 through deletion of histone methyltransferases has previously yielded inconsistent results on ALT activity, likely because of the genome-wide chromatin changes incurred by such approach. To address this ambiguity and elucidate the role of H3K9me3 in ALT, we employed TRF1-KRAB fusion proteins for targeted modulation of heterochromatin at ALT telomeres. Our findings indicate that H3K9me3 promotes ALT, including ALT-associated PML bodies (APBs), C-circles, and mitotic DNA synthesis (MiDAS). Further investigation revealed that HP1, recruited by H3K9me3 marks, is instrumental in increasing ALT activity, as directing HP1 to telomeres via expression of TRF1-HP1 fusion proteins recapitulated these phenotypes.
We then turned to non-ALT cells and found that HP1 enrichment at HeLa telomeres is sufficient to induce robust APB formation and other features of an ALT-like state. We are therefore using this tool to characterize the mechanism by which HP1 promotes ALT activity. Our data suggest that deregulation of constitutive heterochromatin at telomeres may be involved in the activation and the maintenance of ALT.

Chromatin identity crisis: meiotic telomere functions reveal centromere-telomere interactions and interchangeability

Haitong Hou , Ana Lopez Morales , Noah Johnson , Rahul Thadani , Julia Promisel Cooper
University of Colorado Anschutz Medical Campus

Beyond their roles in preventing chromosomal fusion and degradation, telomeres play distinct roles in meiosis, when telomeres gather into a cluster at the nuclear envelope to form the conserved ‘bouquet.’ Not only does the telomere bouquet promote pairing between homologous chromosomes, which is essential for meiotic recombination and chromosome segregation, but also the bouquet creates a unique nuclear microdomain in which telomere factors are concentrated, leading to surprising bouquet functions. We found that the microdomain created by the fission yeast bouquet promotes the local nuclear envelope breakdown necessary for meiotic spindle formation, and promotes de novo centromere assembly, which is necessitated by a tendency of centromeres to become disassembled during meiotic prophase. These observations raise key questions about how telomeres promote nuclear envelope breakdown and centromeric robustness. Here we focus on why meiotic centromeres fall apart and how telomeres promote their reassembly. We found that Spo11, the meiotic DNA double strand break-inducing topoisomerase, and Rec8, a meiosis-specific cohesion, dismantle centromeres. We will outline the pathway from dismantled centromeres to reassembly via co-localization with telomeres, whose heterochromatic nature is essential for centromere reassembly. Recruitment by heterochromatin of the Aurora B kinase confers modification of

centromere factors and the reversal of Spo11/Rec8-mediated centromere destruction. This story highlights trans roles of telomeric heterochromatin and the associated subnuclear microdomain. Our studies extending these observations to human cells highlight dangers of activating facets of the meiotic program in the absence of the full range of cellular safeguards experienced in bona fide (bouquet-containing) meiotic cells.

Understanding the role of TERRA in the regulation of human telomerase

Nicole Bettin [1], Emmanuelle Querido [2], Irene Gialdini [1], Glenda Paola Grupelli [1], Elena Goretti [1], Marta Cantarelli [1], Mart Andolfato [1], Eslam Soror [1], Alessandra Sontacchi [1], Katarina Jurikova [1, 3], Pascal Chartrand [2], Emilio Cusanelli [1]

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In human cells, TERRA transcripts interact with telomerase. Furthermore, TERRA-mimicking oligonucleotides inhibit the activity of human telomerase in vitro. Nevertheless, the mechanisms by which TERRA regulates telomerase in human cells remain to be determined. By performing single molecule RNA FISH, we observed that TERRA transcripts and the telomerase RNA subunit hTR colocalize in the nucleoplasm and at telomeres in HeLa cells during different phases of the cell cycle. Moreover, using live-cell imaging and super-resolution microscopy, we found that endogenous TERRA molecules preferentially localize to long chromosome ends and relocate away from telomeres during telomere lengthening. Consistently, telomere elongation by telomerase correlates with a decline in the number of telomeric TERRA-hTR colocalizing transcripts and increased number of “TERRA-free” telomerase molecules at telomeres. These findings suggest that telomeric TERRA transcripts may interfere with telomerase activity.
To gain insight into the mechanism by which TERRA molecules can regulate telomerase, we depleted TERRA using antisense oligonucleotides and studied hTR localization to telomeres by fluorescence microscopy. TERRA depletion resulted in increased hTR clustering and enhanced localization of hTR to telomeres. Furthermore, hTR single particle and telomere tracking by dual imaging in time lapse revealed extended hTR residence time at telomeres and more frequent long-lasting telomere-hTR interactions in TERRA-depleted cells. Overall, our data support a model in which TERRA molecules contribute to coordinate telomerase access and retention to chromosome ends.

The fatty connection of telomeres

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Fatty acid metabolism, innate immune response and mitochondrial fitness are known to be interconnected. As we identified these signatures to be differentially regulated in isogenic immortalized fibroblast cell lines that are either telomerase- or ALT-positive, we investigated whether lipid metabolism, cytoplasmic telomeric DNA species and/or mitochondrial DNA release were involved in the observed differential induction of the innate immune response. In addition, we found that the telomere maintenance mechanism strongly modulates yet another aspect of cellular metabolism: the resistance of cells to ferroptosis, a cell death mechanism induced by the peroxidation of membrane phospholipids. Importantly, we confirmed our observations in telomerase-positive and ALT-positive sarcoma cell lines. Together, these data bring new light on the increasing connections between telomeres, telomere maintenance mechanisms and cellular metabolism.

Formation of telomeric circles reveals a general mechanism of eccDNA formation at tandem repeats

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IFOM ETS - The AIRC Institute of Molecular Oncology

Extrachromosomal circles made of telomeric repeats accumulate in ALT cells and in a wide range of mutants in genes involved in DNA metabolism. We have shown that single-stranded DNA damage occurring at telomeres is a common route to the generation of telomeric circles. Damaged repeats can interact with each other through base pairing and branch migration, leading to the formation of internal loops (i-loops) visible in two-dimensional agarose gels (a signal commonly known as the t-circle arc) as well as in electron microscopy. I-loops contain a Holliday junction at the base, which can be cleaved leading to excision of a telomeric circle. Because this process is driven by DNA damage occurring at repetitive elements, we asked if it occurs at other repeats in the genome. Using the same experimental conditions developed at telomeres, we found that, upon DNA damage, i-loops accumulate also at centromeric alpha satellite repeats. Electron microscopy analysis of enriched centromeric repeats showed that alpha satellite i-loops are larger than the telomeric ones, likely due to the difference in the size of the repeated unit. Differently from telomeres and alpha satellite repeats, i-loops did not accumulate at interspersed AluI and L1 repeats, indicating that this mechanism occurs preferentially at tandem repeats. Among the multiple conditions associated with i-loop formation, quite surprisingly we found that programmed cell death is associated with a strong accumulation of i-loops both at telomeres and alpha satellite repeats. The possible implications of these findings for telomere metabolism will be discussed.

CRISPR-based screens to illuminate the genetic landscape in response to telomere erosion and senomodulation

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The development of CRISPR-Cas9 technology has been a watershed moment whereby it is now possible to illuminate, in a single experiment, the entire genetic landscape of vulnerability (or resistance) to a particular genetic context or chemical treatment. In our labs, we have employed high-throughput, CRISPR-Cas9 pooled screens of individual gene knockouts in the human pre-B cell line, NALM-6, to query the genetic landscape of cells treated with chemicals that modulate senescence (senomodulators) or induce telomere erosion through telomerase inhibition (BIBR1532). Unexpectedly, cells treated with known pro-longevity senomodulators (resveratrol and pterostilbene) revealed a genetic signature indicative of DNA replication-induced stress, with similarities to the profile observed upon telomere erosion [1,2]. These screens also uncovered a novel regulator of cell fitness, called *C16ORF72/TAPR1/HAPSTR1* (Telomere Attrition and p53 Response Protein 1), which tapers the deleterious activation of the p53-mediated DNA damage response upon genotoxic stress [2]. These data underscore that DNA replication stress is a nexus that integrates cell fitness during aging and cell senescence. We suggest that DNA replication stress may, in some instances, act via a hormetic mechanism; i.e. it is deleterious at high levels but may be beneficial below a certain threshold. We will discuss these findings and their potential future applications, including the potential to harness senomodulators to avert the age-associated transition of functional cells into a non-dividing, senescent state.

[1] Benslimane et al., Molecular Cell 2020. doi: 10.1016/j.molcel.2020.07.010
[2] Benslimane et al., Aging Cell 2021. doi: 10.1111/acel.13331

Characterizing the role of individual shelterin components in telomerase-mediated telomere elongation.

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We present the development of cellular model systems designed to track telomerase activity in situ. Employing this method, we investigated the role of shelterin components in telomerase function. Our results confirm the central importance of TPP1 in telomerase activity and provide insights into the mechanism of POT1-mediated telomerase regulation.

TERRA Dynamics, R-loops and the Induction of Telomere Fragility

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We are interested in understanding how TERRA is recruited to telomeres forming R-loop structures and how TERRA R-loops influence telomere maintenance.
To monitor TERRA-telomere interactions, we employ live-cell imaging in HeLa cells tracking single PP7-stem-loop tagged TERRA molecules expressed from chromosome-internal sites and Halo-tagged TRF1. We find that while most associations of TERRA with chromosome ends are short-lived, longer lasting interactions do occur with higher frequency in cells with short telomeres. We suspect that the long-lasting interactions involve base pairing of TERRA with telomeric DNA in the form of R-loops.
We have also characterized TERRA-mediated R-loop formation in HeLa cells and its effects on telomere maintenance by inducing transcription of endogenous TERRA at chromosome ends using CRISPR activation. TERRA overexpression induced an increase in telomeric R-loops in dependency of the DNA recombinase RAD51 and RAD51-associated protein 1 (RAD51AP1). TERRA R-loops promoted formation of TIFs and increased telomere fragility suggesting that TERRA R-loops interfere with telomere replication. To test whether TERRA R-loop mediated telomere fragility involves replication fork collapse followed by DNA repair, we suppressed several DNA repair pathways. Interestingly, alleviation of telomere fragility required the concomitant suppression of two DNA repair pathways. One pathway involves PrimPol which can reprime DNA synthesis downstream of replication obstacles. The second pathway is Break Induced Replication (BIR), which also contributes to telomere maintenance in ALT cells. Together our results indicate that TERRA R-loops in telomerase-positive cancer cells are sufficient to trigger a major switch in telomere maintenance, from canonical semiconservative DNA replication to BIR.

Autophagy and innate immunity in telomere-mediated tumor suppression

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Cancers arise through the accumulation of genetic and epigenetic alterations that enable normal cells to evade telomere-based proliferative barriers and achieve immortality. One such barrier is replicative crisis, a cell death-dependent program that eliminates checkpoint-deficient cells at risk of malignant transformation. However, the mechanisms that control the onset of this anti-cancer barrier have not been explored, and the implications of avoiding crisis in human cancers remain unclear. Here, we identified the innate immune sensor Z-nucleic acid binding protein 1 (ZBP1) as a regulator of the crisis program. A crisis-associated isoform of ZBP1 is induced by the cGAS–STING DNA-sensing pathway but reaches full activation only when associated with telomeric-repeat-containing RNA (TERRA) transcripts that are synthesized from dysfunctional telomeres. TERRA-bound ZBP1 oligomerizes into filaments on the outer mitochondrial membrane of a subset of mitochondria, where it triggers an autophagy-mediated cell death pathway that eliminates cells that have escaped surveillance by the p53-dependent cell cycle checkpoints. Our study reveals a mechanism for telomere-mediated tumor suppression, whereby dysfunctional telomeres activate innate immune responses and autophagy through mitochondrial TERRA–ZBP1 complexes to eliminate cells destined for neoplastic

transformation. Disruption of this signaling pathway may be the primary mechanism that allows cells to evade crisis-associated cell death and evolve into malignancy.

Processing of oxidative damage at telomeres regulates cellular senescence

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Telomeres are hypersensitive to formation of the mutagenic oxidative lesion 8-oxoguanine (8oxoG), which triggers p53-mediated premature senescence by impairing telomere replication. OGG1 glycosylase initiates base excision repair (BER) by removing 8oxoG opposite C, and MUTYH glycosylase removes A misinserted opposite 8oxoG to enable subsequent repair. Using a chemoptogenetic tool to selectively generate 8oxoG in telomeres, we find OGG1 loss or inhibition, or MUTYH loss, partially rescues telomeric 8oxoG-induced senescence and associated phenotypes, while loss of both glycosylases causes a near complete rescue. Glycosylase deficiency also suppresses 8oxoG-induced telomere fragility and dysfunction, indicating that downstream single-stranded break (SSB) repair intermediates drive telomere replication stress. Exo-FISH reveals increased SSBs in WT cells following 8oxoG induction, but not in OGG1 knock out (ko) or OGG1/MUTYH double ko cells. Preventing SSB intermediates in double ko cells also suppressed telomeric 8oxoG-induced PARylation and PARP inhibitor sensitivity. These data suggest that inefficient completion of 8oxoG BER at telomeres triggers cellular senescence via SSB intermediates which disrupt telomere function. In contrast, we find OGG1 is required to prevent senescence caused by chronic telomeric 8oxoG, whereas MUTYH promotes senescence to prevent chromosomal instability from persistent damage over time. Growth of OGG1 ko cells was more severely impaired by chronic telomere damage compared to WT and MUTYH ko cells. Remarkably, cells lacking both glycosylases continued growing after chronic damage and failed to activate p53 despite increased micronuclei formation. Our data suggest that BER-generated SSBs, and MUTYH functioning at persistent telomeric 8oxoGs, mediate p53 activation and senescence.

The hidden functions and power of telomeric repeats in plants.

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The telomeres of most plant species are composed of telomeric repeats (TTTAGGG), a motif recognized by plant-specific TRB (Telomere Repeat Binding) proteins. TRB proteins possess a

Myb-domain like human TRF1/2, but unlike their human counterparts, TRBs also contain an H1-like domain. TRBs interact with the catalytic subunit of telomerase (TERT) and POT1a/b, colocalize with plant dyskerin, and serve as epigenetic regulators. We have clarified that TRBs emerged in evolution after the transition to a terrestrial habitat, and we have completed characterization of all TRB family members in Arabidopsis [1].
Telomeric repeats are not only localized at the telomeres but also internally, either as a long telomeric DNA tracts called interstitial telomeric sequences (ITSs) or as short repeats (telo-boxes). We have recently helped to show that TRB1 accessibility to telomeres and ITSs may be restricted by H1 [2]. Interestingly, telo-boxes are prevalently localized in gene promoters and they are also recognized by TRBs [3] and they may regulate gene transcription. To elucidate the precise localization of telo-boxes within gene promoters, we developed software named GOLEM (Gene regulatOry eLEMents, www.golem.ncbr.muni.cz). In GOLEM, the distribution and frequency of any motif of interest in relevant plant genomes, in the vicinity of the transcription start site (TSS), may be visualized in relation to the transcript levels of particular genes in different tissues. Novel findings about the function of telomeric repeats and TRB proteins across evolution will be discussed.
[1] Kusova, TPJ, 2023
[2] Teano, Cell Reports, 2023
[3] P.Schrumpfova, TPJ, 2016
Funding:Czech Science Foundation (20-01331X)

Apollo deficiency in humans and mice

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Apollo is a 5'-to-3' DNA exonuclease that interacts with TRF2 and participates in both DNA repair and telomere integrity (van Overbeek and de Lange, 2006, Lenain et al., 2006).
We identified 3 patients who carry a homozygous or compound heterozygous (in combination with a null allele) missense variant affecting the same residue L142 (L142F or L142S) located in the catalytic domain of Apollo. These individuals exhibit the telomere biology disorders dyskeratosis congenita (DC) and its severe variant, Høyeraal-Hreidarsson (HH) syndrome. Patients' Apollo-deficient cells exhibited chromosome instability, impaired DNA repair and signs of telomere fragility that were not associated with global reduction of telomere length (Kermasson et al., 2022).
To further explore the consequence of Apollo deficiency in vivo, we generated a Apollo L142S knock-in mouse model (Noted ApolloL142S/L142S). As observed in the Apollo KO mouse embryonic fibroblasts (MEFs), the ApolloL142S/L142S MEFs exhibit a high frequency of leading-leading telomere fusions, supporting the notion that Apollo L142S/L142S is unable to generate the protective 3' G-overhang at leading telomeres (Wu et al., 2012, Wu et al., 2010, Lam et al., 2010). However, while the Apollo KO mouse model is not viable (Akhter et al., 2010), the homozygous ApolloL142S/L142S mice are viable but present a severe phenotype and reduced life span. This demonstrates that the Apollo L142S mutation represent a severe hypomorphic mutation. Finally, the analysis of the Apollo L142S/L142S mouse model uncovered an unexpected role of Apollo in the development of the immune system. The most recent results will be presented and discussed.

Analysis of telomerase assembly using RNP-MaP

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The mature telomerase ribonucleoprotein (RNP) contains the telomerase RNA (TR) and 12 proteins and is organized into the catalytic and the H/ACA lobes. In addition to folding of TR into its three dimensional structure the 3' end of TR undergoes a sequence of processing events that eventually lead to the formation of the mature 451 nucleotide long telomerase RNA. How 3' end processing is coordinated with TR folding and telomerase RNP assembly is poorly understood. To analyze telomerase assembly in living cells we used RNP mutational profiling, which detects the sites of protein RNA interactions as RNA-protein crosslink induced mutations by high-throughput sequencing. We systematically define the TR-protein interaction network in a variety of different genetic backgrounds, using disease associated TR mutants, and as a function of the 3'-end maturation state to dissect the molecular mechanisms of telomerase biogenesis in human cancer cells.

Widely interspaced Zinc finger (WIZ) induced H3K9 trimethylation is necessary for ALT activity

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Alternative lengthening of telomeres (ALT) is a homologous recombination-driven mechanism of telomere maintenance present in approximately 10-15% of cancer, with most harboring mutations in ATRX/DAXX. However, ATRX loss is insufficient to trigger ALT, indicating the need for additional changes to drive ALT. We identified a Widely Interspaced Zinc Finger (WIZ) as a novel factor that regulates ALT phenotypes. WIZ is known to stabilize the G9a/GLP histone lysine methyltransferase complex, facilitating their interaction with chromatin and leading to H3K9 mono and dimethylation. WIZ exhibits strong telomeric localization in the majority of ALT cell lines tested but not in telomerase-positive lines. Depletion of WIZ in ALT lines results in an expected global loss of H3K9 di and trimethylation. Notably, WIZ loss leads to a significant reduction in ALT phenotypes, including APBs, single-stranded telomeric DNA, and C-circles. Furthermore, ALT lines are sensitive to WIZ depletion by CRISPR/Cas9. Last, we show that expression of a ZF6 domain of WIZ ¬ – required for G9a/GLP interaction – linked to the DNA binding domain of Teb1 (TebDB) for telomeric tethering, was sufficient to rescue ALT phenotypes. In conclusion, our findings suggest that WIZ plays a key role in regulating heterochromatic marks required for ALT accessibility and maintenance.

NanoTelSeq enables accurate analysis of single telomeres and their subtelomeric methylation states in healthy individuals and telomere biology disease patients

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Telomere length measurement is crucial to basic and clinical research and diagnosis. Beyond length, other features associate with telomere function, such as telomeric overhang length, sequence variations and modifications of the telomeric repeats, the state of subtelomeric methylation and TERRA expression. However, current methods are limited in their ability to accurately measure and correlate multiple features at the single telomere level. We have developed a method, termed NanoTelSeq, for measuring the absolute length and composition of individual telomeres by long-read Nanopore sequencing. NanoTelSeq enables the analysis and correlation of telomere length with other features including the identity of the chromosome end and subtelomeric DNA methylation at a single molecule level. We examined telomeres of healthy individuals and patients diagnosed with pulmonary fibrosis, dyskeratosis congenita and Hoyeraal Hreidarsson syndrome, and compared the results of NanoTelSeq with those obtained by telomeric restriction fragment (TRF) analysis. Furthermore, we analyzed the telomeres of ICF patients defective for DNA methylation and correlated their telomere length with the state of their subtelomeric methylation at individual chromosome ends. NanoTelSeq reveals the absolute length distribution and the length and abundance of the shortest telomeres at a resolution that enables precise diagnosis of patients of telomere biology diseases and risk assessment of developing disease in mutation carriers. It also enables the classification of ICF syndrome patients of different subtypes based on telomere length and subtelomeric methylation. Finally, it provides an invaluable tool for studying telomere structure and function in a resolution not possible previously.

Functions of the Saccharomyces cerevisiae CST complex in double-strand break repair

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Following a double-strand break (DSB), resection is an important step that controls the repair pathway choice between homologous recombination and non-homologous end-joining (NHEJ). Telomeres are also subjected to resection, which is counteracted by the Ku and the Cdc13-Stn1-Ten1 (CST) complexes.
The human CST (where C is CTC1) has been involved in DSB processing, by promoting fill-in synthesis, and repair pathway choice, as an effector of the 53BP1-RIF1-Shieldin axis. In

Saccharomyces cerevisiae, the CST complex can localize at DSBs to promote de novo telomere formation. However, whether it contributes to DNA repair is not clear. Additionally, because of its essential function in telomere protection, the genetic approaches to study the question are limited.
Here, we circularized the single linear chromosome of an engineered yeast strain (Shao et al. 2018) to generate a strain without telomeres and where the CST is no longer essential. We then used an inducible CRISPR/Cas9 system (Anand et al. 2017) to create a single targeted DSB. We found that following DSB, survival is mostly NHEJ-dependent and significantly decreased without CST. Using resection assays and CST-Polalpha interaction mutants, we found that CST and Polalpha-primase limit the extent of single-stranded DNA. Analysis of the break site after repair in surviving colonies revealed that the stn1Δ mutant showed a unique mutational signature with less deletions, in particular those involving microhomologies, than wild-type, suggesting a role both in NHEJ and microhomology-mediated repair.
By regulating single-stranded DNA, the CST complex might therefore regulate the choice between multiple DSB repair pathways.

Epigenetic inheritance of a telomere capping defect triggers longevity in C. elegans

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The Protection Of Telomeres 1 (POT1) protein interacts with single stranded telomeric DNA.C. elegans POT1 homologs POT-1 and POT-2 form abundant nuclear foci at telomeres in adult germ cells. However, POT-1 and POT-2 foci vanish from telomeres in 1-cell embryos and gradually accumulate during wild type embryonic development (1). POT-2 fails to interact with telomeres in pot-1 mutants. Children and grandchildren of pot-1 mutants possess wildtype POT-1 protein but display little or no POT-1 or POT-2 at telomeres. This is an example of transgenerational epigenetic inheritance at telomeres, where a phenotype is inherited for more than one generation in the absence of the initiating trigger (lack of POT-1).

Traits that are epigenetically transmitted for multiple generations are hypothesized to improve fitness of future generations. We found that pot-1 mutants and their F1 cross progeny that lack POT-1 and POT-2 at telomeres display moderately extended lifespan. Mutations that disrupt genome silencing marks also elicit loss of POT-1 from telomeres, suggesting that pot-1 mutant telomeres may be in a desilenced epigenetic state that is remembered for multiple generations. We found that lack of pot-1 suppresses the ability of daf-2 insulin/IGF-1 signaling mutants to form dauer larvae, which are a stress resistant developmental state that occurs in response to exogenous stresses. Consistently, RNAi knockdown of TERRA promotes formation of daf-2 mutant dauer larvae. Our data suggest that pot-1 mutants possess a heritable telomere uncapping phenotype that modulates adult longevity and development in response to environmental stress.

1) Lister-Shimauchi et al., Communications Biology 2021.

cGAS-STING activation causes premature aging and cancer in zebrafish.

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Telomere shortening is a hallmark of aging. When telomeres become critically short, they trigger DNA damage responses mediated by the p53 pathway, leading to apoptosis or replicative senescence. To understand the consequences of telomere shortening at the organism level, we use the zebrafish, a model organism that mirrors human telomere erosion during aging. Telomerase-deficient zebrafish (tert) displayed premature aging phenotypes, including reduced fertility, cachexia, increased inflammation, and age-associated diseases. Strikingly, we identified the involvement of the cGAS-STING pathway in tert premature aging. tert mutants show cytosolic DNA and micronuclei resulting in the activation of cGAS-STING pathway and type I interferon-mediated inflammation. Notably, sting tert double mutants suppressed the premature aging of telomerase mutants, mimicking the effects observed in tp53 tert double mutants.
Despite similar telomere lengths between sting tert and tert single mutants, the absence of cGAS-STING activation fails to up-regulate p53 protein, resulting in reduced senescence and

inflammation. Surprisingly, sting tert zebrafish have reduced spontaneous cancer incidence when compared to tert mutants. Consistently, Sting inhibition by H-151 of senescent zebrafish larvae leads to diminished p53 signalling and decreased metastatic progression. Reduction of tumorigenesis through cGAS-STING inhibition challenges the current use of Sting agonists in cancer therapy. We propose that activation of cGAS-STING in an aging microenvironment contributes to chronic inflammation, thereby fostering cancer and metastasis. However, this may change in established tumors maintained by a dormant immune system, where the hyperactivation of cGAS-STING triggers an immune checkpoint and cancer decline.

Consequences of telomere dysfunction in different cell types for kidney fibrosis development

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Consequences of telomere dysfunction in different cell types for kidney fibrosis development

Tubulointerstitial fibrosis associated to chronic kidney disease represents a global healthcare problem. We have previously shown that short and dysfunctional telomeres sensitize the kidneys to develop fibrosis, however, the cellular origin of kidney fibrosis related to telomere dysfunction remains unknown. To identify the specific cell type responsible for this type of renal fibrosis, we induced telomere dysfunction by deleting the Trf1 telomere-binding factor in fibroblasts (COL1a2 positive), pericytes (PDGFRβ positive), epithelial renal tubule cells (CADHERIN-16 positive) and epithelial basal layer cells (P63 positive). We are addressing the functional and histopathological effects including fibrosis in kidney resulting from ablation of Trf1 in these cell types. In this meeting, we will report the characterization of these models which will aid in the development of novel antifibrotic therapies for chronic kidney diseases.

Nuclear F-actin and DNA replication stress regulate the recruitment of human telomerase to telomeres

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While the biochemical mechanism of telomerase is reasonably well characterised, less is known about how it is regulated in human cells. We have previously linked the DNA damage response (DDR) to the recruitment of human telomerase to telomeres, specifically via the regulatory kinase ATR, which coordinates the cellular response to replication stress (1). Furthermore, nuclear filamentous actin (F-actin) is important for DNA replication dynamics following replication stress (2). Given the convergence of these phenomena, we hypothesise that there is controlled interplay between DNA replication, the DDR, and telomere maintenance, which together result in cancer cell immortalisation.

Here we have demonstrated, via fluorescence in situ hybridisation (FISH) and live cell imaging, that nuclear F-actin is important for telomerase recruitment, as inhibition of actin polymerisation decreases the presence of telomerase at telomeres. This occurs in an ATR and

mTOR-dependent manner, employs other regulators of actin structure and function and involves myosin. Specifically, nuclear actin filaments act as sites for telomerase recruitment, potentially due to telomere tethering on F-actin under conditions of replication stress. Telomeres are also re-localized toward the nuclear periphery via F-actin in response to replication stress. Furthermore, the recruitment of telomerase to telomeres occurs in proximity to stalled replication forks. Our data support a model wherein telomeric replication stress, a natural occurrence in the highly repetitive telomere sequence, triggers the DDR and nuclear F-actin polymerization, ultimately resulting in telomere length maintenance.

1. Tong et al. (2015) Cell reports 13, 1633-1646
2. Lamm et al. (2020) Nat Cell Biol 22, 1460-1470

Depletion of the Leishmania spp. TERT and TER telomerase components triggered telomere shortening on different chromosomes

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We have recently shown that the individual depletion of the Leishmania spp. TERT and TER components are detrimental to parasites. The absence of TERT is pleiotropic compared with the TER, inducing telomere shortening, autophagy, DNA damage, DNA replication problems, decreased parasite viability, alterations in the cell membrane structure and composition, and consequent defects in metacyclogenesis that culminated in high reduction or loss of parasite infectivity capacity. The knockout of TER and its overexpression were less drastic, but besides telomere shortening and low viability, induced autophagy, replication stress, and reduced infectivity without affecting metacyclogenesis. To understand the biological differences among these mutants, we perform whole genome sequencing of mutants and the parental lines using Oxford Nanopore. Genomic libraries were constructed using the Native Barcoding Kit, and the PromethION 2 Solo was used for sequencing. The long reads were mapped against the Leishmania major friedlin reference genome using the Minimap2 aligner software. The bigwig files for visualization and further analysis were generated using the DeepTools package. To identify telomeric repeats, the Seqkit package was used, and the JBrowser genome viewer generated the graphs and images. Supported by a decrease in the number of reads detected in the telomeric regions, the mutants showed 15 out of 36 chromosomes with decreased telomere length. The TERT knockouts showed shorter telomeres in the R and L arms of six chromosomes, whereas the TER knockouts presented only two. These findings highlight the existence of different mechanisms regulating telomere length in Leishmania.

Single-molecule imaging of telomerase provides mechanistic insights into cancer-associated POT1 mutations

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Heterozygous dominant mutations in POT1 identified in certain cancers promote various chromosomal abnormalities, such as telomere fragility, end-to-end chromosome fusion, and rapid telomere elongation, events that are known to fuel cancer progression. The later capacity to promote telomerase-dependent telomere elongation was suggested to be the prime driver of cancer progression. The current model posits that cancer-associated POT1 mutations fail to inhibit telomerase access to telomeres, although this model has not yet been validated in vivo. Indeed, some mutants in the POT1 OB-fold domains, like K90E, do not impact POT1 interaction with telomeric ssDNA in vitro, but still result in telomere elongation and telomere fragility in vivo.

We applied single-molecule imaging of telomerase RNA in living cells to measure the impact of cancer-associated POT1 mutations on telomere accessibility for telomerase binding. We observed that overexpression of POT1 increases telomerase probing at telomeres, independently of the capacity of POT1 to bind telomeric ssDNA. We found that expression of mutants POT1-ΔOB, Y223C, D224N or K90E increases telomere accessibility for telomerase interaction and the cumulative dwell-time of telomerase particles at telomeres. However, unlike POT1-ΔOB or D224N mutants, the POT1 Y223C and K90E mutations also increased the residence time of long-lasting telomerase particles at telomeres, which are dependent on hTR base-pairing with telomeric ssDNA. Our data reveal that telomere lenghtening in cells expressing cancer-associated POT1 mutants can arise from the dual impact of these mutations on telomeres accessibility and on telomerase retention at telomeres.

TERRA Nanopore Direct RNA Sequencing and Its Upregulation with Human Aging

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TERRA, a telomeric-containing RNA that is transcribed from subtelomeric regions, poses challenges in understanding its complete sequences. Using Oxford Nanopore direct RNA sequencing and TERRA capture, we annotate TERRA transcripts from different chromosome ends in the T2T-CHM13 reference genome, encompassing hundreds to over a thousand nucleotides of telomeric repeats, predominantly originating from 61-29-37 bp repeat promoters that display enrichment of H3K4me3, RNA pol II, CTCF, and R-loops. We develop a bioinformatic pipeline to quantify chromosome-end-specific TERRA from RNA-seq datasets, revealing a positive correlation between TERRA levels and age in blood and brain tissues. Additionally, TERRA levels increase in fibroblasts derived from old individuals, with abnormal profiles in Hutchinson-Gilford Progeria patients. Single-cell RNA-seq analysis demonstrates TERRA expression in various cell types, displaying increased TERRA in neurons during the early stage of Alzheimer's disease progression. Our study provides a comprehensive analysis of TERRA transcripts, linking TERRA to human aging and diseases.

Regulation of Taz1 functions in telomere replication in fission yeast

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In yeast and mammals, telomeres are one of the most difficult regions to replicate, due to the many obstacles that impede replication fork progression. Thus, replication forks naturally slow down and eventually stop as they approach telomeric repeats. The fission yeast protein Taz1 and its human counterpart TRF1 are known to play crucial roles in DNA end replication (Miller et al., 2006; Sfeir et al., 2009). DNA-bound telomeric proteins also act as barriers to fork progression (Ohki & Ishikawa, 2004; Douglas & Diffley, 2021). To reconcile these seemingly contradictory observations and better understand telomere replication, we further investigated the function of Taz1. We discovered that Taz1 is phosphorylated by the Rad3(ATR) kinase in its dimerization domain. Using Y2H, we showed that the phosphomimetic mutant of Taz1 (S2D), but not the phosphomimetic mutant (S2A), prevents dimerization. Introduced into the S. pombe genome, S2D mutants show slightly longer telomeres. ChIP in synchronized cells revealed that the pattern of Taz1 recruitment to telomeres and subtelomeres in S phase is altered by the S2D mutation. We have shown that Taz1 is also phosphorylated by CdK1 in a domain adjacent to the dimerization domain. I will present our latest data on the regulation of Taz1 functions at telomeres. The current working model suggests that Taz1, bound to DNA, slows the progression of replication forks, giving the cell time to remove obstacles in front of the fork, and then Taz1 is evicted in a phospho-dependent manner to allow the fork to restart.

Chromosome end protection by RAP1-mediated inhibition of DNA-PK

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Telomeres maintain individual, linear chromosomes by inhibiting DNA repair pathways including classical NHEJ (cNHEJ). In spite of this, repair factors including the DNA-dependent protein kinase (DNA-PK), are readily detected at telomeres in mammalian cells. How cNHEJ is avoided at telomeres when DNA-PK is bound remains unclear. Here we show that the Shelterin components TRF2 and RAP1 form a complex with DNA-PK that directly represses its end joining function at telomeres. Biochemical experiments and cryo-electron microscopy reveal that when bound to TRF2, RAP1 establishes a network of interactions with KU and DNA that prevents DNA-PK from recruiting LIG4. In cells, RAP1 is redundant with the APOLLO nuclease in repressing cNHEJ at chromosome ends, demonstrating that inhibition of DNA-PK prevents telomere fusions in parallel with overhang-dependent mechanisms. Together, our experiments show that shelterin can prevent cNHEJ by directly blocking recruitment of end-joining factors to DNA-PK, establishing a molecular mechanism for how stable linear chromosomes can be maintained

Individual cell fate and population dynamics revealed by a mathematical model linking telomere length and replicative senescence

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Progressive shortening of telomeres ultimately causes replicative senescence and is linked with aging and tumor suppression. Studying the intricate link between telomere shortening and senescence at the molecular level and its population-scale effects over time is challenging with current approaches but crucial for understanding behavior at the organ or tissue level. In this study, we developed a mathematical model for telomere shortening and the onset of replicative senescence using data from Saccharomyces cerevisiae without telomerase. Our model tracks individual cell states, their telomere length dynamics, and lifespan over time, revealing selection forces within a population. We discovered that both cell genealogy and global telomere length distribution are key to determine the population proliferation capacity. We also discovered that cell growth defects unrelated to telomeres also affect subsequent proliferation and may act as confounding variables in replicative senescence assays. Overall, while there is a deterministic limit for the shortest telomere length, the stochastic occurrence of non-terminal arrests drive cells into a totally different regime, which may promote genome instability and senescence escape. Our results offer a comprehensive framework for investigating the implications of telomere length on human diseases.

Unraveling the genetic and epigenetic impacts of large-scale chromosomal rearrangements in Arabidopsis thaliana

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Chromosomal rearrangements can result from large-scale structural changes in the genome, such as inversions, translocations, duplications, and deletions. These rearrangements often have significant implications for the plant's genetic makeup and epigenetic regulation. Importantly, it is even possible to simulate large-scale genome re-arrangements mimicking those that occurred naturally during plant evolution. Using CRISPR/Cas9 approach, Arabidopsis thaliana lines with stable reciprocal translocations of chromosome arms (Beying et al., 2020) and inverted chromosome (Rönspies, 2022) were constructed. We studied phenotype, telomere lengths, levels of transcripts and chromatin structure in homozygous generations of these plants.
These analyses are supposed to reveal whether and how chromatin organization is affected in response to changes in genome organization, and whether and how respective changes are inter-related.
Understanding the impact of chromosomal rearrangements in Arabidopsis thaliana can provide valuable insights into the evolutionary dynamics and adaptive potential of plant genomes.

Beying, N.; Schmidt, C.; Pacher, M.; Houben, A.; Puchta, H. CRISPR-Cas9-mediated induction of heritable chromosomal translocations in Arabidopsis. Nat Plants 2020, 6, 638-645.
Rönspies, M.; Schmidt, C.; Schindele, P.; Lieberman-Lazarovich, M.; Houben, A.; Puchta, H. Massive crossover suppression by CRISPR-Cas-mediated plant chromosome engineering. Nat Plants 2022, 8, 1153-1159.

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Ultrabright telomeric foci and telomere clustering in the alternative lengthening of telomeres.

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Ultra-bright telomeric foci (UTs) and telomere clusters (TC) are Fluorescence In-Situ Hybridization (FISH) phenotypes, characterizing the Alternative Lengthening of Telomeres (ALT). Homology driven interactions bridge ALT telomeres to allow telomere maintenance via intra-chromosomal or inter-chromosomal RAD52-dependent, or RAD52-independent, Break-Induced-Replication (BIR). ALT-associated UTs are considered an extreme outcome of multi-telomere clustering that promotes ALT. However, UTs can be triggered by dysfunctional telomeres due to defective shelterin protection in both ALT-positive and ALT-negative cells suggesting that extreme UTs-forming telomere clustering may be distinct from telomere clustering that drives ALT. We applied telomere specific PNA FISH in a panel of human ALT cell lines and classified TC as equal or bigger than 0.4 μm in diameter, while considering UTs assemblies bigger than 0.9 μm. We found that in ALT+ immortalized cell lines UTs are extremely rare, they are limited in numbers per/nucleus, they attract RPA and its counterpart ATRIP and denote cells that have undergone one or more rounds of whole genome duplication. On the other hand, 0.4-0.8 μm TC, are common between ALT cells, involve limited numbers of chromosome termini and frequently colocalize with the recombinase RAD52. In contrast to UTs induction by DNA replication stress, knockout of the exonuclease EXD2, Cyclin-E overexpression, or Aphidicolin exposure did not affect TC. Interestingly ALT repression via reconstitution of telomerase activity suppresses TC, whereas RAD52 knockout increases TC frequencies suggesting that although TC reflect a snapshot of the ALT process, they may also represent failure to complete RAD52-driven inter-chromosomal telomere elongation.

Micronucleus Derived from Chromosome Fusion Is Not a Potent Inducer of cGAS-STING Pathway

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Chromosome fusion, a deleterious consequence of telomere dysfunction, results in mitotic failure and micronuclei (MN) formation, known to interface with the innate immune response. Despite the recognized rupture of MN membranes during interphase and subsequent cGAS accumulation, potentially activating the STING pathway, direct evidence linking MN to cGAS activation has been elusive. We have developed the FuVis2 reporter system, which allows precise visualization of nuclei harboring sister chromatid fusions and the resulting MN. Using FuVis2 equipped with cGAS and STING reporters, we thoroughly assessed cGAS activation by MN in living cells. Our data show that, contrary to expectations, cGAS primarily associates

with MN during mitosis, and this interaction does not lead to STING activation or trigger an interferon response after mitosis. Gamma irradiation activates STING independently of MN formation or cGAS recruitment to MN, challenging the presumed role of MN in cGAS/STING pathway activation. These results suggest a re-evaluation of the impact of MN on cellular immunity, portraying them as largely inert entities with a potential bias towards chromosomal abnormalities. This reassessment highlights the importance of further exploration into the role of MN in telomere biology, chromosomal instability, and the immune response.

Identification of key regulators of DDR signaling and DNA repair at deprotected telomeres

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Besides controlling telomere length, the shelterin complex protects natural chromosome ends from activating ATM- and ATR-dependent DNA damage checkpoint responses and prevents inappropriate repair activities by the homologous recombination (HR), non-homologous end-joining (NHEJ), and microhomology-mediated end-joining (MMEJ) repair machineries. Upon critical shortening or shelterin malfunction, telomeres activate a DNA damage-like response that involves accumulation of DNA damage proteins at telomeres and posttranslational protein modifications. Although several critical components of the telomere damage response have been identified, we still poorly understand the exact nature of the telomere damage signal, how it is sensed, transmitted and terminated. Neither is it well understood what facilitates the execution of DNA repair activities at telomeres. To address this, we developed functional genetic screens to identify factors with a critical role in NHEJ at deprotected telomeres. These resulted in the identification of MAD2L2/REV7, and additional shieldin factors, as key regulators of NHEJ, but also of multiple other factors that we have characterized for their role in telomere NHEJ or are still in the process of studying. More recently we developed genome-wide FACS-based loss-off-function genetic screens to identify factors critical for activation, maintenance or termination of telomere damage response signaling. Both types of screening approaches independently of each other identified different ubiquitin system factors that are each involved in controlling the ATM-dependent phosphorylation of KAP1 upon telomere deprotection. We will present how these factors, each through different mechanisms, affect KAP1 phosphorylation and what the associated consequences are for genome stability.

ZBTB48 causes persistent chromatin bridges in ALT-positive cells

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Telomeres and its associated proteins are essential for genome stability. Previous work had established that ZBTB48/TZAP directly binds to telomeric DNA and acts as a negative regulator of telomere length in both telomerase-positive and ALT-positive cancer cells. In the

latter, ZBTB48 is particularly abundant at telomeres and its overexpression had been reported to cause rapid telomere shortening, but the exact molecular mechanism has not been definitively established. Here, we describe that ZBTB48 overexpression induces chromatin bridge formation as the primary cause of telomere attrition. These bridges strikingly resemble similar phenotypes induced by the dominant-negative TRF2 ΔBΔM mutant, which eventually culminate in chromothripsis. ZBTB48-induced chromatin bridge formation is exclusive to ALT-positive cellular contexts, dose-dependent and leads to concomitant C-circle accumulation while the bridges persist for hours into interphase as observed by live cell imaging. In addition, since ZBTB48 moonlights as a transcription factor, we identified separation-of-function mutants that differentiate between the transcription factor activity and telomeric role to carefully demonstrate that ZBTB48 induces telomeric bridges in cis. Further leveraging on these mutants, we profile protein interactions contributing to the chromatin bridge formation by BioID coupled to label-free quantitative mass spectrometry. Overall, our data demonstrate a profound role of ZBTB48 on telomere stability unique to ALT-positive contexts.

Heterogeneity of the activity of the alternative lengthening of telomeres pathway in neuroblastoma tissue

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Alternative lengthening of telomeres (ALT) is a telomerase-independent telomere maintenance mechanism that exploits aberrant DNA repair and recombination to prevent replicative senescence in cancer cells without reactivation of telomerase. In some tumors, like neuroblastoma, pediatric glioblastoma and leiomyosarcoma, ALT occurs frequently and is linked to high-risk cases and poor outcome. ALT activity manifests itself by the production of single-stranded circles with the telomere C-rich sequences, as detected by the C-circle assay in bulk samples. However, information on the intratumor heterogeneity with respect to ALT activity and its functional implications is lacking. To address this, we have applied our previously developed ALT-FISH method to neuroblastoma tissue sections (Frank et al. 2022 Nucleic Acids Res, gkac113). ALT-FISH detects single-stranded telomeric DNA and RNA repeats via a fluorescence microscopy read-out in single cells. Here, we have performed ALT-FISH on tissue sections from a cohort of 40 neuroblastoma patients in combination with immunostaining of the tumor-cell marker NCAM and DNA. We developed a quantitative image analysis pipeline to assess: (i) The correlation of ALT-FISH signal, C-circle assay results and the detection of ALT-associated PML bodies. (ii) The intra-tumor heterogeneity with respect to ALT activity. (iii) Cell cycle dependent changes in ALT activity as inferred from the cellular DNA content. (iv) The spatial relations of cells with similar/different ALT activities. Our results provide novel insights into the heterogeneity of ALT-positive neuroblastoma and have a number of implications for the evolution of tumor subclones that have acquired ALT as a telomere maintenance mechanism.

smFRET Investigation of Structural Dynamics During T-Loop Formation

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Telomere loop (T-Loop) formation has been suggested as a mechanism of regulating transcription and replication. Formation of T-loop may lead to telomerase blockage and replication fork stalling, which play key roles in telomere shortening and maintenance. However, the molecular mechanism of T-loop formation has not been solved, as techniques to track T-Loop formation have been limited by their resolution and sample preparation methods. Here, we aim to monitor the DNA structural change during T-loop formation using single-molecule Forster resonance energy transfer (smFRET). Telomeric DNA constructs designed to detect the 3' G-rich overhang unfolding by TRF2 showed a stretching of the overhang that does not recover after TRF2 removal. This data suggests that the 3' overhang participates in T-loop formation and remains stretched after. Telomeric DNA constructs designed to detect the overhang looping to the upstream telomeric dsDNA showed that TRF2 causes the overhang to loop back to the very end of a 120 bp telomeric dsDNA, whereas the longer 216 bp dsDNA creates loops of variable size. This data shows that the 120 bp dsDNA construct formed a T-Loop, which is smaller than the reported minimum size. As expected, scrambling the telomeric sequence or removing the dsDNA region eliminates the DNA response from TRF2 incubation. Our work presents a novel system to study the dynamics of TRF2-induced T-Loop formation at the single molecule level. With this system, we proposed a model that describes the DNA structural dynamics during T-loop formation.

The interplay between telomere attrition and EV biology

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Late abstract-Authors list (to be updated)
Yi Gong, Carlos Nogueras-Ortiz, Nikki Noren Hooten, Francheska Delgado-Peraza, Kotb Aly, Mithlesh Termre, Abby Molnar, Wei Zhu, Yongqing Zhang, Deborah Croteau, Myriam Gorospe, Michele Evans, Dimitrios Kapogiannis, Yie Liu

Telomere attrition is a hallmark of aging and associated with human diseases, yet its impact on extracellular vesicles (EVs) remains poorly understood. Here, we investigated the alterations in EV abundance, size, cargo contents, and functional implications in the context of a telomerase reverse transcriptase deficient (Tert -/-) mouse model with progressive telomere shortening. We observed an alteration in circulating plasma EV concentration and size in generation 3 (G3) Tert -/- mice with short telomeres. Furthermore, tissue-specific alterations in EV levels and telomeric DNA cargo were observed. Proteomic analysis revealed differential expression of proteins involved in inflammation and lipid metabolism in plasma- and tissue-derived EVs. Functionally, EVs from G3 Tert -/- mice showed adverse effect on recipient cells. These findings highlight the intricate interplay between telomere dynamics and EV biology, underscoring the potential of EVs as biomarkers and therapeutic targets for telomere-attrition associated diseases.

Regulation of the shelterin complex by phosphorylation of TRF2

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Telomeres are protected by the shelterin complex that suppresses DNA damage response at exposed DNA ends and prevents undesired fusion of telomeres. TRF2 and TRF1 proteins promote folding of the telomeric DNA into a t-loop structure and suppress activation of ATM/ATR kinases, however, the precise mechanisms modulating DDR at telomeres are incompletely understood. We have found that TRF2 is phosphorylated at S410 by ATR upon induction of DNA breaks at telomeres. Phosphorylation of TRF2 impaired recruitment of DNA repair factors 53BP1 and RAD51 to the damaged telomeres and this phenotype was rescued by expression of the non-phosphorylatable TRF2-S410A mutant. Inversely, we found that TRF2 phosphorylation stimulates its interaction with TIN2 in vitro and promotes TIN2 localization at telomeres. We propose that the tightly associated shelterin components reduce the accessibility of the broken telomeric DNA to repair factors. Further, we show that TRF2-pS410 is counteracted by PPM1D phosphatase that interacts with several shelterin components and associates with telomeres. Superresolution microscopy did not reveal significant changes in t-loop formation in PPM1D knock-out cells. On the other hand, PPM1D overexpression reduced the level of TIN2 and TRF2 at telomeres and increased the frequency of telomeric fusions. As PPM1D oncogene is commonly overexpressed in human cancers, the telomeric dysfunction caused by excessive TRF2 dephosphorylation may contribute to genome instability during tumorigenesis. In the summary, we report that shelterin function is inversely regulated by activities of ATR and PPM1D. On the meeting, I will discuss our advances in understanding of PPM1D functions at human telomeres.

Restriction on Ku Inward Translocation Caps Telomere Ends

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Telomere end protection against chromosome end fusions by non-homologous end joining (NHEJ) is essential to genome integrity. The ubiquitous binding of the NHEJ complex Ku to telomere ends requires the NHEJ-promoting roles of Ku to be sharply muted at telomeres. How this is achieved is unknown. It is also unclear how Ku and the end-proximal telomere proteins (Rap1 in the yeast Saccharomyces cerevisiae) co-exist at telomere ends and whether proteins bound to telomere double-stranded DNA closer to the ends have unique roles.
Here, we addressed the role of Rap1 in the control of Ku at yeast telomeres and determined the structure of telomere tips. In vitro, Rap1 and Ku form stable complexes on short DNA duplexes. Solving the structure of a Rap1-DNA-Ku complex reveals how Rap1 constraints the Ku inward translocation needed for NHEJ. Surprisingly, Rap1 and Ku in this stable complex overlap on DNA without interacting. In vivo, a single Rap1 protein bound to a broken end antagonizes NHEJ repair without challenging Ku presence. Nanopore-sequencing of telomere

fusions show that the end-proximal Rap1 at native telomere tips contributes to telomere protection, supporting the importance of Ku control by Rap1. Together, our results uncover a telomere length-independent mechanism of end protection acting by restricting Ku inward translocation, a functional switch preventing promiscuous NHEJ repair at telomeres.

Deep scanning mutagenesis of Shelterin for high-throughput characterization of clinical variants of unknown significance

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Mutations in Shelterin are associated with a spectrum of human disease from cancer to telomere biology disorders such as dyskeratosis congenita. Hundreds of clinically-identified variants of unknown significance (VUS) have been identified in these genes, but delineating which mutations are truly pathogenic versus which mutations have mild or no effect on gene function at this scale has proven to be a challenge. We have therefore developed primary human cell systems for high-throughput VUS characterization of Shelterin proteins, including POT1, TIN2, and TPP1. By engineering locally haploid human embryonic stem cells (loHAPs) through targeted deletions of one full copy of the gene of interest, we can then perform deep scanning mutagenesis of the remaining allele in a bulk population, which can be maintained in culture over the course of several weeks. Because only one allele of the gene remains, this provides a direct genotype-to phenotype readout of VUS impact on cell viability. Mutations which lead to telomere deprotection are quickly depleted from the population, allowing for the isolation of variants which perturb telomere length homeostasis. We will present our progress on characterizing VUS in these proteins as well as analysis of separation of function mutations that highlight how these Shelterin proteins mediate telomere protection and length control.

POT1a humanized mouse models

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The telomeric protein POT1 has been found mutated in several types of human cancer, both sporadic and familial. Several POT1 mutations found in cancer favour acquisition of the malignant features, such as longer telomeres and a higher chromosomal instability. Other POT1 mutations, however, have been found in patients with telomeropathies characterized by having short telomeres. To address the molecular mechanisms underlying the pathogenic effects induced by POT1 mutant variants in humans, we have generated humanized Pot1a knock-in mouse models by introducing these mutations in the Pot1a endogenous locus. In this meeting, we will present the characterization of these models which constitute a useful tool to understand POT1-mediated pathologies and to study therapeutic strategies to treat these diseases.

Replication Fork Proteome Profiling by BLOCK-ID Identifies the Chromatin Reader TRIM24 in ALT

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Chromatin-based DNA damage response (DDR) pathways are vital for the maintenance of genome integrity, including during DNA replication. We have developed a new technique called BLOCK-ID, which allows for proteomic profiling using proximity ligation and visualization of proteins at blocked replication forks generated at LacO arrays bound to Lac repressor (LacI), which is fused to a tagged Bio-ID enzyme. Through BLOCK-ID, we identified and validated many human BRD proteins associated with blocked replication forks. These include the BRD protein TRIM24, which we demonstrate to be involved in replication stress response pathways. Further proteomic analyses identified TRIM24 involvement in Alternative Lengthening of Telomeres (ALT). TRIM24 deficiency results in telomere loss, reduced telomere sister chromatid exchanges and reduced hallmarks of ALT including c-circles and ALT-associated PML bodies (APB). Mechanistically, TRIM24 ALT activity is governed by post-translational modifications (PTMs), including histone acetylation by the histone acetyltransferases p300/CBP, which were found to be required for TRIM24 recruitment to telomeres in ALT cells. Mechanistically, forced tethering of TRIM24 revealed PIAS1 SUMO-dependent interactions between TRIM24 and PML that function to dynamically associate telomeres with APB bodies to support ALT telomere synthesis. In addition, tethering of TRIM24 rescues ALT activity in PML knockout cells in a SUMOylation-dependent manner. Collectively, this study delivers a new methodology for surveying replication stress proteins in human cells and identifies a PTM-driven chromatin pathway reliant on the acetylation effector TRIM24 that promotes ALT through SUMO. This study also provides a rationale for therapeutically targeting this PTM-mediated TRIM24 pathway in ALT cancers.

Cytoplasmic exposure facilitates resolution of non-sister telomere entanglements

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Replication forks stall irreversibly at telomeres lacking the fission yeast telomere protein Taz1 (TRF1/2 ortholog), causing chromosome missegregation. Stalled forks on a single chromosome create entanglements between sister chromatids at mitosis. Moreover, strand invasions can occur between stalled forks on separate, non-sister chromosomes, creating non-sister telomere entanglements. These entanglements are stretched between segregating chromosomes and are resolved in mitosis when cells are grown at temperatures >20°C, but fail to resolve at temperatures ≤20°C, conferring taz1Δ cold sensitivity. Rif1, a conserved DNA replication-repair factor, localizes between segregating chromosomes and inhibits the resolution of entanglements without affecting their formation. Fission yeast cells undergo local nuclear envelope (NE) breakdown between the segregating chromosomes towards the end of the mitosis. We find that mitotic Rif1 delays this localized NE breakdown, delaying the exposure of entanglements to the cytoplasm. We find that rapid exposure to the cytoplasm is crucial for resolution of taz1Δ telomeric entanglements, thus explaining why Rif1 inhibits entanglement resolution. Mechanistically, mitotic Rif1 hinders the clearance of nuclear pore complex components from the midregion, thereby delaying NE breakdown.

To address the types of entanglements whose resolution requires cytosolic exposure, we used circular chromosome containing cells with internal telomere stretches inserted on one or two chromosomes. taz1Δ cells with two telomere stretches lose viability at ≤20°C, while cells with single telomere show no growth defects, suggesting that non-sister entanglements require cytosolic exposure for their resolution. Altogether, our results highlight terminal events of mitotic nuclear/cytoplasmic partitioning as crucial to faithful chromosome segregation.

Engineered telomerase RNA to increase telomere length in human cells

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The RNA component of telomerase (TERC) serves as an essential scaffold for telomerase ribonucleoprotein (RNP) assembly and template for telomere synthesis. Mutations in any one of several genes involved in TERC biogenesis or stabilization impair telomere maintenance to cause degenerative diseases such as dyskeratosis congenita (DC). We hypothesized that transient exposure to TERC as an RNA, akin to mRNA-based therapeutics, might restore defective telomere maintenance. However, degradation by cellular nucleases and lack of incorporation of exogenous TERC RNA into the telomerase RNP represent potential barriers. Moreover, the length of human TERC RNA (451 nucleotides) exceeds chemical synthesis capabilities conventionally used to generate modified RNA therapeutics. Here, we systematically overcome these barriers and demonstrate robust telomere lengthening via a synthetic, modified TERC RNA in human cells including DC patient-derived induced pluripotent stem cells (iPSCs). We found that unmodified in vitro transcribed TERC RNA showed no telomerase activity or telomere lengthening when introduced into cells, despite exogenous TERT overexpression. However, when the synthetic TERC RNA 3' end was enzymatically protected, we found rapid telomere lengthening by kilobases in human cell lines, that was further augmented by TERT. Transient exposure (~72 hours) to exogenous TERC was sufficient to lengthen telomeres in patient iPSCs carrying PARN, TERC, and DKC1 mutations. To our knowledge, these data provide the first evidence of telomerase reconstitution in human cells via exogenously-introduced TERC RNA. By enhancing non-coding RNA stability and function, our strategy enables TERC RNA replacement as a potential therapeutic approach for DC and other telomere biology disorders.

Interplay between lamin B1 and the shelterin protein TRF2 to maintain genome stability

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Telomere maintenance is essential to preserve genomic stability and its deregulation plays important roles in tumorigenesis and premature aging. Lamins are key nuclear envelope components involved in the maintenance of nuclear integrity, regulation of transcription, replication and repair. Elevated levels of lamin B1 (LB1) are observed in some human pathologies and many types of cancer. We recently unveiled that LB1 overexpression leads to telomere instability preceded by a mislocalization of TRF2 and its binding partner RAP1 driven by their trapping to the nuclear periphery by LB1. Moreover, increasing TRF2 levels rescues telomere stability. We also reported that LB1 interacts endogenously with TRF2 and RAP1, indicating that LB1 is a new player in telomere maintenance, which precise physiological role remains to be characterized.
We now uncover that LB1 interacts directly with TRF2, and that LB1 depletion affects TRF2-RAP1 interaction, suggesting a role of LB1 in shelterin stability. Moreover, we show that silencing LB1 leads to rapid telomere losses and accumulation of telomeric phospho-RPA foci, suggesting that telomere replication may be affected. Consistent with a putative function of LB1 in telomere replication, we unveil that LB1 dysregulation interferes with the phosphorylation switch at serine 365 in TRF2 controlling telomere accessibility during S-phase, leading to the inappropriate processing of telomeres by SLX4 complex. These data indicate that LB1 may be involved in TRF2 regulation during telomere replication. Altogether, our data indicate that this new LB1-TRF2 interaction is important for telomere maintenance and thereby genome stability.

Digital telomere measurement distinguishes health and disease

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Telomere length is an important biomarker of organismal aging and cellular replicative potential, but existing measurement methods are limited in resolution and accuracy. Here, we deploy digital telomere measurement (DTM) by nanopore sequencing to understand how distributions of human telomere length change with age and disease. We measure telomere attrition and de novo elongation with up to 30 bp resolution in genetically defined populations of human cells, in blood cells from healthy donors and in blood cells from patients with genetic defects in telomere maintenance. We find that human aging is accompanied by a progressive loss of long telomeres and an accumulation of shorter telomeres. In patients with defects in telomere maintenance, the accumulation of short telomeres is more pronounced and correlates with phenotypic severity. We apply machine learning to train a binary classification model that distinguishes healthy individuals from those with telomere biology disorders. This sequencing and bioinformatic pipeline will advance our understanding of telomere maintenance mechanisms and the use of telomere length as a clinical biomarker of aging and disease.

Mechanisms of telomere protection in the *Drosophila* embryo

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Drosophila telomeres are protected by the Terminin complex and elongated by specific retrotransposons, which encode the RNA template and reverse transcriptase. The Terminin complex comprises rapidly evolving proteins HOAP/HipHop/Moi/Ver/Tea; deficiencies in these proteins lead to telomere fusions. Investigating de novo telomere establishment in embryos, where thousands of telomeres assemble rapidly, provides insights into distinct protection mechanisms in somatic and germline cells. Co- and post-transcriptional regulation of telomeric retrotransposon expression in the germline influences the quality and abundance of transcripts loaded into the embryo, associating with telomeric chromatin and providing a reservoir for telomere establishment during early development, prior to the maternal-to-zygotic transition. Through in vivo analyses in syncytial blastoderm embryos, we demonstrate that disruption of components of the RNA surveillance machinery—such as Ars2, CCR4, and exosome components—results in defective chromosome segregation, persistent anaphase bridges involving telomeres, robust RPA-associated DNA damage, and nuclear collapse/fusion, underscoring the crucial role of telomere transcripts during early development. Our data support a model wherein the accumulation of TERRA-like telomeric retrotransposon transcripts that evade degradation induces damage and stress, leading to chromosome entanglements involving telomeres. This sheds new light on the intergenerational control of telomere state and the intricate relationship between RNA quality control pathways and telomere assembly in the germline.

Silencing of SLFN11 confers replication stress tolerance in ATRX deficient ALT cancers

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Cancer cells must solve the 'end replication problem' to attain replicative immortality and do so by activating one of two telomere maintenance mechanisms: they can either re-express the reverse transcriptase telomerase, which directly extends telomere repeats, or induce alternative lengthening of telomeres (ALT), which relies on telomere recombination. ALT-positive cancers account for approximately 10-15% of all tumours and are strongly associated with mutations in Alpha Thalassemia/Mental Retardation Syndrome X-Linked (ATRX). Since ATRX deficiency alone is insufficient for ALT establishment in cell culture, we exploited CRISPR/Cas9 screens to identify synthetic genetic interactions that cooperate with ATRX loss to drive or attenuate ALT in tumour cells.

Here, we show that the restriction factor SLFN11 functions as a tumour suppressor to prevent ATRX deficient cells from becoming ALT. We show that ATRX deficient cells are exquisitely

sensitive to loss of ssDNA maintenance complexes, CST (CTC1-STN1-TEN1) and 9-1-1 (RAD9A-HUS1-RAD1). While loss of PML suppresses telomere damage to ameliorate cell death in cells deficient in ATRX:CST, SLFN11 loss rescues the synthetic lethality of ATRX:9-1-1 and permits survival of ATRX deficient cells with heightened replication stress and ALT establishment. In this context, SLNF11 loss prevents apoptosis independent of DNA damage checkpoint and p53 signalling, but results in ATR inhibitor resistance. We propose that silencing of SLFN11, which occurs in ~50% of cancers, confers replication stress tolerance and intrinsic chemoresistance in ALT cancers.

Telomeric DNA damage response activation mediates Aβ42 oligomers toxicity in Alzheimer's disease.

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The pathogenesis of Alzheimer's disease (AD) is complex, involving multiple cellular pathways causing macromolecular damage (Scheltens P, Lancet 2021). Recent evidence from both human and mouse model studies highlights the significance of DNA damage in AD. Markers of DNA damage response (DDR) activation have been consistently observed in the brains of AD patients and in AD mouse models (Suberbielle, Nature Neuroscience 2013; Shanbhag, Acta Neuropathological Communication, 2019). Nevertheless, the precise molecular mechanisms leading to DNA damage generation and its causal role in AD pathogenesis remain unclear. We will provide experimental evidence in an AD mouse model (3XTg-AD) that a significant portion of DNA damage and markers of DDR activation accumulates at telomeres in the cortex and hippocampus area. DNA damage was detected by co-localization analysis of DDR markers and telomeric DNA and by increased levels of telomeric damage-induced lncRNA (tdilncRNA) (Rossiello, Nature Communications 2017). Similar observations were made in mouse hippocampal primary neurons and human cortical neurons derived from induced pluripotent stem cells (iPSCs) exposed to Aβ42 oligomers – the main component of amyloid plaques, a hallmark of AD. We will propose a model, based on our unpublished results, providing a mechanism by which Aβ42 oligomers induce telomeric DNA damage. We also show efficacy of antisense oligonucleotide against tdilncRNA (tASO) (Rossiello, Nature Communications 2017; Sepe EMBO report 2022) in reducing the consequences of telomeric DDR activation in neurons. Overall, these results highlight the role of telomere dysfunction in the neuropathology of AD.

Identification of the first poly (ADP-ribose) polymerase in yeasts and its involvement in telomere maintenance

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Poly (ADP-ribose) polymerases (PARPs) are enzymes catalyzing the post-translational addition of ADP-ribose units to proteins, with a role in DNA recombination, repair, and chromosome maintenance. Even though this group of enzymes is common in both eukaryotes and prokaryotes, no PARP homologs have been described so far in ascomycetous yeasts. Analysis of the transcriptome of telomerase-deficient cells of the yeast Yarrowia lipolytica resulted in identification of the gene PYL1 encoding the first candidate of PARP homolog in yeasts. Here, we confirm that PYL1 is overexpressed in Y. lipolytica cells lacking either RNA or catalytic subunit of telomerase. We show that Pyl1 protein is a functional PARP that undergoes auto-PARylation. A search for other in vivo targets of Pyl1 revealed both subunits of the Ku70/80 complex as its major substrates. The overexpression of PYL1 leads to elongation of telomeres supporting the role of Pyl1 in the adaptation of Y. lipolytica cells to loss of telomerase. As this phenotype mimics that of delta ku80 strain, we propose a model of Pyl1-dependent adaptation mechanism to telomere crisis. Based on our observations, we propose that Pyl1 and its homologs we identified in other yeast species represent a distinct class of PARPs thus substantiating a more detailed investigation of their roles in these organisms.

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Telomere length inheritance through the budding yeast sexual cycle

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In humans, short and long telomere syndromes are caused by inheritance of mutations affecting telomere length. These syndromes often show genetic anticipation, where disease has earlier onset and a more severe manifestation in each generation. This is presumably because later generations inherit not only the mutation affecting telomere length, but also abnormal length telomeres. If telomere length alters during human generations, then any changes most likely depend on the number of mitotic and meiotic cell divisions in egg or sperm cell lineages.

Many aspects of telomere length homeostasis are conserved between mammals and yeast. Here we asked if yeast, like humans, can transmit abnormal telomeres through the generations. By following the inheritance of a single telomere, rather than bulk telomeres, we find that if haploid yeast with short telomeres mate with wild-type yeast to create diploids, short telomere lengths rapidly normalize (within 30 cell divisions). However, if yeast with long telomeres

mate with the wild type, long telomeres can persist for more than 200 mitotic cell divisions and be transmitted through meiosis. Furthermore, long telomeres can be transmitted down the generations (sexual cycles), independently of mutations that cause long telomeres.

Our findings suggest that even in yeast there is a complex relationship between telomere length, mutations that affect telomere length and corresponding phenotypes. These findings may have implications for families affected by telomere syndromes.

The DBHS family of RNA/DNA binding proteins regulate telomerase recruitment to the telomere

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Telomerase is a ribonucleoprotein enzyme that maintains telomeric repeats on chromosome ends in continuously dividing cells. Telomere maintenance via telomerase requires the correct assembly of its protein and RNA components, as well as the recruitment of cofactors involved in enzyme maturation, stability, and transport to the telomere. We have identified the Drosophila behaviour/human splicing (DBHS) family of RNA/DNA binding proteins as novel regulators of telomerase recruitment to the telomere. Our data demonstrate all three members of the DBHS protein family (NONO, SFPQ and PSPC1) associate with catalytically active telomerase through the hTR RNA component. Cells lacking the DBHS proteins exhibit a marked reduction in telomerase recruitment, with NONO and SFPQ depletion specifically resulting in hTR sequestration in Cajal bodies, and PSPC1 depletion resulting in reduced levels of coilin, an integral component of Cajal bodies. Rescue experiments using functional domain mutants revealed that DBHS protein-mediated trafficking of active telomerase to telomeres requires a functional RNA recognition motif and the protein-protein interaction NONA/ParaSpeckle (NOPS) domain. Finally, NONO and PSPC1 CRISPR-Cas9 knockout clones passaged over 60 population doublings demonstrated striking telomere shortening, specifically in cancer cells, consistent with the absence of a telomere maintenance mechanism. Our results reveal that telomerase-mediated telomere extension in cancer cells is dependent on DBHS mediated trafficking out of Cajal bodies and shuttling to the telomere.

Insights into compaction of telomeric chromatin: Structured yet dynamic

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Telomeres the ends of our chromosomes play a central role in hallmarks characterizing both ageing and cancer. However, the structural organization of telomeric nucleosomes and its

interaction with shelterin components have remained a mystery. Our recent cryo-EM study showed that telomeric nucleosomes compact into novel columnar stacks, also characterized by an open state, supergrooves and synergistic histone-histone and histone-DNA interactions (Soman et al, Nature, 2022, 609, 1048). We have built upon this work and showed that TRF2 alone can mediate the columnar stacking of telomeric nucleosomes (Wong et al, EMBOJ, 2024, 43,87). TRF2 interaction with the telomeric nucleosome array does not lead to histone eviction and leads to a mechanically and energetically stabilized array. Our investigation into the liquid-liquid phase separation (LLPS) behaviour of telomeric chromatin shows that proteins TRF1, TRF2 and HP1 can mediate phase separation of telomeric chromatin. However, each of these proteins exhibits a signature dynamic phase separation behaviour. Our preliminary LLPS studies complemented by electron microscopy suggest that TRF2 predominantly mediates intra-array compaction and TRF1 inter-array compaction. In tandem, they paint the picture of telomeric chromatin as a dynamic entity with structured columnar elements.

DNA-PK controls Apollo's access to leading-end telomeres

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The complex formed by Ku70/80 and DNA-PKcs (DNA-PK) promotes the synapsis and the joining of Double Strand Breaks (DSBs) during canonical Non-Homologous End joining (c-NHEJ). In c-NHEJ during V(D)J recombination, DNA-PK promotes the processing of the ends and the opening of the DNA hairpins by recruiting and/or activating the nuclease Artemis/DCLRE1C/SNM1C. Paradoxically, DNA-PK is also required to prevent the fusions of newly replicated leading-end telomeres. Here, we describe the role for DNA-PK in controlling Apollo/DCLRE1B/SNM1B, the nuclease that resects leading-end telomeres. We show that the telomeric function of Apollo requires DNA-PKcs's kinase activity and the binding of Apollo to DNA-PK. Furthermore, AlphaFold-Multimer predicts that Apollo's nuclease domain has extensive additional interactions with DNA-PKcs and comparison to the Cryo-EM structure of Artemis bound to DNA-PK phosphorylated on the ABCDE/Thr2609 cluster suggests that DNA-PK can similarly grant Apollo access to the DNA end. In agreement, the telomeric function of DNA-PK requires the ABCDE/Thr2609 cluster. These data reveal that resection of leading-end telomeres is regulated by DNA-PK through its binding to Apollo and its (auto)phosphorylation-dependent positioning of Apollo at the DNA end, analogous but not identical to DNA-PK dependent regulation of Artemis at hairpins.

Transcription stress at telomeres leads to cytosolic DNA release and paracrine senescence

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Transcription stress has been linked to DNA damage -driven aging, yet the underlying mechanism remains unclear. Here, we demonstrate that Tcea1-/- cells, which harbor a TFIIS defect in transcription elongation, exhibit RNAPII stalling at oxidative DNA damage sites, impaired transcription, accumulation of R-loops, telomere uncapping, chromatin bridges, and genome instability, ultimately resulting in cellular senescence. We found that R-loops at telomeres causally contribute to the release of telomeric DNA fragments in the cytoplasm of Tcea1-/- cells and primary cells derived from naturally aged animals triggering a viral-like immune response. TFIIS-defective cells release extracellular vesicles laden with telomeric DNA
fragments that target neighboring cells, which consequently undergo cellular senescence. Thus, transcription stress at telomeres elicits paracrine signals leading to cellular senescence, promoting aging.

Multi-omics analyses unveiled distinctive features of alternative lengthening of telomeres in mouse embryonic stem cells

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In the absence of telomerase, alternative lengthening of telomeres (ALT) is activated, preserving telomere length and extending cellular lifespan. Our previous studies identified two types of ALT survivor cells from mouse embryonic stem cells. The ALT mESC model provides a sequential cell archive before and after ALT activation, allowing us to observe changes over time. Multi-omics analyses (whole-genome sequencing, RNA sequencing, quantitative proteomics, and ATAC sequencing) revealed the distinct characteristics of two types of ALT. Type I cells displayed gene expression signatures linked to chromatin

remodeling, while type II cells showed signatures related to DNA repair. Compared to type II, type I cells accumulated more mutations and exhibited persistent telomere instability. These findings highlight the diverse survival pathways adopted by cells from the same source, providing valuable insights into the plastic nature of cells undergoing crisis. The next important question is what genes are important for ALT initiation. To address this, we introduced single-cell RNA sequencing and compared the characteristics of pre- and post-ALT cells, leading to the identification of a cell cluster displaying the signature of a two-cell state. To elucidate the genomic alterations generated by ALT, we adopted third-generation long-read sequencing to create a haplotype-resolved ALT genome. As a result, we identified over 1000 ALT-specific structural variants. Additionally, we have identified proteins that selectively bind to ALT telomeres. Our ongoing investigation delves into the distinctive functions of proteins unique to each type of ALT, alongside those that function commonly in both types.

RNA-seq analysis of telomerase-deficient *S. cerevisiae* reveals upregulation of distinct cellular pathways during senescence and survivorship

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Akin to what happens during aging in human tissues, senescing yeast cells that lack functional telomerase experience telomere shortening and ultimately enter a G2/M checkpoint. Late in the senescence program, some "survivor" cells escape, maintaining their telomeres by recombination. The gene expression profile during senescence and survivorship is not yet fully known. To investigate it more deeply, we performed RNA-seq on haploid *S. cerevisiae* cells missing the telomerase RNA gene, based on dissecting a heterozygous diploid to generate *tlc1*Δ and wild-type strains. We collected total RNA from five different time points from the *tlc1*Δ strains. The data unveiled distinct pathways, such as autophagy, and novel lncRNA transcripts markedly upregulated in senescence. These results correlate generally well with, but also greatly extend, prior microarray studies. To test upregulated pathways' importance in senescence, we have focused first on the *ATG* autophagy genes. We are testing the hypothesis that autophagy has a key function in inducing cell cycle arrest. This is supported by earlier reports that certain types of autophagy are triggered by genotoxins. Using a functional assay for autophagy, we see evidence of induction of this process as senescence occurs. Overall, our findings provide a deep look at the transcriptional response to short-telomere-induced senescence and identify specific pathways key for checkpoint adaptation for subsequent survivorship.

S. pombe telomerase RNA: secondary structure and flexible-scaffold function

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Little is known about the structure of the telomerase RNA from the 1212 nt fission yeast *Schizosaccharomyces pombe*, TER1. We have derived a robust secondary structure model based on phylogenetics, bioinformatic modeling, as well as genetic and biochemical analyses. We find several conserved regions of the rapidly evolving TER1 RNA are essential based on determining functionality of truncation mutants *in vivo*. Overall, large regions are dispensable without causing senescence. This is similar to budding yeast telomerase RNA, TLC1, and consistent with flexible-scaffold function. We tested if the essential three-way junction (3WJ) works from other locations in TER1, finding that indeed it can, supporting that it is flexibly scaffolded in the RNP. Furthermore, we find that a half-sized Mini-TER1 allele, built from the core of the model, supports catalytic activity with TERT *in vitro*. Overall, we provide an evidence-based secondary structure model for the large fission yeast telomerase RNA and begin to characterize features of its physical organization as they relate to function *in vivo*.

The nucleoside analogue 6-thio-2'-deoxyguanosine (6-thio-dG) sensitize breast cancer cells to ionizing radiation exposure

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About 85% of tumors show a reactivation of telomerase, representing an attractive target for anti-cancer agents. The nucleoside analogue 6-thio-2'-deoxyguanosine (6-thio-dG) is recognized by telomerase and incorporated into de novo telomeres. 6-thio-dG inhibits cells proliferation, increases the DNA damage, induces cell cycle arrest, and leads to cell death. In this study, we investigated 6-thio-dG alone or in combination with ionizing radiations (IR) on breast cancer cell lines. We first assessed the optimal pre-treatment conditions for 6-thio-dG, including concentration and duration of treatment before irradiation on MCF7, HACC-1937, MDA-231 as well as in telomerase-negative human primary fibroblasts, used as a control. Based on these results, combined treatment with 6-thio-dG and X-rays was investigated. MCF7 cells were pre-treated for 72 hours with 6-thio-dG (0.5-1 μM) and irradiated with different IR dosages (1-4 Gy). Surviving fraction experiments indicated that 6-thio-dG synergistically increased radiosensitivity in breast cancer cells. To understand the potential molecular mechanisms behind the radiosensitizing effect, molecular cytogenetic analysis was performed, and the effects of combined treatments on chromosome structure were evaluated. The combined treatment significantly increased frequency of dicentrics and chromosomal fragments, suggesting an interference of dysfunctional telomeres in DNA repair after irradiation. Furthermore, this effect was confirmed in a three-dimensional model using microencapsulation of cells in alginate hydrogels. Cell proliferation and viability assays demonstrated a significant antiproliferative effect with a corresponding increase in cell death, confirmed by elevated expression of cleaved PARP-1. Currently, experiments on C57Bl/6 mice are underway to assess whether this effect is also confirmed in vivo.

The different functions of RTEL1 variants in telomere maintenance

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Hoyeraal-Hreidarsson syndrome (HHS) is a fatal disease, characterized by severe telomere shortening, bone-marrow failure, immunodeficiency and neurodevelopmental defects. HHS is caused by germline mutations in telomerase subunits or accessory factors, and in the helicase RTEL1. RTEL1 has diverse functions in genome-wide and telomere maintenance. Moreover, RTEL1 has been previously shown to participate in the nuclear export of small nuclear RNAs. Human RTEL1 encodes for two main splice variants, 1219aa (RTEL1v1) and 1300aa (RTEL1v2). To distinguish between different functions of the different domains and variants of RTEL1, we used RTEL1-deficient primary fibroblasts derived from an HHS patient carrying heterozygous missense mutations in RTEL1: M492I, located at the center of the helicase domain, and R1264H, located in the C-terminal RING-finger domain. We introduced into the patient fibroblasts ectopic hTERT and then each of the two variants of RTEL1 separately under a tetracycline-inducible promoter. While ectopic expression of WT RTEL1v2 succeeded to elongate the telomeres to normal length, the expression of WT RTELv1 or hTERT alone did

not. We found that RTEL1 deficiency did not affect the in vitro telomerase activity assayed by TRAP, indicating that RTEL1 deficiency did not affect the assembly of the telomerase catalytic core. Interestingly, the RTEL1-deficient patient cells displayed abnormal cytoplasmic accumulation of colocalizing hTR and Telomeric DNA, suggesting a dominant negative effect of RTEL1v1 on telomeres. This finding suggests that RTEL1-deficiency affects the nuclear localization of telomeres and telomerase.

Characterization of the long noncoding RNA TERRA in a vertebrate

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The long noncoding RNA TERRA is an essential component of telomeres, believed to be an evolutionary conserved feature of all eukaryotes. Thus far, TERRA has been extensively characterized mainly in human and murine cultured cells and in yeasts. These studies helped define TERRA dynamics and provided evidence for its fundamental roles at telomeres. However, TERRA functions in complex processes such as development, organismal aging and cancer initiation remain largely unexplored. To fill this gap, we set out to characterize TERRA in zebrafish, a model organism with human-like telomere biology. This make zebrafish an excellent candidate to define what the roles of TERRA and telomere transcription are in a vertebrate.
We show that TERRA is transcribed both at early stages of embryo development and in adulthood. TERRA is present in different organs, with higher expression levels in neuronal tissues. We also developed an approach based on long-read sequencing able to identify TERRA transcription start sites.
Overall, our study profile for the first time the expression pattern and molecular features of TERRA in a vertebrate and generate a framework to study the functional relevance of this RNA.

Characterizing mechanisms and factors in the generation of telomeric ssDNA in ALT-positive cells

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To gain insight into the mechanisms that regulate telomere homeostasis in ALT-positive cells, we used an unbiased and high-resolution genomic approach, S1-END-seq, to detect both secondary structures and the presence of single-stranded DNA (ssDNA) at chromosome ends. Using this method, we can effectively differentiate the telomeres of ALT-positive cells from those of telomerase-positive cells or cells lacking telomere-maintenance mechanisms. ALT-positive cancer cells display a significant accumulation of single-stranded telomeric DNA. Our data suggest that the main source of ssDNA in ALT cells is not extrachromosomal telomeric

repeats (e.g. ecDNA, C-circles) but rather chromosomal loops composed of ssDNA. Furthermore, based on the direct correlation between the presence of telomeric ssDNA and the ALT process, we established a robust high-throughput imaging-based screen to uncover cellular factors and pathways that play a role in either the formation or the suppression of telomeric ssDNA in ALT. In this context, we identified novel factors that provide insights into the mechanism underlying the diverse structures found at telomeres of ALT-positive cells, and we identified the factors responsible for their generation and protection.

Impaired glucose homeostasis in the short-telomere Telomouse.

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Type 2 diabetes (T2D), marked by persistent hyperglycemia, is a common chronic metabolic disease with a prevalence exceeding 20% in individuals aged 65 and above. It stems from pancreatic β-cell failure to meet the increased insulin demand due to peripheral insulin resistance. Despite being a key risk factor, the molecular mechanisms linking aging to T2D remain unclear. Telomere shortening or damage, one of the main hallmarks of aging, can trigger cellular senescence, an increasingly recognized regulator of age-related metabolic disorders by inducing inflammation and insulin resistance. Therefore, we hypothesize that telomere attrition plays a central role in accelerating the development of T2D with age. However, establishing telomere biology as a causative factor in T2D has been challenging thus far, due to model limitations. Here we utilized the Telomouse model, which has human-length telomeres, to assess parameters of prediabetes, both in the natural aging process and under diabetic conditions induced by a high-fat, high-sucrose diet (HFHS). We show that Telomice exhibit age-related insulin resistance, increased fasting blood glucose and glucose intolerance, which are characteristic hallmarks of the pre-diabetes stage. Furthermore, these parameters worsen in later generations of Telomice, correlating with telomere shortening in successive generations. Under HFHS challenge, these metabolic parameters worsen, along with decreased glucose-stimulated insulin secretion, suggesting the involvement of pancreatic β-cell dysfunction. These data confirm that Telomouse emerges as an invaluable tool for exploring the role of telomere biology in age-related metabolic decline and T2D.

A single-cell sequencing-based approach for telomere length analysis reveals telomere length dynamics in the human breast during aging and tumor evolution.

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Telomeres play a critical role in tissue homeostasis and tumorigenesis. Proliferation of cells in the presence of critically short telomeres causes genomic instability, which is thought to play an important role in the genomic evolution of many tumors. However, technical and clinical limitations make it difficult to study the dynamics of telomere length in human tissues during tumorigenesis. To gain new insights into telomere length dynamics during tissue aging, tumor initiation, and tumor evolution, we developed and validated a method for inferring mean telomere length from single-cell DNA sequencing (scDNA-seq) data, allowing simultaneous characterization of mean telomere length and genotype in single cells. We combined this computational method with a single-cell DNA+ATAC-seq co-assay to analyze the cell-type specific telomere lengths in normal breast tissue and breast tumor samples. Using this method, we identified distinct patterns of telomere length in the epithelial, stromal, and immune compartments of the human breast. We found that, contrary to our expectation, breast tumor cells did not show consistently shorter telomere lengths compared to their appropriate diploid comparison cells. However, we observed that tumor cell subclones – defined by genomic copy number aberrations – often showed marked differences in telomere lengths from one another, even within the same tumor. Our observations raise new questions about telomere length dynamics in breast cancer initiation and evolution. By reconstructing phylogenetic trees for tumor subclones, we hope to uncover new insights into the dynamics and role of telomere length in tumor evolution.

Clinical and molecular features associated with immunodeficiency in telomere biology disorders.

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Telomere biology disorders (TBD) result from germline mutations in telomere associated genes that cause excessive telomere shortening. TBD are associated with bone marrow failure, liver and lung fibrosis. Immunodeficiency has been described in the severe phenotype of dyskeratosis congenita, but is not well characterized in broad spectrum and in large cohorts of TBD patients. We report the clinical and molecular features of immunodeficiency in TBD. Clinical records were reviewed for 88 TBD patients. Laboratory data included: blood counts, B, T and NK subsets, and immunoglobulin levels. A significant infection was defined as the presence of opportunistic, recurrent, and/or infections that required hospitalization. Immunodeficiency was defined by CD8 ≤ 0.178 cell/μL, CD4 ≤ 200 cells/μL, and CD19 ≤ 60 cells/μL.

TERT and TERC were the most prevalent germline mutations. Thirty-two patients experienced clinically significant infections (36%), categorized as opportunistic (n=7, 8%), recurrent (n=15, 17%), and those requiring hospitalization (n=20, 22%). CD4 immunodeficiency correlated with infections requiring hospitalization, CD8 immunodeficiency with opportunistic infections and those requiring hospitalization, and CD19 immunodeficiency with recurrent infections. Additionally, decreased absolute lymphocyte count was correlated with solid cancer. No relationship was found between underlying germline mutation and immunodeficiency, but splicing factor somatic mutations were associated with decreased CD3 and CD8 counts.

In conclusion, immunodeficiency is common and are associated with increased risk of clinically significant infections.

Processing and initiation of genome instability at the shortest telomere in the absence of telomerase in Saccharomyces cerevisiae

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Telomere shortening in the absence of telomerase leads to replicative senescence, a powerful tumor suppressor mechanism, but is accompanied of genomic instability, which in turn promotes oncogenesis. To solve this paradox, we devised a system in Saccharomyces cerevisiae called FinalCut to define the structure of telomeres at the point of dysfunction and the fate of cells carrying them.
FinalCut generates a single telomere of a given short length in cells in which we conditionally inactivate telomerase. Structural analysis reveals a sharp transition to an unprotected state at a precise short length. Monitoring single-cell proliferation capacity supports a mainly deterministic model of the first telomere in such switch being sufficient to abruptly stop cell divisions. Conversely, genomic instability, namely chromosomal rearrangements, were found to stem from the shortest telomere in senescent cells.
Our findings shed light on the crucial and intricate process of handling the shortest telomere in cells, establishing a mechanistic link that explains the contrasting role of replicative senescence in oncogenesis.

Investigating proteins that associate with TIN2 via the region mutated in dyskeratosis congenita and related telomere biology disorders

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Pathogenic variants in the dyskeratosis congenita (DC)-region of TIN2 result in markedly short telomeres and severe, early-onset disease. The effect of DC-mutant TIN2 on telomere length is restricted to telomerase-positive cells and is dosage-dependent, but how it restricts telomere maintenance remains unknown. We hypothesize that DC mutations impair or enhance the binding of protein(s) to TIN2 that then promote or block telomerase-mediated telomere elongation, respectively. To identify potential factors, we expressed TIN2(254-322)-GFP fusion (which spans the TRF1 binding motif and DC region) without (wild type; WT) or with a DC mutation or GFP alone in 293T cells. We performed GFP-pulldowns followed by liquid

chromatography-tandem mass spectrometry analysis. Of the 300-400 proteins significantly enriched ≥10-fold in the TIN2(254-322)-WT or -mutant IPs over GFP alone, most showed more enrichment with mutant TIN2(254-322). Notably, Tel2, which along with TTI1/2 promotes PIKK biogenesis, was enriched 20- and 28-fold in the WT and mutant IPs, respectively. Its association with TIN2 was validated in co-IP assays of transiently expressed proteins, showing a greater association with the TIN2S(short) than TIN2L(long) isoform. Tel2 overexpression decreased TIN2L protein level, whereas TIN2S was less affected. Tel2-interacting protein TTI1 was also significantly enriched (12- and 18-fold in WT and mutant, respectively). Its binding to TIN2S was also greater than to TIN2L in co-IPs; however, TTI1 overexpression did not reduce TIN2S or TIN2L protein levels. The role of TRF1- and impact of TIN2-DC mutations on these findings will be examined. We will also present investigations on additional hits, such as GNL3L.

A novel role for BRAF in telomere biology

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Shelterin complex protects eukaryotic telomeres from degradation, unwanted DNA repair activities at chromosome ends and plays an active role in the maintenance of genome stability. Growing evidence indicates that the shelterin factor TRF1 is a stemness marker, is essential for the pluripotency induction and is upregulated in several human cancers.
BRAF, is a master kinase of the ERK-signaling cascade, a well-known mitogenic pathway that promotes cell proliferation in the presence of external growth factors. Oncogenic lesions affecting BRAF encoding gene frequently occur in a plethora of human malignancies and result in a continuous proliferative cue.
Here, we describe an unanticipated function for BRAF in telomere biology. We show for the first time that BRAF depletion in murine models as well as in vitro results in a post-transcriptional regulation of TRF1 in several organs and in primary murine embryonic fibroblasts. Importantly, we disclose that BRAF displays telomere-binding properties and co-localizes with TRF1. Finally, we also find that BRAF physically interacts with and directly phosphorylates TRF1, thus strongly indicating that BRAF-mediated TRF1 regulation occurs in a direct fashion.
Collectively, our findings reveal an unprecedented non-canonical function for BRAF in telomere biology, which may be a promising target for the development of novel successful anti-cancer therapies.

Identification of factors involved in the excision of telomeric i-loops as eccDNA

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Our lab has shown that, in the presence of single-stranded damage, telomeric repeats form internal loop (i-loop) structures through base pairing and branch migration. I-loops are a substrate for the generation of extrachromosomal telomeric circles. Indeed, we have shown that incubation of i-loop-containing telomeric DNA with a cellular extract leads to the formation of telomeric circles, in a Mg++ and ATP-dependent manner.
I plan to use this biochemical assay as a tool to identify the activities and conditions that promote excision of i-loops and accumulation of eccDNA. Since mature i-loops contain a Holliday junction (HJ) at the base, we anticipate that they may become a substrate for structure-specific endonucleases, such as the HJ resolvases Slx4-Mus81, and Gen1. These proteins, however, are involved in multiple steps in DNA repair, and deletion of the respective genes might affect different steps in i-loop formation therefore contributing to eccDNA accumulation indirectly. To address their specific contribution to the excision step of pre-existing i-loops, I will provide i-loop-containing DNA to different cellular substrates, including ones that were immuno-depleted of the candidate proteins. In parallel, I will also test ALT and non-ALT cell lines for possible differences in their relative i-loop excision activity. Finally, based on the results with the depleted extracts, I will attempt to reconstitute the excision reaction with purified proteins.

Telomere reprogramming to test the compatibility of different telomeric repeats with telomere-binding proteins in Saccharomyces cerevisiae

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Understanding how telomeres and telomere-binding proteins (TBPs) co-evolve is one of the unanswered questions in telomere biology. One possibility is that TBPs have adapted to the rapid evolution of their target DNA. Indeed, when examining the group of TBPs in Ascomycetes, we find that they form a highly diverse group of DNA-binding proteins. Another possibilty is that the ability to maintain telomeres has not led to significant changes in the TBPs properties. These two possibilities are not necessarily mutually exclusive. Robustness may have facilitated changes in telomeric repeats, subsequently allow for the fine-tuning of TBPs. Nevertheless, a systematic investigation of this issue is currently lacking. To explore the evolutionary trajectories from ancestral telomeres to the highly diversified telomeres in ascomycete fungi, we developed a pipeline for telomere reprogramming in S. cerevisiae. Telomere reprogramming was achieved by introducing mutations to the RNA subunit of telomerase, encoded by the TLC1 gene, using scarless CRISPR-Cas9 genome editing. This resulted in a collection of strains with a mutated template domain of TLC1, each possessing a different non-native sequence. Subsequently, we conducted a phenotypic analysis of the strains with reprogrammed telomeres. Using this approach, we employed S. cerevisiae as a living test tube for investigating evolutionary questions related to the telomeric complex.

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Fragmentation patterns in human telomeric cell-free DNA

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The chromatin structure of telomeres in vivo is a matter of intense investigation. Here we show plasma cell-free DNA (cfDNA) as an unexpected means to study telomere structure in humans. Blood plasma cfDNA telomeric abundance (CDTA) significantly decreases with healthy human aging. Blood serum CDTA decreases significantly in age matched Acute Myeloid Leukemia donors compared to healthy human donors. Acute genotoxic stress in cells results in dramatic loss of cell-free telomeric protections over short treatment times. In human blood plasma we observe two classes of protections: subnucleosomal (40-70 bp) and nucleosomal (≥150 bp). Our sequencing libraries capture DNA separated into single strands, in a strand-specific manner. Surprisingly, the subnucleosomal telomeric fragments are highly enriched for the C-rich strand. Our results suggest specific accessibility in the C-rich strand pointing to possible models of protein binding on telomeres. Finally, to get true lengths of telomeric sequences in plasma we develop an approach to enrich and perform long read sequencing of telomeric cfDNA. Taken together, we demonstrate that cfDNA offers a new avenue to study telomeric chromatin structure in the context of aging and cancer.

Genome and telomere integrity depend on type I ESCRT subunits

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The ESCRT complex, dedicated to membrane repair, consists of types I, II, and III subunits, primarily assembling at multivesicular bodies, the intercellular bridge, and sites of nuclear rim rupture. ESCRTs III are known determinants of nuclear integrity at the rim, while information on ESCRTs I and II at the nucleus is still limited.

In previous research, AKTIP, crucial for telomere and genome integrity at the nuclear rim, was identified. Leveraging similarities between AKTIP and ESCRT I TSG101, we used AKTIP to identify new nuclear ESCRTs I and explored their correlation with telomere integrity.

Super-resolution microscopy revealed TSG101 at the nuclear rim and VPS28 in the nucleoplasm. FRET analysis showed TSG101's proximity to lamin A and AKTIP. Depletion of these ESCRTs I led to reduced circularity and nuclear bleb formation. TSG101 and VPS28, like AKTIP, were crucial for genome and telomere integrity. TSG101 played a more significant role in telomere integrity, while VPS28 impacted global DNA damage. Preliminary data on ESCRT I depletion over a progeroid background indicated AKTIP and progerin were epistatic in inducing global DNA damage, whereas VPS28 and progerin were not, causing cumulative DNA damage.

In summary, this study establishes ESCRT I presence at the nuclear rim and nucleoplasm,

highlighting their crucial role in preserving genome, telomere, and nuclear integrity. The findings suggest nuclear rim ESCRTs are specifically associated with telomere damage, while nucleoplasmic ones relate more to global genome damage.

Telomerase activity and proliferative capacity in activated T-lymphocytes in telomere biology disorders

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The telomerase enzyme is essential for telomere length maintenance. Several known mutations in telomerase-associated genes lead to pathological telomere shortening, resulting in Telomere Biology Disorders (TBD) including bone marrow failure, idiopathic pulmonary fibrosis, and dyskeratosis congenita. The TBDs have a clinically heterogeneous picture with anticipation where more severe symptomatology can be seen for each generation. Assessing the pathogenicity of novel single nucleotide polymorphisms (SNPs) in telomerase-associated genes involves genetic and bioinformatic analysis and telomere length measurements. Some SNPs are shown to be mutations, but many are classified as variants of unknown significance (VUS) according to the ACMG criteria. Cloning of SNPs with in vitro analysis may help the classification but is time-consuming. Therefore, a functional analysis of telomerase activity in cells from patients could provide additional support when assessing the pathogenicity of a VUS.

We have set up a functional analysis of telomerase activity in blood cells. In 100 healthy controls and five TBD patients with TERT mutation, mononuclear cells were stimulated with Phytohemagglutinin. The proliferative capacity of the T-lymphocytes was evaluated through S-phase measurement by flow cytometry and telomerase activity was measured with TRAP. The telomerase activity was reduced in patients compared to controls (p

Thus, although the proliferative capacity of the T-lymphocytes from TBD patients was not significantly reduced, they had lower telomerase activity. The functional analysis of T-lymphocytes may be of value in the examination of suspected TBD and may reduce VUS frequency in the genetic analysis.

Structural studies of TRF1-Tankyrase interaction

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Tankyrase (TRF1-interacting ankyrin-related ADP-ribose polymerase) catalyzes the addition of branched-ADP molecules to specific targets in a process known as PARylation. This important post-translational modification regulates multiple processes within cells, including telomere maintenance. In particular, TRF1 has been shown to be PARylated by tankyrase. PARrylated TRF1 is released from telomeric DNA and consequently ubiquitinated and degraded by

proteasome. Removal of TRF1 favours the access of telomerase, upregulating telomere elongation and is essential for resolving sister telomeres during mitosis.

Misregulation of this process can disrupt shelterin dynamics and lead to telomeric malfunction which is associated with human diseases, including premature aging syndromes, telomerase-associated disorders and cancer. Despite its relevance, the structural and mechanistic information about human shelterin regulation is limited. In our group, we are trying to get insights on this important shelterin regulators that are key for chromosome end protection and for developing new therapy strategies. To achieve this aim, we use cryo-electron microscopy, among other techniques, to reveal more structural details about tankyrase and its interaction with TRF1.

Dissecting a mechanism of telomere end protection against NHEJ

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Telomeres cap chromosome ends to prevent end-to-end fusions driven by the Non-Homologous End Joining (NHEJ) pathway. In the yeast Saccharomyces cerevisiae, Rap1 binds to telomeric repeats, recruiting Sir4 and Rif2, which collectively inhibit NHEJ at telomeres through distinct mechanisms. Sir4, together with the histone-binding protein Sir3 and the histone-deacetylase Sir2, is essential for the spreading of sub-telomeric heterochromatin. Interestingly, despite the presence of Sir4 in heterochromatin, NHEJ remain proficient [1]. Reciprocally, Sir4 inhibits NHEJ in absence of Sir3 [2]. Altogether, these observations suggest a context dependent regulation of Sir4 NHEJ-inhibitory function. In addition, Sir4 interacts with the core NHEJ factor Ku to promote sub-telomeric heterochromatin, telomerase recruitment and telomere tethering at the nuclear periphery. Specifically, a Ku-binding motif in Sir4 N-terminal region interacts with the Yku80 subunit of Ku [3]. Our aim is to dissect the modes of Sir4 recruitment and its NHEJ inhibitory effect mechanism at chromosome ends. We show that the Sir4 C-terminal coiled-coil dimerization domain interacts with the Yku70 subunit, both in vivo (Y2H) and in vitro (BLI). By identifying critical residues within this domain essential for the interaction with Yku70, we observed that missense mutations in these residues lead to a partial loss of Sir4-dependent NHEJ inhibition without affecting Rap1-mediated Sir4 recruitment at telomeres. We are now exploring the molecular mechanism through which the interaction between Sir4 coiled-coil and Yku70 can impact NHEJ.

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Evolution of yeast telomeres is shaped by the flexibility of telomere-protecting systems

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The two principal components of telomere-protecting system – telomere-binding proteins (TBPs) and telomerase are responsible for the stable maintenance of chromosomal ends. DNA-binding flexibility of TBPs and structural flexibility of telomerase are also crucial for accepting the changes in primary sequence of telomeric repeats, occurring in some phylogenetic branches. In ascomycetous yeasts, this evolutionary process resulted in an extremely variable set of telomeric sequences associated with diverse TBPs. To investigate this process in greater detail, we compared the DNA-binding properties of four different TBPs (Tay1 from Yarrowia lipolytica, Rap1 from Saccharomyces serevisiae, Rap1 from Candida parapsilosis and Taz1 from Schizosaccharomyces pombe) along with SpTeb1 (a non-TBP homolog of Tay1 in S. pombe) using the bacterial 1-hybrid system and electrophoretic mobility shift assay. Here, we show that the overall flexibility of these proteins in binding to various DNA substrates suggests a common basic mode of evolution in both budding yeasts (Sachcaromycotina) and fission yeasts (Taphrinomycotina). We also reprogrammed the telomeres of Y. lipolytica by expression of variant versions of telomerase RNA to demonstrate that telomerase complex in this species accepts some degree of variability of telomeric repeat sequences. Taken together, we suggest the flexibility of all components of yeast telomere-protecting systems provide guidelines shaping the evolution of telomeric repeats and telomere-binding protein complexes.

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CRISPR/Cas9 Approach to Unravel the Molecular Basis of Telomeric DNA Damage and the Disparity in DNA Damage Responses between Genomic and Telomeric Regions

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Telomeres play a crucial role in aging and cellular senescence. The presence of the shelterin at telomeres can trigger different DNA damage responses compared to other parts of the genome. Differences in telomeric DNA damage responses can be attributed to the specific methods used to induce DNA damage. In this study, we examined the mechanism involved in telomere-targeting CRISPRs using transient experimental setups and an inducible Cas9 cell model. We found that CRISPR/Cas9 efficiently induced DNA damage and activated the G2 DNA checkpoint. Pharmacological inhibition of DNA damage response kinases abrogated the checkpoint, indicating the involvement of the DNA damage response cascade. Phosphorylation of H2AX by ATM and ATR kinases played a crucial role in transmitting the responsible for DNA damage response. Although both CHK1 and CHK2 kinases were activated, the regulation of WEE1 by CHK1 determined whether cells underwent prolonged G2 delay or progressed into mitosis. Notably, we observed telomere removal and reduced telomere length. DNA damage and repair were active in a telomere-length independent manner, instead of following an aging-like telomere erosion pattern. CRISPR/Cas9 was further utilized to investigate the disparities in DNA damage responses between genomic and telomeric regions. We found that both types of double-strand breaks initiated similar DNA damage responses, but genomic double-strand breaks induced additional micronuclei. Moreover, we observed anaphase bridges and nuclei envelope rupture during interphase, leading to the accumulation of genome

instabilities and reduced proliferation. These findings enhance our understanding of telomere biology and its implications in double-strand breaks.

PML promotes organization of nuclear f-actin to facilitate ALT

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To preserve genomic integrity, proliferating cells must enact a program of telomere maintenance. In a subset of tumors telomeres are lengthened through a DNA-templated process termed Alternative Lengthening of Telomeres or ALT. In ALT, dysfunctional telomeres cluster at nuclear PML bodies, forming ALT-associated PML bodies or APBs. There, the ALT mechanism co-opts homologous recombination machinery to synthesize new telomere DNA. ALT is associated with replication stress, and stalled telomeric replication forks are thought to be a major substrate for ALT. Polymerization of nuclear actin fibers has been shown to be an essential part of the replication stress response. We wished to ask whether nuclear f-actin might be important in the ALT mechanism. Using immunoprecipitation-mass spectrometry we found that PML protein associates with actin, actin nucleators, and myosin II subunits. Knock-down of actin nucleation factors impacts levels of C-circles—extrachromosomal telomere sequence that is a hallmark of ALT activity—implying a functional importance in the ALT mechanism. Super-resolution images of ALT cells demonstrate that PML protein decorates nuclear actin fibers. In live-cell imaging using cell lines with fluorescent markers for actin, telomeres, and PML protein we observe that actin fibers polymerize dynamically in tangent to PML bodies. Cells lacking PML only rarely form nuclear actin fibers, and in the absence of PML, the nuclear f-actin network appears disorganized. Based on these results we propose a model in which PML bodies serve as a platform for nuclear f-actin regulation, playing a critical role in organizing the nucleus to facilitate ALT.

Structural characterization of small-molecule inhibitors of human telomerase

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Telomerase is considered a prime therapeutic target for cancer through the application of telomerase inhibitors; however, no small-molecule telomerase inhibitors have entered clinical trials. We have developed two structurally and mechanistically distinct small-molecule telomerase inhibitors. 'SBC-1' binds reversibly and competes with DNA substrate binding. 'NU-1' inhibits telomerase irreversibly through covalent modification of an hTERT cysteine thiol (1). Remarkably, both compounds completely inhibit telomerase in a direct (non-PCR) primer extension activity assay. We have achieved a cryo-EM structure of the catalytic lobe of human telomerase to ~2.4 Angstrom; this is enabling us to refine the two telomerase inhibitors

through structure-based drug design, by acquiring cryo-EM data on the complexes of [telomerase + inhibitor]. The complete telomerase inhibition achieved by these small molecules has also allowed us to demonstrate synergy with chemotherapeutic drugs against cell lines in vitro. Research from our labs has revealed an interdependence between telomerase recruitment to telomeres, the DNA replication stress response, and a nuclear F-actin fiber network. We hypothesize that this interdependence can be exploited to identify and mechanistically define synergistic combinations of telomerase inhibitors with standard-of-care chemotherapeutic agents.

(1) Liu Y; Betori RC; Pagacz J; Frost GB; Efimova EV; Wu D; Wolfgeher DJ; Bryan TM; Cohen SB; Scheidt KA; Kron SJ (2022) Targeting telomerase reverse transcriptase with the covalent inhibitor NU-1 confers immunogenic radiation sensitization. Cell Chemical Biology 29:1517-1531.

Subtelomeric sequences stabilize the shortened telomeres in N. castellii ALT cells.

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Telomeres in the budding yeast Naumovozyma castellii are maintained by telomerase at a mean length of 300 bp. Disruption of telomerase causes progressive telomere shortening. However, telomerase-deficient N. castellii cells maintain their telomeres by the recombination-dependent Alternative Lengthening of Telomeres (ALT) mechanism. The ALT pathway is effectively activated as the telomeres shorten, thus bypassing senescence and allowing for sustained long-term growth. Intriguingly, N. castellii ALT telomeres are sporadically elongated with long repeated arrays of terminal sequences, TelKO elements, containing both a subtelomeric region and a short stretch of telomeric sequence (ca. 50 bp). We have identified two different TelKO elements that are maintained at chromosome ends, indicating that the terminal subtelomeric region is key to the lengthening and stabilization of the ALT telomeres. To study the underpinnings of this ALT mechanism, we investigated the stability of the telomere structure through subsequent generations of ALT cells. Analysis of large numbers of ALT clones from different passages, showed that drastically shortened telomeres are stably maintained through many generations. After the initial resection, the shortened telomeres exhibit a uniform structure that is faithfully transferred during extensive passaging. Our results show that the DNA sequence of the subtelomeric part of the TelKO element is highly conserved throughout the series of experiments. Notably, the telomere-binding protein Rap1 can bind to the subtelomeric sequence in EMSA. This indicates an important functional role of the TelKO element in providing stability to the shortened ALT telomeres, possibly by incorporating the distal subtelomeric sequences into the telomeric chromatin structure.

Characterization of the oligomeric state of the C. glabrata CST complex.

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The yeast CST complex, formed by Cdc13, Stn1 and Ten1, is involved in telomere replication, DNA repair and in chromosome cohesion. The better characterized functions of the complex are telomere capping and telomere replication regulation. For telomere capping, Cdc13 specifically binds to telomeric ssDNA regions in a sequence specific way. Additionally, the complex directly interacts with the Est1 subunit of the telomerase and to the catalytic subunit of the DNA polymerase alpha (Polα). The direct binding to telomerase, involved in the elongation of the G-strand of the telomeres, and to Polα, in charge of the fill-in of the C-strand, places the CST components in a central role in the regulation of telomere replication. Structures of the human CST complex revealed decameric structures when the complex binds to DNA but monomeric structures in several states of binding to Polα. Our recent work using the CST complex from Candida glabrata has given some insights in the dimerization of Cdc13 when it interacts with telomeric DNA. However, while Stn1 and Ten1 are very well conserved across species, Cdc13 does not resemble its mammalian counterpart CTC1 either in sequence or domain organization. Due to the lack of structural information in the yeast CST complex it remains unclear whether Cdc13 is dimeric when it binds the ST complex to interact with Polα or how known post-translational modifications of the complex could be affecting its conformation. We are using biochemical and structural approaches to determine the oligomerization state of Cdc13 when bound to the ST complex.

Understanding the Full Lifespan and Dynamics of the Telomerase RNP in Saccharomyces cerevisiae.

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Telomere maintenance and accurate telomerase assembly is important for cellular function. This has been emphasized in human cells where mutations affecting telomere maintenance can result in life-threatening diseases, such as Dyskeratosis congenita. Unfortunately, the precise localization, assembly and overall dynamics of the telomerase RNP are not fully understood, resulting in limited patient diagnosis. Recent results from our lab yielded a comprehensive model of the assembly and maturation of a fully functional telomerase RNP in Saccharomyces cerevisiae (Neumann et al.; Cell Reports 2023). In brief, these results show a rapid export of a stabilized yet inactive telomerase complex, followed by RNA trimming and an extensive swap of protein components on the RNP in the cytoplasm. This is re-imported into the nucleus where the RNA (TLC1) is irreversibly modified to ensure stable nuclear localization and consequently preventing re-export into the cytoplasm. Although, this model has shed light on the assembly and maturation of a fully functional telomerase RNP, questions concerning the remarkable longevity of TLC1 remain. Therefore, we further investigate the RNP composition during the late stages of the telomerase RNP. We use an inducible RNA untagging switch to enrich for RNPs that are at a very late stage of their "lifespan". Those molecules will be assessed by MS to establish whether there is something specific on the RNP that causes it to fail at some point (specific factor lost or protein PTMs for example). Such information will be extremely useful if we wish to influence the lifespan of telomerase in cells.

OGG1 and MUTYH repair activities promote telomeric 8-oxoguanine induced cellular senescence

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Telomeres are hypersensitive to formation of the common oxidative lesion 8-oxoguanine (8oxoG), which impacts telomere stability and function. 8oxoG DNA glycosylase (OGG1) and MutY-homolog (MUTYH) initiate base excision repair (BER) at 8oxoG to remove the lesion or prevent mutation. Here, by exploiting a unique tool that selectively targets telomeres with 8-oxoG formation, we investigated the role of telomeric 8oxoG processing by the BER glycosylases in cellular senescence induction. We found that OGG1 loss or inhibition, or MUTYH loss, partially rescued telomeric 8oxoG-induced premature senescence and associated proinflammatory responses, while loss of both glycosylases caused a near complete rescue. Glycosylase deficiency also suppressed 8oxoG-induced telomere fragility and dysfunction, indicating that downstream single-stranded break (SSB) repair intermediates impair telomere replication. Preventing BER initiation suppressed poly(ADP-ribosyl)ation and conferred resistance to the synergistic effects of the poly(ADP-ribose) polymerase (PARP) inhibitor Olaparib on 8oxoG-induced senescence. However, we found that after chronic telomeric 8oxoG damage, OGG1 activity is essential for preserving cell growth, whereas MUTYH promotes senescence to prevent chromosomal instability from unrepaired lesions. Our studies reveal that inefficient completion of 8oxoG BER at telomeres triggers cellular senescence via SSB intermediates which disrupt telomere function.

ZCCHC8 mutations associated with telomere biology disorders affect both hTR biogenesis and telomere stability

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ZCCHC8 is a factor of the NEXT complex that participates in the modification and degradation of RNAs by addressing them to the exosome. NEXT has been shown to participate to the maturation of the human RNA telomerase component hTR. The involvement of ZCCHC8 in hTR biogenesis has been confirmed by the identification of a heterozygous missense mutation in ZCCHC8 that cosegregated with telomere biology disorder (TBD) and short telomeres.
Here we report 4 novel missense heterozygous ZCCCHC8 mutations in 8 patients exhibiting short telomeres and TBD manifestations ranging from pulmonary fibrosis in adulthood to Høyeraal-Hreidarsson (HH) syndrome in childhood. We introduced the HH mutation in the HT1080 human cell lines (ZCCHC8 KI/WT). ZCCHC8 KI/WT cells exhibited impaired hTR biogenesis and reduced telomerase activity. Furthermore, independently of the telomerase defect, ZCCHC8 KI/WT exhibited telomere fragility and instability that were absent in

ZCCHC8 haploinsufficient or KO cell lines. Hence, the ZCCHC8 missense mutation exerts a dominant negative effect on both hTR biogenesis and telomere integrity. We developed Zcchc8 KI and KO mouse models that further support the notion of a dominant negative effect of the Zcchc8 missense mutation. Our last results obtained in these cellular and animal models will be presented and discussed.

hTERC Template Mutation Impairs Telomerase Processivity Resulting in DKC

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Human telomerase, the ribonucleoprotein complex responsible for telomere elongation, is at its core comprised of a non-coding RNA subunit (hTR encoded by TERC) and the catalytic protein telomerase reverse transcriptase (hTERT). The template region of hTR is reiteratively reverse-transcribed by TERT to replenish telomeric repeats at the ends of chromosomes. Here we describe a TERC mutation identified in a patient with dyskeratosis congenita (DKC), a rare genetic disorder associated with abnormally shortened telomeres caused by mutations in telomerase subunits or other factors required for telomere maintenance. By isolating telomeric sequences from WGS data of the affected individual, we find that mutant sequences constitute approximately 10% of telomeric repeats. Pattern analysis of wild-type and mutant repeats indicates a striking difference in telomerase processivity in vivo. Biochemical experiments confirmed this finding and provided mechanistic insights into the defect caused by the template mutation. Healthy family members lacking the TERC template mutation have inherited mutant telomeric repeats from previous generations; however, it is unclear how or if this will impact their health as they age. Analyzing telomeric sequencings via short-read WGS is non-trivial as the threshold for identifying telomeric sequences is poorly defined, it is impossible to differentiate between DNA replication errors and sequencing noise, and positional information on the read's location within the telomeric tract is unattainable. Third-generation long-read sequencing allows for the complete sequencing of native telomeres but has a significantly higher error rate than NGS and presents other unique challenges to overcome.

The fatty connection of telomeres

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Fatty acid metabolism, innate immune response and mitochondrial fitness are known to be interconnected. As we identified these signatures to be differentially regulated in isogenic immortalized fibroblast cell lines that are either telomerase- or ALT-positive, we investigated whether lipid metabolism, cytoplasmic telomeric DNA species and/or mitochondrial DNA release were involved in the observed differential induction of the innate immune response. In addition, we found that the telomere maintenance mechanism strongly modulates yet another aspect of cellular metabolism: the resistance of cells to ferroptosis, a cell death mechanism induced by the peroxidation of membrane phospholipids. Importantly, we confirmed our observations in telomerase-positive and ALT-positive sarcoma cell lines. Together, these data bring new light on the increasing connections between telomeres, telomere maintenance mechanisms and cellular metabolism.

Study of telomere length dynamics and oxidative stress-mediated biomarkers in Psittacidae species with different longevity.

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Telomeres, conserved DNA sequences at chromosome ends, naturally shorten with age, exacerbated by environmental challenges or reproduction. Telomere length (TL) varies among species and within organisms, and their telomere dynamics through life have shown to be an estimate of life expectancy. However, a lot is still to investigate how the different life traits, stress conditions, and longevity strategies of these species take part in TL dynamics. Birds, particularly psittacine, are gaining prominence as new aging models over the years because of their unique characteristics. This study explores changes in erythrocyte telomere length (TL) measured by qPCR and oxidative stress markers in plasma, such as Total Antioxidant Capacity (TAC) and Lipid peroxidation products (TBARS), of long- and short-lived Psittaciformes over four years. Long-lived birds consistently exhibited longer TL than short-lived ones ($p=0.012$) but experienced a more pronounced TL shortening rate than short-lived birds (p

Heterogeneity of Telomere Maintenance Mechanisms in Pediatric High-grade Glioma
and Therapeutic Implications

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Pediatric high-grade gliomas (pHGGs), including diffuse intrinsic pontine glioma (DIPG), are highly aggressive tumors with dismal prognosis. Cancer cells overcome replicative senescence by mainly activating telomere maintenance mechanisms (TMMs) through telomerase activity or using alternative lengthening of telomere (ALT) pathway. Although ALT phenotype is more prevalent in pHGGs, the molecular pathway and the prognostic significance of ALT activation are not well understood in pHGGs. Here, we report the heterogeneity of TMMs in pHGGs and their association with genetic alterations. We did not observe ATRX/DAXX or TP53 mutations in our ALT DIPG tumors, indicating that in contrast to non-DIPG pHGGs where ATRX mutations and ALT association occurs with a high frequency, ATRX/DAXX mutations are not relevant to ALT pathway in DIPG. Interestingly, Some DIPG tumors harbor either both ALT and telomerase activity or no canonical TMM. Finally, our data indicate that sensitivity to ATR and CHK1 inhibitors is not specific to pHGG ALT-positive cells. Moreover, contrasting with previous studies, both CHK1 inhibition and aberrant spread of H3.3 S31P in pHGG cells were not specific to ALT cells, suggesting that CHK1 kinase activity and H3.3 S31 phosphorylation are not required for pHGGs ALT cell survival. Together, these data indicate the inter- and intratumor heterogeneity of TMMs and the variability of ALT determinants in pHGGs. Elucidating the molecular pathway of ALT is a prerequisite for the development of new therapeutic strategies to improve outcome in this vulnerable group of patients.

THIO-induced Telomere Dysfunction Leads to Cytoskeleton Remodeling

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High-risk pediatric brain tumors, including diffuse intrinsic pontine glioma (DIPG) are essentially incurable and remain the leading cause of cancer-related death in children. We have previously shown that the incorporation of 6-thio-dG (THIO) into telomeres leads to TIFs formation, cell death and tumor growth inhibition. While the majority of THIO treated cells died by apoptosis, survivor cells emerged after an extended drug holiday. Recent studies have shown that downregulation of SLC43A3, an equilibrative nucleobase transporter that mediates the cellular uptake of extracellular purine nucleobases, is associated with THIO resistance in lung cancer cells. However, our RNA-seq data analysis of patients' DIPG tumors indicates that SLC43A3 is highly expressed compared to normal tissue specimens.
To better understand the mechanism of THIO resistance in high-risk brain tumor patient-derived cells, we generated resistant cells after a prolonged exposure to sub-lethal doses of THIO. Our data indicate that resistant cells acquired new properties related to cytoskeletal gene expression and remodeling, displaying a distinct adherent spread morphology compared to

parental neurosphere cells. We observed a focal expression of F-actin demonstrating adherent morphology. The comparative analysis of gene expression relative to parental cells indicated an upregulation of cytoskeletal remodeling markers vimentin (VIM), fibronectin (FN1) and metallomatrix gene COL1A1. Furthermore, the epithelial-mesenchymal transition (EMT) master regulator TWIST1 was upregulated in the resistant cells. TWIST1 is known to induce the expression of both VIM and COL1A1.
Together, our data suggest that THIO-induced telomere dysfunction leads to EMT pathway activation, resulting in cytoskeleton remodeling and gene expression reprogramming.

Evolutionary Divergence in Hymenoptera and Lepidoptera Telomerase RNAs: A Shift from the Norm in Animal TR Biogenesis

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Unlike typical animal TRs, which are transcribed by RNA Polymerase II, the TRs in Hymenoptera and Lepidoptera insects display characteristics similar to TRs in plants and ciliates, transcribed by RNA Polymerase III under the control of type 3 promoters. This significant deviation in TR biogenesis challenges the previously assumed monophyletic origin of TRs in animals and suggests an evolutionary shift in TR type and biogenesis within Arthropods. This discovery not only questions the uniformity of TR evolution across animals but also highlights the evolutionary flexibility in telomere biology, particularly in the context of insect genomic stability and telomere maintenance mechanisms.

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Telomere Targeting using Small Molecule Mediated G-Quadruplex Stabilization in Cancer Cells

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Telomeres present at chromosome ends undergo progressive shortening during replication, consequently initiating cellular senescence and apoptotic pathways in eukaryotic somatic cells. However, cancer cells exhibit increased telomerase activity, promoting telomere extension and cell immortality. Targeting this process, we explore telomeric G-quadruplex (G4) stabilization using small molecules as a potential therapeutic strategy. G-quadruplexes are unique non-canonical conformations adopted by guanine (G)-rich nucleic acids sequences. Planar G-quartets associated via Hoogsteen type hydrogen bonds stack to form the folded secondary G-quadruplex structure and stabilized by monovalent metal cations such as Na+ or K+. The aim of telomeric G4 stabilizing molecules is to inhibit telomerase activity and hinder telomere

elongation specifically in cancer cells. Despite the identification of numerous molecules with potential, the selective recognition of particular G-quadruplex motifs poses a critical challenge that constrains the translational progress of these stabilizing ligands into clinical investigations. We have employed a molecular library of rationally designed triazolyl (TIQs) ligands with side-chain functionalization to selectively stabilize the telomeric G-quadruplexes. A series of biophysical and biochemical investigations of these ligands showed that two ligands effectively stabilize telomeric G4 via π-π stacking and induce apoptosis. They also arrest cell cycle progression, trigger DNA damage and increase the generation of reactive oxygen species further propelling the cancer cells towards apoptosis. The downregulation of telomerase component, hTERT and effective inhibition of telomerase activity emerged as critical components of the study. Multi-faceted TIQ ligands capable of inducing a graded inhibitory response in telomerase activity may provide an alternative strategy for cancer therapy.

Telomerase activation under the p21-promoter induces cardiomyocyte proliferation and heart regeneration

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Cardiovascular diseases are the leading cause of death worldwide. This high mortality rate ultimately results from the adult heart's inability to regenerate after a myocardial infarction. In response to injury, the vast majority of adult cardiomyocytes do not divide, and therefore lost cardiomyocytes are not replaced. However, neonatal mammalian cardiomyocytes, including those in human, possess the capacity to divide, thereby contributing to the regeneration of the neonatal heart following injury. The transition to a non-regenerative state in cardiomyocytes concurs with a change in their nucleation and ploidy status, from being mononucleated and diploid to binucleated and polyploid. These changes also coincide with the inactivation of telomerase, rapid telomere erosion, appearance of telomere fusions, and an increased number of p21-positive cardiomyocytes.

To examine the role of telomere shortening in cardiomyocyte polyploidization and heart regeneration, we have recently generated mice expressing telomerase (Tert) under the p21Cdkn1a promoter. In these p21Tert/Tert mice, telomerase remains active beyond the initial week of life and telomeric shortening is slowed. Additionally, we observed a delay in the binucleation process, an increased number of proliferating cardiomyocytes and a regeneration response beyond the first days of life. These findings imply that modulating telomerase activity could have a beneficial effect in the treatment of myocardial infarction.

Orphan nuclear receptors promote human cell immortalization by activating ALT

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Orphan nuclear receptors (NRs), such as COUP-TF1, COUP-TF2, TR2, TR4, and EAR2, are implicated in ALT cancers by binding to variant TCAGGG telomeric repeats. However, how these orphan NRs function in ALT development remains unclear. Recently, we found that telomeric targeting of orphan NRs in fibroblasts promotes ALT phenotypes and activity via ALT-associated PML bodies. Here, through tethering of COUP-TF2 to telomeres of SV40-transformed primary fibroblasts, we reveal that orphan NRs promote cell immortalization by activating the ALT pathway. The immortalized clones are telomerase-negative and possess multiple ALT features such as long telomeres, APBs, telomere clustering, telomeric DNA synthesis, and C-circles. The ALT activity is dependent on the RAD52-POLD-BTR-mediated break-induced replication pathway. Intriguingly, these immortalized clones still express the most commonly silenced genes in ALT cells – ATRX and DAXX, suggesting an ATRX/DAXX loss-independent mechanism for immortalization. Previous studies demonstrate that a considerable proportion of cancers have no mechanism for ATRX deactivation, posing a need for a novel tool for ATRX/DAXX loss-independent ALT cancer subtypes. Our study bridges this gap and highlights orphan NR-mediated immortalization as a novel model for understanding these ALT cancer subtypes. Moreover, our transcriptome analysis revealed multiple cell physiological changes upon immortalization. The altered pathways that potentially contribute to the immortalization will be discussed. Collectively, we report that telomeric targeting of orphan NRs promotes cell immortalization by ALT in fibroblasts and present this phenomenon as an alternative mechanism for ALT immortalization that is independent of ATRX/DAXX loss, thus highlighting a novel model for ALT cancer subtypes.

Telomere maintenance and structural variants: the impact of ALT on complex genomic rearrangements

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Cancer cells must overcome progressive telomere erosion in order to achieve unlimited replicative potential. Critically short telomeres can induce chromosomal fusions, structures associated with chromatin bridges and the development of complex genomic rearrangements (CGRs). Still, the broader impact of telomere dynamics on structural variation remains to be elucidated.
Alternative lengthening of telomeres (ALT) employs break-induced replication (BIR) to elongate short telomeres via recombination and conservative DNA synthesis. Interestingly, BIR appears to also occur on chromosomes that experienced bridge breakage and it leads to their segregation into micronuclei, compartments where CGR have been proven to take place. This project investigates the effect of telomere maintenance on the formation and repertoire of structural variants (SVs). The main hypothesis is that ALT, intrinsically elevating recombination rates, leads to chromosomal instability production of CGRs.
Analyses carried out on the PCAWG dataset highlighted a correlation between ALT(-related) phenotypes and chromothripsis, a type of CGR, in cancer samples. Therefore, we started by characterising the landscape of SVs in isogenic wild-type and ATRX-KO cell models. While the absence of ATRX caused an overall increase in micronuclei, this was not accompanied by an increase in the complexity of the detected rearrangements, hinting at the necessity of additional factors to introduce CGRs, beyond the genome-wide chromatin decompaction occurring in the absence of ATRX.
By further describing CGRs in models with different genetic backgrounds and telomere

maintenance mechanisms, we aim to finally identify what determines emergence and complexity of de novo structural variants in an ALT context.

A structural role for Topoisomerase II in resolving telomere entanglements in late anaphase

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Topoisomerase II (Top2) is essential for chromosome segregation in mitosis through its decatenation activity. Top2 is enriched at replication forks and regions where replication-associated lesions often arise, such as centromeres and rDNA. It is unknown how Top2 activity is targeted and regulated on the chromosome and whether it could have, independently of decatenation, a "structural" role. In fission yeast, replication stress at telomeric regions results in entanglements between telomeres, which must be resolved for the survival of the daughter cells. We find that a Top2 mutant, Top2-191, has, compared to the wild-type, an improved activity that favors entanglement removal and rescues viability. This activity is independent of DNA cleavage and ATPase activities,which can be inactivated without affecting entanglements resolution. Top2-191 has an improved DNA bridging ability and its phase separation property is involved in Top2 localization to the chromosome and is essential for Top2-191 suppression. In late anaphase, telomere entanglements stretched along the midzone are transiently exposed to the cytosol through nuclear envelope breakdown (NEBD), which favor their resolution. We show that Top2-191 resolution activity is restricted to late anaphase when cytosol exposure occurs. We therefore hypothesize that Top2, in response to cytosol exposure, undergoes phase separation at entangled telomeres and holds them in a conformation that protects telomeres against further damage, and possibly limits its own catenation activity. Our data suggest that Top2 fulfills a regulated structural function at telomeres to favor chromosome segregation that could be relevant to higher eukaryotes, where NEBD occurs in mitosis and in interphase.

Does RECQL5 play a role in protecting telomere integrity?

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RECQL5 is a member of the RECQ helicase family whose members execute essential roles in DNA replication, transcription, DNA repair and chromatin remodeling. While the five RECQ helicases share several domains, each has unique features that allows it to carry out specialized functions. RECQL1-4 have been shown to play protective roles at telomeres, regions that pose challenges to the replication apparatus. Whether RECQL5 is important for telomere integrity in mammalian cells is yet unknown. Due to its unique combination of interaction domains, we hypothesize that RECQL5 may function at telomeres in resolving transcription-replication conflicts, regulating telomeric DNA:RNA hybrids and controlling levels of homologous recombination. Our preliminary findings show that RECQL5 loss-of-function (LOF) exerts a strong effect on proliferation of human primary fibroblasts, which is significantly rescued by hTERT expression. Moreover, telomere length in fibroblasts expressing RECQL5-shRNA for two months is significantly shorter compared to control fibroblasts. Further supporting a role for RECQL5 at telomeres, we found that GFP-RECQL5 colocalizes with TRF2 in nuclei of

human cells. We will further explore whether knock-down of RECQL5 induces telomeric-DNA damage foci, and elevates fragility and recombination at telomeres. Our study is expected to determine whether RECQL5 is a crucial player that safeguards chromosome ends, and through such action protects the stability of the genome.

Biochemical studies reveal a possible correlation between the strength of the Apollo-TRF2 complex and longevity

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Reforming the T-loop after replication requires the presence of a 5' overhang particularly on the DNA generated by leading strand synthesis. Contrary to classical double-strand breaks, generating the single strand end of telomeres does not involve the MRN complex but necessitates the APOLLO protein (also named SNM1 and encoded by DCLRE1B). This protein is recruited to telomeres through an interaction between the TRFH domain of TRF2 and the terminal part of APOLLO.
Amazingly, two articles reporting genome comparison between long-lived and short-lived animals1,2 have observed adaptative signatures in DCLRE1B around the TRF2 binding domain. Further analysis of sequences of other short-lived species also revealed notable differences in this region suggesting a possible link between variations in the affinity of the TRF2-Apollo complex and longevity. To address this question, we analyzed the interaction between the TRFH domain of TRF2 and the C-terminal domain of Apollo in different species with different longevities: human, naked mole rat (the longest living rat, 30 years), killifish (the fish with the shortest longevity, 8 months), zebrafish (5 years), the Galapagos tortoise (200 years) and the painted turtle (40 years). Our results reveal an inverse correlation between affinity and longevity. Indeed, the shorter the longevity in giving species, the stronger the interaction. This suggests that the strength of the affinity of Apollo for TRF2 could constitute an important parameter in determining life span.
1- V. Quesada et al., Nat. Ecol. Evol. 3, 87–95 (2019).
2- S. R.R. Kolora et al., Science 374, 842-847 (2021).

Loss of SETD2 facilitates induction of ALT pathway telomere maintenance in ATRX deficient cancers through ROS-induced replication stress

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The alternative lengthening of telomeres (ALT) pathway is a telomerase-independent mechanism for immortalisation in cancer cells. Activation of ALT-pathway activity requires two events: mutational loss of ATRX and a poorly understood second event. We recently demonstrated that artificial induction of DNA-protein complexes (DPCs) could act as the second driver, but the endogenous origins of DPCs in ALT-cancers remained obscure. Here, we demonstrate that excessive reactive oxygen species (ROS) is a second causative factor in the evolution of ALT-telomere maintenance. We found that elevated ROS levels led to accumulation of R-loops (DNA:RNA secondary structures) and also formation of DPCs. Further, we identified mutational loss of SETD2 as one potential source of elevated ROS in ALT-positive gliomas. Combined loss of ATRX and SETD2 led to a robust induction of ALT hallmarks and associated telomere elongation that could be reversed by RNase H1 overexpression and antioxidant treatment. Our data provides a complete model of ALT telomere elongation, redefining our understanding of this critical cancer pathway.

Structural studies of Candida glabrata Cdc13 by cryoEM

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Telomere protection and maintenance are crucial for genome integrity and thus, cell survival. Cdc13, the largest CST complex component in yeast, participates in both processes. It binds to telomeric ssDNA, which caps telomere ends and protects against chromosome fusions; and, as part of CST, inhibits G-strand elongation by telomerase and recruits the Polα complex to synthesize the complementary C-strand during telomere ends replication. Cdc13's architecture, essentially a concatenation of OB folds, allows it to encompass several molecular functions: binding to different ssDNA substrates and interacting with and recruiting other telomeric proteins. The currently available information of Cdc13 architecture and its oligomeric states is limited by fragmented structures of the polypeptide in different, apparently contradictory, oligomerization states. We recently proposed a homodimerization mechanism mediated by OB2 and OB4 using functional assays, also showing that Candida glabrata Cdc13 requires dimerization and the concerted use of all OB folds for optimal ssDNA binding and secondary structure unfolding (Coloma and González-Rodríguez et al., 2023). We have now initiated cryoEM experiments to determine the structure of full-length Cdc13. This structure will allow us to understand the structural basis of dimerization and ssDNA binding, and hopefully provide much-expected insights into the evolutionary history of Cdc13 and CST. We will present the challenges faced during this project and the latest advances towards determining the structure of full-length Cdc13.

Human hnRNPA1 reorganizes telomere-bound Replication Protein A

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Human replication protein A (RPA) is a heterotrimeric ssDNA binding protein responsible for many aspects of cellular DNA metabolism. Dynamic interactions of the four RPA DNA binding domains (DBDs) with DNA control replacement of RPA by downstream proteins in various cellular metabolic pathways. RPA plays several important functions at telomeres where it binds to and melts telomeric G-quadruplexes, non-canonical DNA structures formed at the G-rich telomeric ssDNA overhangs. Here, we combine single-molecule total internal reflection fluorescence microscopy (smTIRFM) and mass photometry (MP) with biophysical and biochemical analyses to demonstrate that heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) specifically remodels RPA bound to telomeric ssDNA by dampening the RPA configurational dynamics and forming a ternary complex. Uniquely, among hnRNPA1 target RNAs, telomeric repeat-containing RNA (TERRA) is selectively capable of releasing hnRNPA1 from the RPA-telomeric DNA complex. We speculate that this telomere specific RPA-DNA-hnRNPA1 complex is an important structure in telomere protection.

Investigating the role of TERRA R-loops in telomerase regulation

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TERRA transcripts localize at telomeres, both in cis and in trans, through the interaction with TRF1 and TRF2 proteins as well as by direct base-pairing with the C-rich telomeric strand forming RNA:DNA hybrids, also called R-loops. R-loop formation at telomeres has been demonstrated to be essential in ALT cancer cells as it can support homologous recombination-mediated telomere maintenance. While the involvement of telomeric R-loops in ALT cell lines is well established, their potential roles in telomerase regulation remain to be defined. As the 3' end of TERRA transcripts is complementary to the template region of hTR, TERRA R-loops at telomeres may regulate telomerase recruitment at telomeres and consequently its activity.
By performing single-molecule RNA FISH combined with IF, we observed that a fraction of telomeric TERRA molecules colocalizes with hTR. In this project, we will use this approach to study the telomeric localization of hTR in HeLa cells upon ectopic expression of RNaseH1, or its mutant version unable to dissolve R-loops, as well as upon silencing of endogenous RNaseH1 by siRNAs to promote telomeric R-loops accumulation. Finally, we aim to investigate the localization dynamics of TERRA and hTR in living cells upon R-loops deregulation by exploiting engineered human cancer cell lines that express endogenous TERRA or hTR molecules tagged with the MS2 sequence. Our approaches aim to contribute to understanding the potential roles of telomeric R-loops in telomerase activity regulation.

Clonal signatures of telomere biology disorders

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The clonal landscape of telomere biology disorders (TBD) have been reported in small studies, but the role of clonal hematopoiesis (CH) associated with compensatory or malignant disease mechanisms in a large cohort of patients with a broad spectrum of ages and disease phenotypes are still uncertain. We characterized the clonal landscape of a cohort of 207 TBD patients by multi-omic analyses. CH was predominantly observed in symptomatic patients (83%) and associated with the acquisition of recurrent somatic mutations in PPM1D, POT1, TERT promoter (TERTp), U2AF1-S34, and/or TP53, or chromosome 1q gain (Chr1q+). CH highly correlated with the underlying germline defect and aging; CH frequency was higher than in age-matched healthy individuals. CH in TERTp, TP53¸ and MDS-related genes associated with poorer overall survival, while Chr1q+, and splicing factor genes or TP53 mutations significantly increased the risk of hematologic malignancy, regardless of the clonal burden. U2AF1-S34 and Chr1q+ were driver clonal events associated with subsequent acquisition of MDS-related mutations (maladaptive CH). CH in POT1, PPM1D, or TERTp were found in independent clones and did not correlate with malignancy but rather with DC atypical phenotypes. Functional studies demonstrated that U2AF1-S34 mutations compensate aberrant TP53 and interferon (IFN) pathway activation, both known to induce hematopoietic stem and progenitor cell (HSPC) exhaustion in TBD.
The clonal signature of TBDs attempts to overcome the cellular replication defects caused by TBD germline mutations, and can be used as a biomarker of cancer development, poor overall survival, and progressive bone marrow failure in TBD patients.

Telomerase-mediated targeting of telomeric G4s via the 6-thio-dG as a potential treatment strategy for Melanoma

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Malignant melanoma is the most aggressive skin cancer and the survival rate of patients suffering from metastatic melanoma is less than 10 %. One obstacle facing unlimited proliferative potential is the inevitable process of telomere shortening after DNA replication needed for cell division. Germ cells employ the enzyme complex, telomerase to sustain telomeres; cancer cells, including melanoma, exploit this telomere maintenance mechanism, which remains repressed in somatic cells, to overcome proliferative limitation. Targeting telomeres as a therapeutic approach has been followed up in several studies in mouse and human melanoma cell lines.
Modified nucleotides, like 6-Thio-2'-Deoxyguanosine (6-thio-dG) offer a promising prospect for resistant tumors. It relies on the telomerase to incorporate modified substrate into the telomeric 3'-overhang, disrupting telomere maintenance, which results in DNA breaks. The resulting dysfunctional telomeres lead to the activation of anti-tumor immune response. The underlying molecular mechanism of 6-thio-dG on telomere maintenance has not been addressed yet.

Our preliminary observations show that G-quadruplex (G4) structures are the driving force of telomere-induced DNA damage after 6-thio-dG treatment. Telomeric oligonucleotides harboring 6-thio-dG do not fold to G4s In vitro. Assessment of cell viability and metaphase FISH upon 6-thio-dG treatment revealed the sensitivity of A-375 and LOX-IMVI melanoma cell lines with increased telomere loss in comparison to normal fibroblasts. We hypothesize that 6-thio-dG serves as a substrate for telomerase and once incorporated into telomeres, induces dysfunctional telomeres and thus, impairs G4 formation at telomeres. Consequently, G4s cannot exhibit their structural protection and induction of DNA damage is favored.

Inhibiting the telomere processing Apollo/SNM1B nuclease

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The human enzyme Apollo/SNM1B is a 5′-3′ exonuclease implicated in two aspects of genome integrity, both the response to DNA damage, where it is proposed to play a role in double-strand break (DSB) and interstrand crosslink (ICL) repair and telomere maintenance, where it resects leading strand telomeres, generating 3′ overhangs that allow the protective t-loop to form.

As Apollo plays a critical role in the response to DNA damage, compounds that inhibit it might have utility as standalone anti-proliferative agents in certain tumour types or could be used as adjuncts to genotoxic chemotherapeutics, potentiating their effects or preventing resistance from arising. This targeting of Apollo could also elicit senescence or loss of tumour cell viability due to telomere attrition.

A high-throughput screen guided initial Apollo inhibitor development, with iterative rounds of optimisation reliant on understanding the structure-activity relationships of these compounds. This led to the discovery of a highly potent Apollo inhibitor, which displays impressive target selectivity. Structural studies demonstrated that this compound interacts with both the active site and the phosphate-binding pocket of Apollo, forming an extensive network of hydrogen bonds. To combat a lack of cell permeability, a prodrug analogue of this compound was generated, which was cytotoxic across both malignant and non-malignant (hTERT-immortalised) cell lines. p53 status was found to be a significant determinant of sensitivity to this compound, revealing an under-appreciated aspect of the biology of Apollo. This compound, therefore, has potential anti-cancer utility and provides a tool to investigate the poorly understood roles of Apollo.

HOT1 acts as a DYNLL1-mediated dimer to influence telomeric chromatin organization.

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Telomere homeostasis is a highly regulated, dynamic process maintained by a coordinated network of proteins. One such factor is HOT1 (HMBOX1), which directly binds to TTAGGG repeats and positively regulates telomere length in telomerase-positive cells. In ALT-positive cells, HOT1 associates with telomeres at a particularly high frequency, however, its functional implication remains unclear. To address this, we first interrogated ALT hallmarks and found that, while not affecting APB formation, HOT1 depletion led to a significant reduction in C-circle levels. To understand how HOT1 affects ALT activity, we initially searched for HOT1 interaction partners and identified DYNLL1, which acts as a 'molecular velcro' to facilitate the homodimerization of its target proteins. Indeed, both DYNLL1 depletion and mutations in the HOT1-DYNLL1 interaction domain led to a significant reduction in HOT1 colocalization with telomeres, suggesting that HOT1 homodimerization is required for its in vivo association. Finally, we compared the native telomere chromatin composition in the presence or absence of HOT1 using chromatin immunoprecipitation coupled to mass spectrometry (ChIP-MS). Here, loss of HOT1 led to a decrease in telomeric H3K9/14ac signal with a concomitant increase in H3K9me3 signal. In addition, HOT1 knock-out caused dissociation of the NuRD complex member CHD3 from telomeres. In sum, these findings demonstrate HOT1's impact on telomeric chromatin organization and the ALT pathway.

Sleeping kidnapers inside human cells – extra-telomeric functions of telomerase in the pathogenesis of Epstein-Barr virus

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The Epstein-Barr virus (EBV), a widespread human γ-herpesvirus, is intricately linked to a diverse range of lymphoid and epithelial malignancies. To perpetuate EBV presence in host cells and facilitate tumorigenesis, EBV strategically regulates its lytic cycle, selectively expresses latent viral proteins through viral transcription factors Rta and Zta, and relies on telomerase for maintaining telomere length. The expressed viral oncoprotein LMP-1 activates TERT, the catalytic component of telomerase. Beyond its conventional role in stabilizing telomeres, TERT also exerts extra-telomeric functions that impact EBV-driven tumorigenesis.

Specifically, TERT preserves EBV latency by modulating the NOTCH2/BATF pathway, which in turn negatively affects the expression of Zta - the master regulator of the EBV lytic cycle. Inhibiting TERT triggers a complete EBV lytic cycle, leading to the demise of EBV-infected cells. Short-term TERT inhibition induces cell cycle arrest and apoptosis. Importantly, TERT inhibition sensitizes EBV-positive tumor cells to antiviral therapy and enhances the pro-apoptotic effects of chemotherapeutic agents.

We combined cell immunolocalization studies, molecular biology approaches, biophysical methods, and structural analyses (i) to reveal the proximity of Zta-Rta in model EBV positive

cells, (ii) to describe DNA binding affinity of Rta and Zta, and (iii) characterize the structure of Rta-RRE DNA complex in vitro.

Understanding extra-telomeric functions of TERT and DNA binding mechanisms of Zta and Rta offer promising avenues for therapeutic intervention in EBV-associated malignancies.

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Measurement of telomere variant repeats by monochromatic quantitative PCR

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The canonical telomere repeat sequence 5'-TTAGGG-3' is disrupted by the presence of telomere variant repeat (TVR) sequences distributed throughout the telomere repeat region. These findings were recently measured using telomere reads extracted from whole-genome sequences and single-molecule real-time sequencing. Here we describe a quantitative polymerase chain reaction (QPCR) method that can be used to measure TVRs such as TCAGGG, TGAGGG and TAAGGG. The simple and rapid fluorescence-based single-plex QPCR specifically detected individual TVRs within canonical telomere repeats, but was less efficient to detect clusters of TVRs confirmed by artificial QPCR DNA targets. The method was applied to quantify TVRs in DNA isolated from human and canine cell lines and tissues, as well as in the rolling circle amplification (RCA) products of the c-circle assay used to quantify alternative lengthening of telomeres (ALT) activity. TVRs were increased in DNA after RCA, indicating that c-circles harbor TVRs. DNA from ALT cells with elevated TVRs was pretreated with restriction enzymes that recognize TGAGGG, and QPCR with these digests resulted in specific TVR reduction. TVR content measured by QPCR correlated with values obtained from public WGS datasets of human cell line models (n=7) using ALT and telomerase as the telomere maintenance mechanism (TMM). In addition, DNA isolated from human astrocytoma (n=2) and canine sarcoma (n=9) FFPE cancer tissues was analyzed for TMM status and TVRs in first pilot experiments to be presented. We propose that the less laborious method of TVR measurements by QPCR may have future implications in scientific and clinical analyses.

Functional analysis of mutations via locally haploid human pluripotent stem cells

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Classifying variants of unknown significance (VUS) and the impact of single amino acid changes is an ongoing challenge in human genetics. We recently developed the locally haploid

(loHAP) human pluripotent stem cell (hPSC) system to conduct detailed structure-function analyses of genes and evaluate VUS. LoHAPs contain a precise deletion of one copy of a gene, allowing for CRISPR/Cas9 mutagenesis on the remaining wild-type allele and creating a haploid setting where mutations can be directly assessed. Here, we use the BRCA2 as a proof of concept for our pipeline and subsequently generate TRF1 loHAPs. Mutations in the BRCA2 gene are associated with sporadic and familial cancer, cause genomic instability, and sensitize cancer cells to poly(ADP-ribose) polymerase inhibition (PARPi). Using BRCA2 loHAP hPSCs and fibroblasts differentiated from them, we annotated essential regions in the protein and identified loss-of-function mutations. Finally, we show this pipeline can effectively separate hypomorphic and functional alleles using PARPi, fluorescence-activated cell sorting (FACS), or additional separation of function assays. Based on these preliminary experiments, we have engineered TRF1 LoHAPs. TRF1 binds double-strand TTAGGG telomeric repeats and is required to localize the shelterin complex to the telomeres. Moreover, TRF1 is also highly upregulated in human pluripotent stem cells (hPSCs) relative to somatic cells, suggesting the protein is essential for maintaining pluripotency. Therefore, we will use the loHAP editing pipeline to uncover the function of TRF1 and its domains in regulating telomere length in hPSCs and differentiated cell types.

HHS patient cells with homozygous RTEL1 mutations exhibit accelerated telomere shortening and disrupted nuclear morphology associated with cytoplasmic localization of nuclear factors

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Hoyeraal-Hreidarsson syndrome (HHS), a clinically severe variant of dyskeratosis congenita, is characterized by accelerated telomere shortening and diverse symptoms including bone marrow failure, immunodeficiency, neurodevelopmental defects, and premature death. HHS is caused by germline mutations in components of telomeres and telomerase, and in the helicase RTEL1. RTEL1 was reported to play diverse roles in telomeric, as well as non-telomeric, genome stability. Several domains have been identified in RTEL1, and HHS-causing mutations were found in most of them. To further understand the functions of RTEL1, we established HHS patient fibroblast cultures from two patients, each containing a different homozygous mutation – R957W, located at Harmonin 1 domain, and R1264H, located at the C4C4 domain. Examination of the phenotypes upon the ectopic expression of hTERT and TET-inducible WT-RTEL1 revealed that the viability of the patient cells was rescued by hTERT expression, while WT-RTEL1 expression further suppressed their slow growth and elongated their telomeres, consistent with the role of RTEL1 in telomere elongation by telomerase. Surprisingly, the cells exhibited aberrant localization of nuclear factors in the cytoplasm, including human Telomerase RNA (hTR) and the nucleolar factor Nucleolin. Additionally, the cells displayed increased cellular compartments deformations. Our results indicate that RTEL1 deficiency not only compromised telomere elongation by telomerase, but also impaired the nuclear integrity, leaking nuclear components to the cytoplasm. The contribution of these cellular aberrations to inflammation processes and to the disease etiology and symptoms is yet to be investigated.

Histone Variant H3.3 Mediates cGAS-STING Pathway Activation upon Telomere Deprotection

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Molecular mechanisms for inducing cellular senescence in response to telomere deprotection has been linked to DNA damage response. Recently, the innate immune response activated by endogenous DNA through the cGAS-STING pathway is found to mediate telomere dysfunction-induced cellular senescence. By the expression of a dominant-negative TRF2 mutant, TRF2ΔBΔM, we modeled the telomere uncapping in telomerase immortalized BJ cells for investigating the molecular mechanism controlling the process. We show that depletion of cGAS rescued the proliferation defects, suggesting the activation of cGAS-STING response mediates the cell proliferation defects driven by telomere uncapping. On the other hand, the histone variant H3.3 is deposited to telomeres by the chaperon ATRX-DAXX complex and H3.3 mutations are found in glioblastoma cancer cells. Previously, our lab also described that ATRX, DAXX, and H3.3 contribute to the activation of cGAS-STING response due to the formation of extrachromosomal telomere repeats (ECTRs). During this meeting, we will discuss the regulatory mechanism of H3.3 on cGAS-STING activation following telomere uncapping.

Landscape of genetic vulnerabilities in ALT cancers

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Alternative lengthening of telomeres (ALT) is a cancer-specific telomere elongation mechanism that relies on homologous recombination pathways to mediate telomere extension and sustain replicative immortality. ALT+ tumours are a major cancer type of unmet need, have a universally poor prognosis, and lack targeted therapies but are readily definable in patients using established ALT markers. Here, we identify novel regulators essential for ALT cancer survival. We performed multiple CRISPR screens in a panel of ALT and no-ALT patient-dervied cell lines, using a focused library designed against the ALT telomere proteome, which we characterised by PICh. This approach identified ~25 high-priority targets that cause ALT specific cell death through suppression or hyperactivation of ALT activity. Loss of viability resulted in either apoptosis, autophagy, or senescence, dependent on the target and tumour cell type. A drug screen performed on pre-existing compounds confirmed the ALT-specificity of our genetic results. Mechanistic analysis of several targets confirmed co-localisation with ALT telomeres and APBs, and higher expression in ALT+ Undifferentiated Pleomorphic Sarcomas. These data were further integrated with three orthogonal ALT genome-wide CRISPR/Cas9 screens: 1) in-silico, 2) isogenic ATRX loss of function screen, and 3) an ALT activity phenotypic screen. This integrated approach revealed two targets that connect telomeres and a distinct metabolic pathway. Our work provides mechanistic insights into ALT vulnerabilities, potentially identifying new therapeutic opportunities and biomarkers.

Induction of telomere fragility by TERRA

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TERRA is transcribed at a large number of telomeres. Whereas TERRA is repressed during S phase in telomerase-positive cells, unscheduled expression of TERRA during S phase and extensive changes in telomeric chromatin have been observed in ALT cancer cells that maintain telomeres by homologous recombination. ALT telomeres are also characterized by increased telomere fragility, which is thought to stem from replication stress and DNA repair. To understand how TERRA affects telomere replication and repair during S phase, we utilized a CRISPR-mediated gene activation system to induce TERRA transcription in HeLa cells. TERRA induction led to an increase in telomeric R-loops, formation of Telomere Dysfunction Induced Foci, and a strong increase in telomere fragility. Depletion of RAD51 or RAD51AP1 suppressed the formation of TERRA R-loops and TERRA-induced telomere fragility. RNaseH1-overexpression, which degrades R-loops alleviated telomere fragility. These demonstrate that R-loops cause replication stress at telomeres and telomere fragility. To test the hypothesis that TERRA R-loop-mediated telomere fragility is caused by replication fork collapse followed by DNA repair, we suppressed several DNA repair pathways. We identified two DNA repair pathways whose co-depletion alleviated telomere fragility. One pathway involves PrimPol which reprimes DNA synthesis downstream of replication obstacles. The second is Break Induced Replication, which contributes to telomere maintenance in ALT cells. Our results reveal discrete steps of how TERRA manipulates telomere maintenance. First, TERRA forms R-loops in a RAD51 and RAD51AP1-dependent manner. Second, TERRA R-loops lead to replication fork collapse. Third, the damaged telomeric forks are repaired by DNA repair pathways.

The biophysical environment of telomeres at the nuclear envelope

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In addition to their classically recognized functions in protecting chromosome ends, telomeres have distinct and crucial roles in ensuring proper chromosomal segregation in meiosis. Fission yeast chromosomes undergo dramatic positional reorganization in meiosis, resulting in telomeres localizing at the nuclear envelope beneath the centrosome (spindle pole body or SPB in fission yeast) to form a highly conserved structure known as the telomere bouquet. Our lab has demonstrated that the telomere bouquet is necessary for initiation of local nuclear envelope breakdown (NEBD), which is required for SPB insertion, meiotic spindle formation, and proper chromosomal segregation. Many questions are yet to be answered regarding this non-canonical function of telomeres, such as what telomere feature confers the promotion of NEBD? It is possible that the biophysical environment surrounding the telomere, and in turn the proteins being recruited to this area, are crucial in initiating this step in meiosis. To address this issue, we are studying the molecular dynamics of various proteins that are associated with the telomere, including shelterin and kinases implicated in triggering NEBD. Through single molecule localization microscopy (SMLM), we are beginning to elucidate the activity of Taz1 and Rap1 in both mitosis and meiosis. Preliminary data suggests that there is one population of Taz1, stably bound to the telomere, but Rap1 comprises both a stably bound and a more diffuse and dynamic population that could point to condensate formation. These studies will enhance our understanding of the biophysical environment and diffusion properties of proteins near the telomere and nuclear envelope.

Functional characterization of Apollo deficiency in murine and human cellular models

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Apollo is a 5'-to-3' DNA exonuclease participating in both DNA repair and telomere integrity via its interaction with TRF2.
We identified 3 unrelated patients who carry a homozygous or compound heterozygous (in combination with a null allele) missense variant affecting the same residue L142 (L142F or L142S) located in the catalytic domain of Apollo. These individuals exhibit the telomere biology disorders dyskeratosis congenita and its severe variant, Høyeraal-Hreidarsson syndrome. Apollo-deficient cells from patients exhibited spontaneous chromosome instability, impaired DNA repair and signs of telomere fragility that were not associated with global reduction of telomere.
We generated by CRISPR/Cas9 several human and murine cellular models including Apollo KO HT1080 cell line, tansformed patients' cell lines, and mouse embryonic fibroblasts (MEFs) obtained from ApolloL142S/L42S KI mouse model. We used these cellular models to determine the impact of Apollo deficiency on DNA repair, genome stability and telomere integrity. The generation of Apollo L142S KI HT1080 cell lines as well as tagged at the endogenous Apollo locus with a NeonGreen or FLAG tag is ongoing. These cellular models will be of great interest for a better understanding of Apollo's pleiotropic functions in genome stability and telomere integrity. Our most recent results will be presented.

RPA copes with replication stress at telomeres: missense mutations in RPA2 gene cause telomere biology disorders

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Human telomere biology disorders (TBD) are heterogeneous disorders caused by inherited loss-of-function mutations in telomere-associated genes. These disorders are linked to diverse clinical manifestations, including bone marrow failure, myelodysplastic syndrome, pulmonary fibrosis, skin manifestation, and various premature aging phenotypes. We identified a heterozygous rare missense variant in RPA2 gene in two distinct patients exhibiting short telomeres and clinical signs of TBD, such as idiopathic pulmonary fibrosis and premature aging phenotypes. This mutation is located in the C-terminal part of RPA2. Importantly, the patient mutation does not modify the biochemical properties of recombinant RPA complex. However, CrisprCas9 knock-in cell lines carrying RPA2 mutation exhibit telomeric abnormalities and short telomeres. Importantly, our findings also reveal that interaction of RPA2 with the the ubiquitin ligase RFWD3 is impaired. RFWD3 is a component of the

Fanconi anemia (FA) pathway that polyubiquitinates RPA to promote eviction of RPA and thus facilitates homologous recombination-dependent fork restart. Strikingly, mutation in RPA2 prevents the efficient ubiquitination of RPA by RFWD3 and causes an accumulation of RPA2 at telomeres. This suggests that the balance between RPA and POT1 could be affected. Changes in RPA-to-POT1 switch may alter DNA replication and/or telomere capping provoking telomere defects. Collectively, our study provides insights into the underlying molecular mechanisms of RPA in telomere biology and defines RPA2 as a new genetic driver of TBD.

Transient two-cell like state coincides with type II ALT acquisition in telomerase-deficient mouse embryonic stem cell

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Proper telomere maintenance is a requirements for cell survival. Telomerase-knockout mESCs underwent senescence and cell death as they go through generations, but rare population can survive by acquiring telomerase-independent telomere maintenance mechanisms known as alternative lengthening of telomeres(ALT). Although transcriptomic analysis of ALT models can unveil crucial gene signatures for ALT maintenance, it does not reveal the factors responsible for the acquirement of ALT. Relevant ALT models have not been available to compare the states before and after ALT activation. Our previous studies demonstrated that mESC ALT survivors are established into two types: type I(DKO741) which maintained telomeres by inserting unique sequences called mTALT and type II(DKO301) which maintained by canonical telomere repeats. I tried to compare samples before and after ALT acquisition through single-cell transcriptome analysis. We found that cells were distinguished into clusters for ALT type and generation, and these clusters exhibited unique gene expression patterns. Focusing on type II samples, gene-set enrichment analysis showed stress response and DNA damage repair increase in telomeric crisis, but decreases following ALT stabilization. Interestingly, a small cluster consists of a mixture of pre- and post-ALT cells, and this group displayed a signature of 'two-cell state' including Zscan4 family, Tcstv3, Nelfa, Dppa2 which are associated with transient ALT activation during early embryogenesis. We are attempting to determine whether Zscan4 is a key player in type II ALT acquisition in our mESC model. This study will contribute to enhancing our knowledge of the process acquiring ALT mechanisms.

Live tracking of TERRA in cancer cells

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Previously we showed in fixed telomerase-positive cancer cells that TERRA can associate post transcription in trans with telomeres. Intriguingly, TERRA associates preferentially with short telomeres forming RNA:DNA hybrids that are mediated through a RAD51-dependent homology search mechanism[1]. Yet, it remains elusive how TERRA is preferentially recruited to short telomeres nor do we know about the dynamics that underlie this interaction. To address

these points, we developed a live-cell imaging approach. We employ the PP7-stem-loop system for tracking TERRA and visualize telomeres with endogenously tagged Halo-TRF1. Using live cell microscopy, deep-learning tools for spot detection and single particle tracking analysis we determined telomere and TERRA dynamics in telomerase-positive HeLa cells with short or long telomeres. We find that TERRA molecules are much more mobile than telomeres. When we analyze colocalization of TERRA with telomeres, we find that most associations are transient. However, a subset of the colocalization events are longer-lasting, especially in cells with short telomeres. We are currently modulating RNH1 expression levels to interrogate whether the longer-lasting interactions rely on TERRA RNA:DNA hybrids. We are also performing depletion studies to identify the roles of TERRA-associated factors in TERRA dynamics. Consequently, we hope to obtain novel insights into the TERRA recruitment machinery and TERRA function, as well as RNA:DNA hybrid dynamics.

[1] Feretzaki, M., Pospisilova, M., Valador Fernandes, R., Lunardi, T., Krejci, L., and Lingner, J. (2020). RAD51-dependent
recruitment of TERRA lncRNA to telomeres through R-loops. Nature 587.10.1038/s41586-0202815-6.

Target identification for the Alternative Lengthening of Telomeres (ALT) pathway: Towards a tumor-agnostic ALT-targeting therapy

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Cancer cells evade replicative senescence, and hence telomere shortening, by activating telomere maintenance mechanisms. The majority of cancer cells (85-90%) activate the enzyme telomerase (TEL) to safeguard genomic stability. In contrast, 10-15% of cancers achieve immortalization via a telomerase-independent mechanism referred to as the Alternative Lengthening of Telomeres (ALT) pathway, which depends on homologous telomeric recombination. However, the underlying mechanisms causing ALT still remain elusive. Although generally rare, a higher frequency of ALT is found in mesenchymal malignancies. These aggressive tumors are often refractory to chemotherapy and, as a result, have a poor prognosis. Thus, there is a high medical need to develop an ALT-targeting tumor-agnostic therapy for patients suffering from these tumors. To unravel the molecular alterations that characterize ALT, we used genome-wide CRISPR/Cas9 knockout screening in IMR90-derived syngeneic SW26ALT and SW39TEL cells. Comparative analysis of the essential genes in both syngeneic cell lines identified several hits, including well known ALT-specific alterations, but also a variety of new ALT essential genes. These hits are currently being validated in vitro in a panel of established ALT+ and TEL+ cell lines. In addition, their molecular mechanism and synthetic lethality in the ALT pathway are being elucidated. Finally, these newly identified targets will be explored for their potential therapeutic use. In summary, genome-wide CRISPR knockout screens are performed to enhance the knowledge of ALT and to identify targets for the development of an ALT-targeting tumor-agnostic therapy that will tackle many orphan diseases relying on the ALT pathway and lacking treatment standards.

Exploring the role of telomeric transcripts in the cytoplasm of ALT cancer cells

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Telomere transcription produces a lncRNA called TERRA, contatining subtelomeric and telomeric sequences. In human, TERRA localizes to telomeres and participates in the regulation of telomere length, replication and response to telomere dysfunction. TERRA has other roles in telomerase-negative cancers: approximately 15% of tumors maintaining their telomeres via alternative lengthtening of telomeres (ALT). ALT cancer cells have increased levels of TERRA, which stimulates the recombination at telomeres, presumably in an R-loop-dependent manner. Interestingly, data from mouse and human cells demonstrate that TERRA may have other, extra-telomeric roles, as it associates with non-telomeric loci in mice and its depletion leads to the accumulation of DNA damage outside telomeres in human telomerase-positive cells.
Apart from its nuclear roles, TERRA has been observed in the cytoplasm: in budding yeast, it shuttles to the cytoplasm in cells undergoing diauxic shift; in mammals, an extracellular, 200 nt-long RNA containing telomeric repeats was found in exosomes eliciting inflammatory response. TERRA in senescing fibroblasts has been recently proposed to be involved in the signalling of telomere attrition to the cytoplasm and triggering inflammation.
In our work, we explore the presence of TERRA in the cytoplasm of human ALT cell lines. Using smFISH combined with immunofluorescence, dCas13-based imaging and cellular fractionation, we demonstrate the presence of TERRA in the cytoplasm of ALT osteosarcoma cell lines, explore its connection to telomeric DNA damage and study the factors influencing its cytoplasmic localization. Moreover, we describe its protein interaction partners and speculate about the role of TERRA in stress granule formation.

The Potential Regulatory Role of Telomeric Overhang Structure in Telomere Looping

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When a telomere forms a T-loop, the G-rich ssDNA overhang needs to interact with the upstream telomeric dsDNA. However, multiple lines of research have shown that the telomeric ssDNA overhang forms the non-canonical secondary structure G-quadruplex (G4). Stable G4 cannot be unfolded by a full-length C-rich complementary strand or the shelterin protein POT1,

therefore may prevent the overhang from interacting with other shelterin proteins and dsDNA. It has been shown that G4-interactive ligands may reduce T-loop formation, but it is unclear how G4 and T-loop interact with each other. The strand invasion during T-loop formation may happen with the part of the overhang that does not form a stable G4 due to protein or RNA binding. In this study, we used a set of fluorescently labeled DNA that allows studying the internal structural dynamics during T-loop formation using single-molecule FRET (smFRET). Combining smFRET, electron microscopy, and biochemistry methods, our data suggests that telomeric G4 undergoes a slow refolding process until reaching the final stable state. The formation of a T-loop is allowed with a weak G4 but blocked by a stable G4. We have demonstrated how the dynamics of nucleic acid secondary structures affect telomere looping. We also propose a model in which the slow process of forming a stable G4 in telomeric G-rich ssDNA serves as a biological timer.

Third-generation sequencing-based studies on alternative lengthening of telomeres in mouse embryonic stem cell model

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Telomeres, essential structures capping linear chromosomes, are elongated by telomerase in most cells. However, certain cancers adopt the alternative lengthening of telomeres (ALT) pathway. In a previous study, we found that one of our telomerase knockout mouse embryonic stem cells (mESC) model escaped replicative senescence through type I ALT, utilizing non-telomeric sequences. Here, to elucidate genomic alterations generated during telomere crisis and ALT activation, we constructed haplotype-resolved ALT genome using third-generation sequencing. ALT cells protected the end of chromosomes through either ALT telomere or end-to-end chromosome fusion. Various types of genomic rearrangements, including structural variants, single-nucleotide variants (SNVs) and chromothripsis, were distributed across the entire genome. Notably, we identified over 1000 ALT-specific structural variants, including 5 duplications. The increased expressions of the two genes within the duplicated region, Dpf3 and Rgs6, were revealed to contribute to the maintenance of ALT telomere stability. Furthermore, we identified an additional sequence suitable for serving as the template for ALT telomeres. Our genomics-based approach will contribute to understanding the activation and maintenance of type I ALT at the genome-level.

The impact of telomeric components on a reconstituted human replisome

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In dividing cells, telomeric DNA is maintained from one cell cycle to the next by semi-conservative DNA replication. Genetic studies have shown that telomeres are a challenging substrate for the replication fork and a potent source of replication stress. However, which components are responsible for this effect, and which aspects of the replication process are affected has remained unclear.

To address these questions, we have taken a reconstitution approach in which we combine eleven purified replication factors to assemble a human replisome capable of leading and lagging strand synthesis at in vivo-like rates. Combining this system with human telomeric repeats, we find that leading strand synthesis and replication rate is largely unaffected by C- or G-rich telomeric sequence in isolation. In contrast, telomere-bound shelterin acts as a potent replication barrier, with the TRF1 subunit preventing replication fork progression and leading strand synthesis on telomeric DNA. We will present experiments examining how Shelterin is overcome by accessory proteins working alongside the replisome, and transmission electron microscopy images that examine the organisation of Shelterin-stalled forks. Our work has important implications for understanding how telomeres are copied semi-conservatively, and how the human replisome responds to blocks and barriers found throughout the genome.

Telomere end processing in Trypanosoma brucei

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Telomeres, the nucleoprotein complexes at chromosome ends, are essential for genome integrity and chromosome stability. In the protozoan parasite Trypanosoma brucei that causes sleeping sickness in humans, we found that telomere proteins also play important roles in the pathogenesis of this parasite. While proliferating in the mammalian host, T. brucei evades the host immune response by regularly switching its major surface antigen, VSG, which is expressed exclusively from subtelomeric loci in a strictly monoallelic manner. We have shown that telomere proteins regulate the VSG switching rate and are essential for VSG monoallelic expression. T. brucei telomere is maintained by telomerase, but the details of telomere end processing are poorly understood. OB fold-containing ssDNA binding proteins play important roles in coordinating telomere G- and C-strand syntheses in mammalian, yeast, and plant cells, but T. brucei does not seem to have any OB fold-containing telomere-specific ssDNA binding proteins. We recently demonstrated that PolIE, an A type DNA polymerase, is essential for telomere maintenance in T. brucei. PolIE suppresses telomerase-mediated G-strand extension and promotes the C-strand fill-in process. We have also found that the T. brucei Apollo and EXO 1 homologs play important roles in telomere G- and C-strand processing. Finally, we have now identified PrimPol-like protein 2 (PPL2, a translation DNA polymerase), when depleted by RNAi, also exhibits an elongated telomere 3' overhang phenotype. Our data indicate that PolIE, PPL2, and Apollo/ExoI play important roles in telomere end processing in T. brucei. We are actively investigating the underlying mechanisms of their functions.

A whole genome arrayed phenotypic screen for modulators of ALT activity

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Cancers evade replicative crisis by maintaining telomere repeats and gaining replicative mortality. Most cancers re-activate telomerase to elongate their telomeres while ~15% of cancers utilise the Alternative Lengthening of Telomeres (ALT) mechanism to extend telomeres by recombination. ALT is a mechanism occurring in cancer types with unmet need and is thought to be absent in normal tissues. We carried out a high-content arrayed whole-genome CRISPR-knockout screen using telomere native-FISH as a readout for ALT activity to identify novel ALT regulators. In parallel to this screen, 2 orthogonal screening approaches were undertaken to identify genetic vulnerabilities of ALT cancers – a whole genome CRISPR KO screen for ATRX deficiency and a targeted telomere proteome screen. Through data integration and mechanistic studies, we have uncovered three distinct hit categories that kill ALT+ cells by 1) hyper-activating ALT, 2) inactivating ALT, or 3) killing ALT+ cells independent of ALT activity modulation. Within these classes, we reveal novel genetic interactions and dependencies of ALT cancers including opportunities for repurposing of drugs already in the clinic.

Prognostic role of TERRA levels in peripheral blood mononuclear cells from elderly colorectal cancer patients: an exploratory study

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This study investigates the role of TElomeric Repeat-containing RNA (TERRA) in peripheral blood mononuclear cells (PBMC) as senescence and prognostic biomarker in colorectal cancer (CRC) patients.
TERRA transcripts originating from chromosomes 1q-2q-10q-13q (TERRAch1-2-10-13), 15q (TERRAch15), 20q (TERRAch20), and XpYp (TERRAchXY) were assessed in a cohort of 47 elderly (≥70 years old) CRC patients at surgery (baseline) and 12 months after tumor resection (follow-up). TERRA levels were quantified by RT-PCR and were analyzed in relation to other senescence biomarkers (including T-cell immunophenotype, thymic output, telomere length, and senescence-associated secretory phenotype, SASP), as well as clinical outcome.
At baseline, TERRA levels correlated with telomere length (TERRAch1-2-10-13, rs=0.279, p=0.081; TERRAch15, rs=0.298, p=0.061; TERRAch20 rs=0.257, p=0.109; TERRAchXY rs=0.366, p=0.02). Notably, patients with higher TERRAch15 levels (above the median value) demonstrated significantly lower risk of a negative event (relapse, progression, or death) (hazard ratio: 0.369 p=0.028) and displayed significantly lower levels of CD8 cells, including senescent (CD8+CD28-CD57+, p=0.029), naïve (CD8+CD45RA+, p=0.043), recent thymic emigrants (CD8+CD45RA+CD31+, p=0.04) cells, and higher CD4/CD8 ratio (p=0.039) compared to patients with lower TERRAch15 expression. At follow-up, TERRA levels inversely correlated with SASP markers. Specifically, TERRAch1-2-10-13 and TERRAch20 showed an inverse correlation with IL8 (rs=-0.407, p=0.048 and rs=-0.447, p=0.042, respectively), and TERRAch20 and TERRAchXY inversely correlated with CXCL-1 (rs=-0.467, p=0.033 and rs=-0.608, p=0.002, respectively). Overall, these findings suggest that

TERRA levels in PBMC could be useful for monitoring "biological aging" and a promising prognostic marker for elderly colorectal cancer patients.

Telomere dysfunction mediates poor outcome in acute intestinal GVHD

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Among myelodysplastic syndrome patients ≥40, shorter recipient pre-transplant telomere is associated with inferior survival independent of clinical and genetic factors. In a competing risks analysis, short telomere length was associated with non-relapse mortality (sHR 1.57, 1.20-2.06, p=0.001) but not with relapse, and this effect was evident only in patients receiving myeloablative conditioning regimens (p=0.002). Intestinal mucosal injury during transplant is an important driver of acute intestinal GVHD, which is a major cause of early NRM. We found that survival among MDS patients who developed severe acute GVHD was significantly worse in patients with shorter telomere length. Given that enterocyte telomere attrition is accelerated in acute GVHD, we hypothesized that shorter recipient telomere length limits canonical Wnt-dependent intestinal regeneration and lead to worse acute GVHD survival. To determine whether baseline recipient telomere length modifies the severity of tissue injury, we performed multiplexed serum proteomic analysis on patient samples obtained before and after conditioning. Serum levels of REG3α, a validated marker of intestinal mucosal injury that is predictive of NRM from acute GVHD, was elevated after conditioning only among patients with short lymphocyte telomere length. We next evaluated the impact of impaired telomere maintenance on GVHD severity and survival in an MHC-mismatched mouse aGVHD model. Aged G2 Terc-KO mice had significantly more weight loss (p

Consequences of ribonucleotides in telomeres

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Ribonucleotides (ribos) inserted by DNA polymerases constitute the most prevalent form of genomic DNA damage. Left unrepaired, ribos can induce genomic instability and are associated with multiple human diseases such as Aicardi-Goutières syndrome and cancer. While the prevalence and impact of ribos has been established across the bulk genome, their presence and impact at telomeres remains unexplored. The gap in knowledge on the effects of

telomeric ribos is due, in part, to a lack in the ability to selectively insert ribos into telomeres. We have overcome this limitation by generating a telomerase variant that inserts ribos as efficiently as deoxyribonucleotides. This is a powerful tool we have begun using to selectively elevate ribo levels at telomeres, since telomerase functions specifically at telomere ends. With this tool, we have begun to explore the effects of ribos on the cell and specifically the telomeres. Our preliminary results suggest that ribos have negative consequences on the telomeres.

Telomeres, replicative senescence and genomic instability : the RNA link

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The mechanisms involved in genome instability increase in the absence of telomerase remain to be established. More specifically, the structure(s) of short versus dysfunctional telomeres, which either trigger permanent replicative senescence or potentially promote genome instability, respectively, remains unclear. Also, whether ALT and increase of genomic instability are mechanistically linked remains unknown.

The telomeric non-coding transcript TERRA was found to be at the heart of ALT mechanisms in both yeast and mammalian cells. TERRA has been involved in the promotion of ALT through the putative formation of R-loops at the shortest telomeres in telomerase negative cancer cells. R-loops were proven to promote genome instability in various places of the genome. This suggests that R-loops could trigger both genome instability and ALT.

In this project, we aim at understanding the origins of the rare initial events of genome instability and senescence escape through ALT triggered in the absence of telomerase. Our central hypothesis is that the first telomere reaching a short length in the absence of telomerase initiates both genome instability and ALT. The mechanisms involved are possibly inherent to the processing of the critical short telomere occurring along to the consecutive prolonged cell divisions at the onset of replicative senescence and/or through the accumulation of R-loops. To test these models, our strategy is to leverage the unique multidisciplinary tools we established in S. cerevisiae to circumvent the rarity of genome instability, the heterogeneity of telomeres, and the asynchrony of replicative senescence.

The Single-Stranded DNA-Binding Factor SUB1/PC4 Alleviates Replication Stress at Telomeres and is a Vulnerability of ALT Cancer Cells

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To achieve replicative immortality, cancer cells must activate telomere maintenance mechanisms. In 10-15% of cancers, this is enabled by recombination-based alternative lengthening of telomeres pathways (ALT). ALT cells display several hallmarks including heterogeneous telomere length, extrachromosomal telomeric repeats and ALT-associated PML bodies. ALT cells also have high telomeric replication stress (RS) that is enhanced by fork-stalling structures (R-loops, G4s) and altered chromatin states. In ALT cells, telomeric RS promotes telomere elongation but above a certain threshold becomes detrimental to cell survival. Manipulating RS at telomeres has thus been proposed as a therapeutic strategy against ALT cancers.

Through analysis of genome-wide CRISPR fitness screens, we identified ALT-specific vulnerabilities and characterized SUB1, a ssDNA-binding protein as a novel factor involved in telomere maintenance. SUB1 depletion induces RS at ALT telomeres and profoundly impairs ALT cell growth without impacting telomerase-positive cancer cells. During RS, SUB1 is recruited to stalled forks and ALT telomeres via its ssDNA-binding domain. This recruitment is potentiated by RPA depletion, suggesting that these factors may compete for ssDNA. The viability of ALT cells and resilience towards RS also requires ssDNA-binding by SUB1. SUB1 depletion accelerates cell death induced by FANCM depletion, triggering unsustainable levels of telomeric damage specifically in ALT cells. Finally, combining SUB1 depletion with RS-inducing drugs rapidly induces replication catastrophe in ALT cells. Altogether, our work identifies SUB1 as a new ALT susceptibility with important roles in the mitigation of RS at ALT telomeres and suggests new therapeutic strategies for a host of still poorly managed cancers.

Role of the E2-variant ubiquitin conjugating enzyme AKTIP in chromatin conformation, telomere stability and aging.

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Telomere damage, including telomere shortening, plays a critical role in loss of tissue homeostasis associated with aging. Epigenetic modifications are emerging as key regulators of telomere stability and contribute to the aging process.
AKTIP, a highly conserved gene encoding an E2-ubiquitin variant enzyme (UEV), is known to be involved in telomere maintenance, DNA replication, and aging across various species, including Drosophila and mammals. Through survival and climbing analyses on dAKTIP mutant flies, we observed a significant reduction in lifespan and an increase in age-dependent locomotor decline. The analysis of different aging markers displayed that loss of dAKTIP exacerbates the age-related reduction of H3K9 trimethylation and leads to elevated levels of the cell-death marker caspase DCP-1. These observations highlights dAKTIP significance in aging-related processes.
Affinity purification/mass spectrometry (AP/MS) experiments showed that dAKTIP interacts with 4 components of the Nucleopore Complex indicating a possible functional interaction of

dAKTIP protein with the Nuclear Envelop to maintain chromatin organization inside the nucleus and telomere stability. Interestingly, even though AKTIP is an E2 UEV that lack the catalytic cysteine, AP/MS revealed that dAKTIP also interacts with ubiquitin, four Cullins and two COP9 signalosome components, indicating that dAKTIP has also a role in the ubiquitination machinery.
Together these results not only advance our understanding of the molecular mechanisms governing telomere maintenance and chromatin organization, but also highlight the importance of AKTIP in epigenetic mechanism crucial for aging.

LIG3-dependent repair of telomeres damaged by the G4-binder pyridostatin

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To study the molecular mechanisms underlying G-quadruplex (G4) induced replication stress at telomeres, we treated telomerase positive cancer cells with the G4 ligand pyridostatin (PDS). We found in HEK293E cells that PDS triggers accumulation of fragile telomeres, which is indicative of replication stress. To characterize the molecular changes at telomeres that were triggered by PDS, we purified telomeric chromatin by 2-Step QTIP (Glousker et al. EMBOJ. 39:e104500 (2020)). Most strikingly, we identified that PDS triggers accumulation of DNA Ligase III (LIG3) at telomeres. LIG3 has been implicated in alternative nonhomologous endjoining (ALT-NHEJ; also called microhomology-mediated end joining) and base excision repair. Experimental depletion of LIG3 reduced the frequency of fragile telomeres triggered by PDS while increasing the frequency of telomere-free ends (TFE). These results suggest that PDS promotes telomere double strand breaks presumably during DNA replication, which are repaired by LIG3-dependent ALT-NHEJ. Consistent with this notion, POLQ inhibition which cooperates with LIG3 in ALT-NHEJ also reduced telomere fragility in PDS treated cells. Curiously, POLQ loss did not impact on TFE levels under PDS treatment. Thus, while POLQ appears to contribute to telomere repair other repair proteins may cooperate with LIG3 in order to seal broken telomeres.

MRX Inhibition by Rap1 at Telomeres

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Since early studies, repetition at telomeres has been viewed as a way to increase local protein concentration for end protection and length homeostasis. Here we investigated if the nature of the repetition (how the motifs are arranged) and the consequential dense DNA covering by telomere proteins [1] could be important for the control of the SMC-like MRX (MRN in mammals) complex at telomeres.
We set up an in vivo assay to assess the inhibition of MRX functions by Rap1 in S. cerevisiae. We found that the strength of this inhibition is proportional to the number, the density and the affinity of Rap1 proteins bound on DNA. It requires Rap1 close proximity to the end. In an in

vitro assay using purified proteins, Rap1 bound in tandem also inhibits MRX-dependent NHEJ in proportion to the tandem length and to its proximity to the end. Nanopore-sequencing of telomere fusions shows that the density of Rap1 binding sites in the end-proximal 100-150 bp of native telomere sequences contributes to the strength of telomere protection against MRX-dependent fusions.
These results show that tight Rap1 binding in tandem and the resulting full coverage of telomeric DNA inhibit MRX action, likely by steric hindrance. This mechanism counteracts MRX ability to capture ends beyond terminal obstacles. Since the dense covering of DNA by proteins and MRN control are conserved features of telomeres, this mechanism could contribute to telomere end protection and length homeostasis in other species.

[1] Analikwu*, Deshayes* et al. https://doi.org/10.1101/2023.10.29.564563

Single-molecule analysis of replication fork progression through mammalian telomeric repeats

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Telomeres are hard to replicate regions that behave like fragile sites in metaphase spreads. Telomere replication constitutively requires shelterin function and many specialized factors involved in the replication stress response. Using 2D-gels and electron microscopy on purified telomeres, we have found that telomere replication is associated with fork stalling and a nearly two-fold increase in replication fork reversal. Here we monitor replication fork progression at mouse telomeric repeats with molecular combing. In order to obtain a high number of replicating telomeric molecules, necessary for this analysis, we have included an enrichment step through restriction digestion and fractionation in sucrose gradient.
We found a ~30% reduction in fork speed at telomeric repeats compared to the bulk DNA of the same sample. By consecutive labeling with two thymidine analogs, we detected a nearly 3-fold increase of fork stalling events at telomeric repeats. Conditional deletion of the shelterin component TRF1 results in a 2-fold reduction of replicating telomeric fibers, as observed in previous studies. Surprisingly, TRF1 deletion did not affect fork speed at TTAGGG repeat tracts, nor did it increase the frequency of stalled forks inside the telomeric repeats.
We detected replication initiation events both at bulk DNA and at telomere-enriched fragments, however these events were 2-3-fold more frequent at telomeres. Deletion of TRF1 nearly abolished these events at telomeric repeats.
Based on these data we are considering a model where one important function of TRF1 in telomere replication is to promote replication initiation events at telomeric repeats.

The riddle of TERT inside the mitochondria

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Telomerase Reverse Transcriptase (TERT) is now recognized to have some extra-telomeric roles and to be able to localize inside the mitochondria. In a previous work, we showed that TERT has a protective function against oxidative stress and a role in preserving mitochondria

functionality. To better understand the putative mechanism of action and the reason of TERT translocation into mitochondria, we transduced two osteosarcoma cell lines (U2OS and SaOS-2), normally lacking TERT expression, with HA-tagged TERT protein, obtaining TERT overexpressing cells. It is important to note that SaOS-2 cells, differently from U2OS, are normally characterized by the expression of the Telomerase RNA Component (TERC). We showed clearly and unambiguously the presence of TERT inside mitochondria independently from the presence of TERC, taking advantage of Western blot and electron microscopy. In SaOS-2 cells, TERC was found in mitochondria, but not in their matrix where TERT is localized, indicating that TERT function in the mitochondria is independent from its canonical TERC counterpart.
Since it is known that in the nucleus TERT is involved in gene expression regulation of different pathways, we have hypothesized a similar function in the mitochondrion. Mitochondrial chromatin immunoprecipitation (mIP) showed the binding of TERT to several mtDNA sequences; hence, we decided to study the possible implication of this binding on mitochondrial DNA replication and transcription, by measuring mtDNA copy number, BrdU incorporation and mitochondrial mRNA levels. Furthermore, to shed lights on our results, it would be interesting to investigate a TERT broad involvement in mitochondrial biogenesis.

A screen for candidate telomere elongating chemicals in human cell culture identifies Sirtuin-active compounds modulating telomere length.

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Reduced telomere length can lead to stem cell senescence and premature aging syndromes such as Telomere Biology Disorders. The discovery of Telomerase activating compounds holds considerable promise in restoring telomere length in such syndromes and other age-related diseases. Here, we present the results of a screen for telomere length mediating and Telomerase activating small molecule compounds in human cell culture. We confirmed the results of Mannherz and Agarwal of a telomere length increasing effect of Thymidine supplementation. We also identified several compounds that increased telomere length, apparently through Telomerase activation via Sirtuin activity. The discovery of Telomerase activating compounds holds considerable promise in restoring telomere length in age-related diseases. Previous studies associated healthy and longer telomeres to the deacetylase Sirtuin proteins activity in strict dependency to high NAD(+) level (Amano H et al., 2019). On the other hand, decrease of telomere length and sheltering protein complex loss is associated with DNA-damage repair mechanism, activated by low Sirtuin activity in response to low NAD(+) (Chen et al., 2014) (Amano H et al., 2019). Here, we screened a library of compounds and identified active chemicals that enhance Sirtuin proteins activity. This effect contributes to maintain telomere and shelterin complex homeostasis during stem cell differentiation. This study aims to characterize small molecules in treatment of Telomere Biology Disorders underlying possible molecular pathways for age-related diseases.

A model of the dynamics of individual telomere lengths

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Long-read telomere sequencing allows for the accurate measurement of the length of individual telomeres. This method has the potential to greatly increase our knowledge of telomere length dynamics on the individual telomere level. However, due to the complex interplay of replication rates, telomere loss during replication, and Telomerase activity, interpreting and quantifying hypotheses of the underlying mechanisms responsible for observed changes in individual telomere lengths is not trivial. Here, we present a model and simulation of the dynamics of individual telomere lengths in human cell culture models. The simulation is directly integrated with the Telo-seq telomere sequencing workflow recently published by Schmidt et al., supporting both the non-mapping route (when a telomere-to-telomere reference genome is not available) as well as the mapping route (when a telomere-to-telomere reference genome is available) of the workflow. Given an initial cell count and Telo-seq measurement, our simulation constructs a model of the cell population that reflects these measurements. Given parameters such as replication rates, telomere loss per cell division, and Telomerase activity, we then forward-simulate the cell population for the duration of the experiment. A final cell count and Telo-seq measurement at the end of the experiment are then compared to the results of the simulation. We present a method based on Approximate Bayesian Computation (ABC) to estimate probability distributions of the unknown model parameters that can explain the Telo-seq measurements. Our method allows to leverage telomere sequencing to evaluate difficult-to-test hypotheses about individual telomere length dynamics.

Computational methods for assessment of telomere length dynamics and maintenance mechanisms in health and disease

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Computational analysis of DNA and RNA sequencing datasets offers a robust approach to studying telomere biology in. We have a number of tools for analyzing telomere length and the presence of telomeric repeat variants in whole genome sequencing (WGS) data and applied those to study telomere dynamics in aging and cancers.

Nonetheless, we acknowledge that merely understanding telomeric sequence content and length falls short of comprehensively elucidating the molecular dynamics of telomere maintenance mechanisms (TMM) in health and disease. The molecular drivers behind the selective activation of either the telomerase-dependent (TEL) or alternative lengthening of telomeres (ALT) pathways are poorly understood. Additionally, there's a significant demand for more precise experimental assays for TMM phenotyping. In this study, we've undertaken a literature-based reconstruction of signaling pathways involved in both the ALT and TEL TMMs. We've employed gene expression data for computational assessment of TMM pathway activities, comparing the the results with experimental assays. Our bioinformatics analysis, which incorporates pathway topology and comprehensive visualization of molecular interactions, yields more details regarding molecular interactions behind TMM activation than traditional methods.

Our methodology differs from experimental assays in its ability to detect subtle activations of TMM pathways in healthy tissues as well. We've discovered heightened ALT and TEL

pathway activities in testis and identified specific genes and pathways that may initiate TMM activation. This novel approach paves the way for systematic investigations of TMM activation patterns in various cancers and healthy tissues, contributing to the discovery of pathway-based molecular markers with potential diagnostic value.

Telomere end protection in mouse embryonic stem cells

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Telomeres play a critical role in "end protection", preventing the ends of linear chromosome from activating DNA damage response (DDR) pathways. TRF2 is critical for end protection and acts by inhibiting the DDR and sequestering telomere ends within a T-loop. Loss of TRF2 in somatic cells triggers DDR and rapid telomere-telomere fusions mediated by end joining. Whilst TRF2-dependent end protection is observed in numerous somatic cell types, we and others recently showed that telomeres remain protected in pluripotent embryonic stem cells that lack TRF2. Importantly, this unique attribute of pluripotent embryonic stem cells is lost during pluripotency exit, when the TRF2-dependent telomere end protection is restored. These findings challenge current dogma and reveal fundamental differences in "end protection" between pluripotent and somatic cell states. However, the timing and the mechanisms governing this switch are unknown.

To better understand how pluripotent stem cells maintain and protect telomeres, we focused on the shelterin protein TRF1. Notably, TRF1 deletion causes early embryonic lethality (blastocyst stage), whilst TRF2 embryonic lethality occurs later. TRF1 is also highly expressed in pluripotent stem cells (ESCs and iPSCs) and is a direct transcriptional target of the pluripotency factor Oct3/4. Furthermore, TRF1 and TRF2 have distinct roles at telomeres and their divergent N-terminal domains confer different binding behaviour in a chromatin environment. Here, we show evidence that a specific domain of TRF1 is sufficient for end protection and T-loop formation in pluripotent embryonic stem cells.

Targeting telomeric DNA damage response as a therapeutic strategy for hematopoietic and immune dysfunction caused by telomere shortening

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Inactivating mutations in genes involved in telomere biology are associated with several genetic disorders (such as Dyskeratosis Congenita), usually characterized by critically short or/and dysfunctional telomeres. When critically-shortened or dysfunctional, telomeres become unable to bind telomere-capping proteins that protect them from being recognized as DNA damage, initiating a DNA-damage response (DDR) leading to cellular senescence (Rossiello, NCB, 2022).
We previously reported that dysfunctional telomeres are actively transcribed to generate telomeric non-coding RNAs (tncRNAs), which are key to recruit a full set of DDR factors at damaged telomeres, fueling DDR and causing cellular senescence initiation and maintenance. Sequence-specific telomeric antisense oligonucleotides (tASOs) target these tncRNAs and effectively inhibit tDDR activation in cultured cells and in mouse models (Rossiello, Nat Comm, 2017; Aguado, Nat Comm, 2019; Sepe, EMBO Rep, 2022).
The Terc-/- mouse model, which lacks the RNA component of telomerase (Blasco, Cell, 1997), shows telomere shortening in the hematopoietic organs and recapitulates features of hematopoietic and immune dysfunction. We established an in vivo tASOs administration protocol to assess its effects on this phenotype.
tASOs treatment could effectively improve hematopoietic fitness of hematopoietic stem and progenitor cells of Terc-/- mice and restore normal cell composition of bone marrow and spleen, including the myeloid-to-erythroid ratio. RNAseq analysis showed that tASOs treatment could restore altered biological pathways mainly involved in inflammation. Collectively, our results indicate that inhibiting DDR at damaged telomeres in vivo could have beneficial effects on the hematopoietic/immune impairment caused by telomere shortening.

Mechanistic differences between disease-related mutations of human Telomerase RNA

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Telomerase is a ribonucleoprotein complex, at its core composed of Telomerase Reverse Transcriptase (hTERT), the catalytic protein, and Telomerase RNA (TR), which harbors the template sequence used by the enzyme. This complex replenishes telomeric repeat sequences that are lost during normal cell division due to the "end replication problem". Mutations in various components of telomerase, as well as in factors involved in telomerase biogenesis, have been identified in patients affected by telomere biology disorders (TBDs). These mutations accelerate telomere shortening, leading to the premature depletion of stem cells in various organ systems. Here we compare the effects of diseases-associated mutations within two distinct regions of hTR on telomerase function in vitro: those in the template region versus those near the 5'-end. Telomerase containing hTR template mutations showed severely impaired processivity, while mutations near the 5' end of hTR reduced telomerase activity without affecting processivity. Our results show that even mutations within the same telomerase component, namely hTR, can affect the function of this enzyme in different ways. A better understanding of the pathological mechanism of individual mutations will help in the development of mutation-specific treatments for TBDs.

A platform to identify and validate novel vulnerabilities in ALT cancers at Artios

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The Alternative Lengthening of Telomeres (ALT) pathway is a telomere maintenance mechanism required for replicative immortality in approximately 10-15% of all cancers, but with greater incidence of up to 50-100% in tumours of mesenchymal origin such as soft tissue sarcomas. These cancers represent an area of unmet clinical need, but despite an increased understanding of the molecular mechanisms underlying ALT biology and improved strategies to identify ALT tumours, to date there are no specific ALT-targeted therapies.
ALT is a DNA damage response-driven process, mediated by homology directed repair mechanisms. Activation of ALT in cancer cells is associated with a range of molecular features, which include ALT-associated PML bodies (APBs), C-rich single-stranded telomeric DNA (ssTelo-C) formation and C-circle enrichment. These can be used to measure ALT in tumours and potentially function as both patient stratification and pharmacodynamic biomarkers.
Here we describe Artios' ALT platform, which comprises a suite of tools and assays allowing identification, validation, and prosecution of drug discovery targets, aimed specifically at identifying and treating ALT-utilising tumours. We demonstrate that this established platform can be used to identify novel ALT factors, enhancing our understanding of ALT biology, and driving development of new, ALT tumour-targeting medicines.

The Leishmania spp. PinX1 orthologue preserves the canonical domains and specifically interacts with the TERT telomerase component

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Parasites of the Leishmania genus cause leishmaniasis, a neglected tropical disease that, depending on the host immune system and parasite species, has different clinical outcomes. The existing treatments show low efficacy, high toxicity, and cost, leading to parasite resistance. Telomeres, the physical ends of eukaryotic chromosomes, have been considered potential drug targets due to their role in maintaining genome stability and cell proliferation. Leishmania telomeres, similar to other eukaryotes, are maintained by telomerase and regulatory proteins. One of these proteins, PinX1, a natural telomerase inhibitor, has an orthologue in Leishmania spp. (LPinX1). Our goal was to perform structural and functional studies to characterize LPinX1. Here, we used amino acid sequence alignments (TCoffee and MAFFT), AlphaFold2, computer simulations (GROMACS), and molecular docking (Gramm

docking web server), to predict the tridimensional structure of LPinX1 and study its interactions. Our analyses revealed that LPinX1 presents some genus-specific amino acid substitutions but preserves the canonical N-terminal G-patch and a least conserved C-terminal Telomerase Inhibitory Domain (TID). The tertiary structure analysis unveiled an alpha-helical structure with disorder regions, and the molecular docking showed the interaction between LPinX1 and the LTERT telomerase component. Biophysical assays (Circular Dichroism and Fluorescence Spectroscopy) were performed using a purified and correctly folded recombinant LPinX1. The recombinant LPinX1 specifically interacted with LTERT, confirming the docking analysis. Our results provide valuable insights for further studies on LPinX1's role in Leishmania telomerase regulation.

The role of SLX4IP in telomere maintenance

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To achieve replicative immortality, 10- 15% of tumors activate a recombination-based telomere maintenance mechanism called alternative lengthening of telomeres (ALT). ALT-positive tumours are often associated with a poor prognosis. The molecular mechanisms underlying ALT induction and maintenance are not well understood but involve changes in chromatin structures and increased telomeric replication stress. Even though a certain level of telomeric replication stress is required for enabling telomere extension, it needs to be finely balanced. Hence, additional replication stress may result in impaired survival of ALT cells. We previously characterized SLX4IP as a negative regulator of ALT, where it helps to balance telomere recombination and prevents the accumulation of telomere recombination intermediates. Here, we will present our ongoing experiments that characterize additional functions of SLX4IP at ALT telomeres. In particular, we show that SLX4IP responds to telomeric replication stress upstream of recombination intermediate processing. We find that SLX4IP loss is synthetic lethal with the translocase FANCM, which promotes fork reversal at stalled replication forks arising from DNA secondary structures such as G-quadruplexes (G4s) and R-loops. SLX4IP itself interacts with and is epistatic with the flap endonuclease XPF, which cleaves G4-containing DNA flaps in preparation of both alternative end-joining and recombination. Together, our data suggest that SLX4IP prevents R-loop- and G4- associated replication stress at ALT telomeres.

At the Crossroads of Replication and Recombination: How SLX4IP Balances the Alternative Lengthening of Telomeres Pathway

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Telomere maintenance is crucial for the sustained proliferation of cycling cells. The Alternative Lengthening of Telomeres (ALT) pathway is a telomerase-independent telomere maintenance mechanism that critically relies on replicative stress to initiate recombination-mediated extension of telomeric DNA.

Striking the right balance between replication stress and DNA recombination is essential for effective ALT function. Both insufficient and excessive levels can be counterproductive to telomere extension. Our research is centered on deciphering how this equilibrium is maintained, particularly through the role of the genome maintenance factor SLX4IP.

In previous work, we have elucidated how SLX4IP accumulation at ALT telomeres plays a decisive role in modulating telomere recombination processes. We demonstrated that SLX4IP interacts with the genome caretakers SLX4 and BLM to ensure effective processing of telomere recombination intermediates. The absence of SLX4IP leads to unchecked telomere recombination, impairing effective telomere extension.

Here, I will present our newest insights into the regulatory functions of SLX4IP within the ALT pathway. I will specifically address how SLX4IP balances replication stress levels and helps to deal with DNA secondary structures in a pathway that is parallel to DNA translocase FANCM, which is another key negative regulator of ALT.

Together, our findings indicate that SLX4IP limits ALT at multiple levels including replication stress and DNA recombination, to ensure balanced and productive ALT activity.

Proteomic Landscape of Type 2 ALT Maintenance in Mouse Embryonic Stem Cells

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Alternative Lengthening of Telomeres (ALT) is a non-canonical pathway for telomere maintenance in the absence of telomerase activity. Our previous studies have delineated two distinct subtypes of ALT survivors derived from mouse embryonic stem cells. Type 1 ALT survivors exhibit telomere elongation through the utilization of unconventional repeat sequences called mTALT, while type 2 ALT survivors utilize the canonical telomere repeat. ALT is a conserved phenomenon throughout evolution, observed in a spectrum ranging from yeast to human cancer cells. Despite ALT's ubiquity, the mechanisms governing activation and maintenance of ALT remain poorly understood mostly due to the lack of control samples before ALT activation. Our ALT mESC model can demonstrate various ALT stages, starting from the early stage where ALT isn't initiated, to later stages where ALT is activated and stabilized. Thus, the primary objective of this study was to elucidate the underlying mechanisms of type 2 ALT maintenance in mESCs. We conducted a comprehensive analysis of the proteomic landscape surrounding telomeres in ALT-stabilized cells by Proteomics of Isolated Chromatin Segments (PICh), followed by a comparative examination of telomere-proximal proteins in cells before ALT activation and after ALT stabilization. We discovered that various proteins including DDR factors, FANC proteins, LEM domain-related proteins, and VRK2 were significantly up-regulated upon stabilization of ALT. Currently, we are investigating the impact of these diverse factors on the maintenance of type 2 ALT. Our studies will contribute to the advancement of our understandings on ALT maintenance mechanisms and the intricate telomere environment associated with ALT.

Investigating the molecular mechanisms of telomere length homeostasis in *S. cerevisiae*

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Elucidating the telomere-length maintenance system in yeast can help better understand related processes in humans, such as aging and cancer. As in human stem cells, *S. cerevisiae* telomeres are maintained within a homeostatic length range. The Counting Model, developed by the Shore and Blackburn labs, proposed a positive correlation between the "count" of Rif1/2 proteins bound to Rap1 on a given yeast telomere and the length of that chromosome end. Conversely, short telomeres were shown to be more prone to extension by telomerase. Our lab showed that the telomerase RNP subunit Ku recruits the enzyme to telomeres by binding Sir4, which the transcriptional silencing field had shown competes with Rif1 and 2 for binding telomere-coating Rap1. We also found that Sir4 is enriched at telomeres in *rif1*Δ and *rif2*Δ strains. Altogether, these results provide a sound model for how Rif proteins may inhibit telomerase action at long telomeres: blockage of Sir4-Ku recruitment. We are now further investigating the negative-feedback system that regulates telomere length in *S. cerevisiae* using a range of genetic and molecular-biological approaches. In particular, we are examining the interplay between Sir4-Ku recruitment and inhibition by Rif proteins, interrogating the model and identifying the quantitative parameters underlying telomerase maintenance of chromosome ends in *S. cerevisiae*.

Oxidative stress and telomere length during replicative senescence in Saccharomyces cerevisiae

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Replicative senescence is triggered when telomeres reach critically short length and activate permanent DNA damage checkpoint-dependent cell cycle arrest. Studying it is a challenge due to inherent heterogeneity, where additionally, redox imbalance was shown to affect telomere biology in mammalian cells. Filling a knowledge gap in budding yeast, we have recently demonstrated that increased total ROS levels is a feature of senescence. Here we investigate the sources of ROS in this context. To do this, we test the involvement of key enzymes in budding yeast redox metabolism in the ROS increase in the absence of telomerase. NADPH oxidase (Yno1) is located in the endoplasmic reticulum and has been described as one of the main sources of ROS, producing superoxide (O_2•-) in a NADPH-dependent manner. Preliminary analysis of yno1Δ mutant telomerase-negative cells revealed an acceleration of replicative senescence as assessed in population senescence assays and an increase in the total ROS level compared to YNO1 controls. To establish whether this acceleration in senescence in liquid culture is due to telomere shortening or an increase in cell death, assays using our custom microfluidic device are being conducted to track the consecutive cell divisions from telomerase removal to cell death and establish the timeline of events.

Auxin-inducible degron in telomere research: Pros and cons

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Telomeres safeguard chromosomal ends against replicative erosion and undesirable recognition by double-strand break repair machinery. Their function is facilitated by a number of telomere-associated proteins, participating on their maintenance. In order to elucidate the mechanisms behind cellular response to a disruption of these components, a method for their fast, regulated and reliable depletion is required. To this end, we evaluated the auxin-inducible degron, a tool for protein knock-down set off by the addition of fytohormone auxin, in Saccharomyces cerevisiae. We found that the system provides rapid depletion of degron-tagged proteins, with major signal reduction within ten minutes after addition of auxin. We prepared a collection of S. cerevisiae strains with various tagged telomere-associated proteins, and confirmed their depletion followed by early changes in telomere state. However, not all targets seem to be prone for tagging, some having reduced function even in absence of auxin. The auxin-inducible degron also displays variable effectiveness based on the cultivation conditions. The effectiveness of depletion is dependent on the carbon source and the system is less potent at temperatures above 28 °C, although the latter shortcoming is largely averted by using its modified variants. Our results point out factors to be considered when applying auxin-inducible degron to telomere studies in yeast.

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The TeloDIAG: Insights into Glioma Pathogenesis and Diagnosis Through Telomeric Analysis

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Gliomas represent the most prevalent primary tumors of the central nervous system. Diffuse gliomas are categorized into subtypes with different prognoses, including Oligodendroglioma (OD), IDH-mutant Astrocytoma (A), and Glioblastoma (GBM). Telomere maintenance mechanism (TMM)-related markers serve as diagnostic parameters according to the WHO 2021 classification. OD and GBM activate telomerase through TERT promoter mutation, while Astrocytoma utilizes the alternative lengthening of telomeres (ALT) characterized by the loss of the ATRX protein.
Based on these characteristics, we have developed the 'TeloDIAG,' an integrated diagnostic tool, incorporating the C-circle assay, a teloPCR, and IDH1/2 status. The TeloDIAG has enabled the identification of a novel category of GBM using the ALT mechanism, as well as the reclassification of tumors with discordant histo-molecular parameters.
Furthermore, we propose enhancing ATRX testing, typically performed via immunohistochemistry, with ALT functional testing, particularly in ATRXwt Astrocytomas.
Finally, our findings indicate that TeloDIAG reveals distinct trajectories of TMM acquisition between OD and GBM.

Function of Telomere Heterochromatin In Alternative Lengthening of Telomeres

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In cancer cells lacking active telomerase, the Alternative Lengthening of Telomeres (ALT) mechanism is pivotal for telomere maintenance. ALT cells have particular chromatin features, including an enrichment for the heterochromatin mark H3K9me3. However, the function of H3K9me3 in ALT remains elusive. Employing targeted fusion proteins, we selectively increased H3K9me3 levels locally at ALT telomeres, and found that it led to a significant rise in ALT-associated promyelocytic leukemia (PML) bodies, C-circles, and mitotic DNA synthesis (MiDAS). Since heterochromatin protein 1 (HP1) is the main reader of H3K9me3, we further asked whether the increase in ALT activity is due to the recruitment of HP1. Indeed, tethering of HP1 to ALT telomeres phenocopied H3K9me3 enrichment in terms of increases ALT activity.
Given HP1's known interaction with the nuclear antigen SP100, our ongoing investigations aim to discern whether the observed increase in ALT activity is attributable to the HP1-SP100 interaction or linked to HP1's role in liquid-liquid phase separation. These discoveries illuminate the intricate dynamics at play in ALT telomeres, providing valuable insights into the complex landscape of cancer-associated telomere biology.

The yeast Hog1 MAPK controls telomere homeostasis during stress adaptation

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Maintaining telomere length is essential for cell survival. Disruption of this length equilibrium is a hallmark of most cancers and it is directly associated with aging. Recent studies have linked environmental signals with telomere shortening and elongation. However, while the complexes regulating telomere length and its interplay with the replication cell cycle machinery are well described, the molecular mechanisms that integrate environmental cues to telomeres remain elusive. Here we aim to characterize the molecular mechanisms governing telomere biology during osmostress-adaptive responses, using Saccharomyces cerevisiae as a model organism. We focus on deciphering the role of the yeast homolog of p38, the Hog1 SAPK (Stress-Activated Protein Kinase) in regulating telomere length upon stress conditions. An increase in extracellular osmolarity leads to the activation of Hog1 that modulates gene expression and cell cycle progression, both of which are required to ensure cell adaptation and to maximize cell survival. By applying genome-wide chromatin association studies (ChIP-seq), we found that Hog1 binds to virtually all telomeric regions upon osmostress and there is a subsequent recruitment of RNA Pol II. Hog1 binding to telomeric regions was shown to be Rap1-dependent, thus we assessed the role of Rap1-associated proteins in mediating this interaction. Remarkably, we also showed that impaired telomere regulation impacts cell fitness upon osmostress. Therefore, our work focuses on obtaining an in-depth understanding of the interplay between osmostress responses and telomere length regulation.

Human genetic variation in TERT: insights into disease susceptibility at the population level

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TERT encodes the enzymatic component of the telomerase complex and is the most commonly mutated gene among patients with telomere biology disorders. TERT is composed of four major structural domains with distinct functions: telomerase essential N-terminal domain, Telomerase RNA binding domain, reverse transcriptase domain, and C-terminal extension domain. The prevalence, characteristics, and functional effects of TERT rare missense variants in the population have not been systematically studied. We analyzed germline TERT variant calls from three independent population-level databases (gnomADv4, UK Biobank Touchscreen Duration cohort, AllofUs) comprising 1.45 million individuals. At the population level, nonsynonymous TERT variants are predominantly single nucleotide variants (SNVs) (94%) that result in missense substitutions (~90%) and classified as variants of uncertain significance (VUS) (96%) by ACMG criteria. We hypothesized that high regional missense constraint within TERT reveals structural elements that are essential for biological function. Among the total cohort, missense constraint, derived as the ratio of observed (O) missense variants to the number of possible SNV substitutions (P) in a given region (O:P ratio), is greater in the major structural domains compared to the unstructured linker region (p

A high-throughput screening assay for telomere length and dynamics in S. cerevisiae based on luminescence

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We developed a new way to quickly and easily assess telomere length and dynamics inspired by the method that cells use to assess their own telomere length. The system currently allows a semiquantitative determination of telomere length by two color luminescence in the yeast Saccharomyces cerevisiae. We use genomic protein tagging with luciferase and yellow fluorescent protein of two telomeric proteins and measuring bioluminescence resonance energy transfer (BRET) between the fusion proteins.

Our aim is to combine the system with an inducible telomerase to allow screening at different starting points of telomere length as well as with and without influence of telomerase.

Investigating the role of TERRA in the activation of the Alternative Lengthening of Telomeres pathway

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Alternative Lengthening of Telomeres (ALT) is a telomere maintenance mechanism based on homologous recombination, active in telomerase-negative tumors. A hallmark of ALT cells is the elevated expression of the telomeric lncRNA TERRA. Several studies indicate that TERRA promotes telomere recombination in ALT through the formation of telomeric RNA-DNA hybrids. ALT cells are also characterized by the relocation of telomeres into promyelocytic leukemia (PML) bodies, forming the ALT-associated PML bodies (APBs). These structures provide a recombinogenic environment essential for ALT. Intriguingly, TERRA was shown to be enriched at APBs and to physically interact with PML in ALT cells. However, whether TERRA is the initial trigger of ALT remains to be determined. To test whether TERRA is required for ALT activation, we induced ALT in telomerase-positive cells by depleting the histone chaperone ASF1 and characterized TERRA levels and localization. Our preliminary results show that TERRA colocalization with telomeres increases during early stages of ALT, concomitant with increased PML bodies formation but prior to APBs detection. We hypothesize that TERRA localization at telomeres represents an early event of ALT activation, which may lead to APBs nucleation. We are currently investigating this possibility by testing the interactions between TERRA, telomeres, and APB components during ALT activation. Furthermore, we aim to test the importance of TERRA in ALT activation by depleting TERRA during ALT induction and study the impact on the emergence of ALT hallmarks. Our experiments will help define the potential function of TERRA as an initial trigger of ALT.

Hypoxia induces Alternative Lengthening of Telomeres pathway activity in ATRX-deficient cells

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Telomere maintenance in some cancers is achieved through the telomerase-independent Alternative Lengthening of Telomeres (ALT) pathway. The ALT-pathway can be activated under various conditions and often includes mutational loss of ATRX. However, this has been shown to be insufficient in isolation and so other cellular event must also be implicated. It has previously been shown that excessive accumulation of DNA:RNA hybrid structures (R-loops) and/or formation of covalent DNA-protein complexes (DPCs) can be other important driving factors.

Here, we demonstrate that exposure of ATRX-deficient cells to low oxygen conditions (hypoxia; oxygen

This work presents important mechanistic insights into the endogenous origin of excessive R-loops and DPCs in ALT-positive cancers. It suggests that a hypoxic tumour microenvironment might be an important driving factor in telomere maintenance in ATRX-deficient cells.

Inhibition of the telomeric DNA damage response as a therapeutic strategy for idiopathic pulmonary fibrosis

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During aging, telomeres become progressively shortened in proliferating tissues and accumulate DNA damage in non-proliferating ones, activating the DNA damage response (DDR) pathways. This causes the establishment and maintenance of cellular senescence and senescence-associated secretory phenotype (SASP), which sustain inflammation. Telomere dysfunction is indeed a driver of age-related diseases (Rossiello, Nat Cell Biol 2022).
Damage-induced non-coding RNAs (dincRNAs) are generated at DNA double-strand breaks, including dysfunctional telomeres. These transcripts are essential for DDR activation, and their sequence-specific targeting with antisense oligonucleotides (ASOs) results in site-specific DDR inhibition (Francia, Nature 2012; Michelini, Nat Cell Biol 2017; Pessina, Nat Cell Biol 2019).
Telomeric ASOs (tASOs) targeting telomeric dincRNAs (tdincRNAs) generated at dysfunctional telomeres effectively inhibit DDR signalling in a mouse model of acute telomere dysfunction (Rossiello, Nat Comm 2017), improve the pathological phenotypes and extend the lifespan of a Hutchinson-Gilford Progeria Syndrome mouse model (Aguado, Nat Comm 2019), and prevent the telomere dysfunction-dependent lung expression of ACE2, the Sars-CoV-2 receptor (Sepe, EMBO Rep 2022).
Idiopathic Pulmonary Fibrosis (IPF) is a disease whose familial forms are associated with mutations in telomere-biology genes, including telomerase (Rossiello, Nat Cell Biol 2022).
We used a late generation telomerase-deficient mouse model (Terc-/-) as a genetic model of IPF (Piñeiro-Hermida, JCB 2020). Systemic treatment of Terc-/- mice with tASOs reduced DDR activation at telomeres, improved lung architecture, reduced inflammation, and decreased

lung fibrosis.
Given the wide impact of telomere dysfunction in age-related disorders, this therapeutic approach might represent a novel and versatile strategy for many diseases.

Telomere length homeostasis: *S. cerevisiae* to humans

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In humans, the Shelterin complex protein TPP1 has been shown to recruit the TERT catalytic subunit of telomerase to telomeres, although how telomerase is preferentially brought to short ends is unclear. Research in the model organism *S. cerevisiae* has led to a paradigm for telomere-length homeostasis provided by the Counting Model. There are two telomerase-recruitment pathways in budding yeast, mediated by Cdc13-Est1 and Ku. Our lab recently showed that the Ku pathway acts by binding telomere-associated Sir4. We also proposed that Ku-Sir4 comprises a length-sensing mechanism, with Sir4 being outcompeted on long telomeres by Rif1 and Rif2 (Sir4 competes with Rif1/2 for binding telomeric Rap1). Could a similar molecular mechanism help sense short telomeres in humans and recruit telomerase? Multiple publications report Ku binding to human telomerase RNA, hTR. As a first step in examining if Ku represents a recruitment mechanism in humans, we are establishing human Ku binding to human telomerase RNA (hTR) using the CARRY-two hybrid (C2H) system. This necessitates overcoming the challenge of studying a human heterodimer by a two-hybrid system in yeast. Our initial results do suggest detectable weak binding. Meanwhile, to more deeply understand the molecular mechanism of yeast telomere homeostasis, we are structurally and functionally investigating Rap1 binding to Rif versus Sir proteins. Overall, by learning the molecular mechanisms of negative-feedback regulation of telomere length in yeast, we aim to provide a basis for the human system.

The nucleic acids-binding protein SUB1 supports the viability of ALT cancer cells

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Immortal cancer cells bypass senescence and divide indefinitely by activating mechanisms that buffer telomere shortening. Most cancers reactivate the reverse transcriptase telomerase, yet about 15% of them elongate their telomeres through the Alternative Lengthening of Telomeres (ALT) pathway. ALT is a specialized branch of break-induced replication (BIR), a homology-directed repair mechanism activated at damaged telomeric DNA. This damage is induced by replication stress (ALT-specific Telomeric Replication Stress; ATRS) and must be kept at a level that supports enough BIR yet without strongly activating DNA damage checkpoints that would cause cell death. This balance is attained by the counteracting activities of molecular triggers and alleviators of ATRS. We have identified the transcriptional regulator SUB1, a multifunctional nuclear protein capable of binding to dsDNA, ssDNA, G-quadruplex structures and RNA, as essential for ALT cell survival. We show here that SUB1 depletion in ALT cells, but not in telomerase-positive cells, leads to cell death in a short period of time. Moreover,

SUB1 depletion exacerbates ATRS, ALT features and telomere fragility. By using chromatin immunoprecipitation, we confirmed that SUB1 associates with telomeres and this association increases when cells are treated with drugs inducing replication stress. We also show that SUB1 binds to telomeric ssDNA and TERRA RNA in vitro. Our work reveals that SUB1 is an ATRS alleviator capable of restricting telomeric replication stress and thus fine-tuning ALT activity. We propose that SUB1 represents an attractive target for the development of novel therapeutic protocols to cure ALT cancers.

In vivo roles of phosphorylation-dependent TRF1 regulation

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The telomere-bound shelterin complex is essential for chromosome-end protection and genomic stability. We showed that human and mouse TRF1 are subjected to PI3K/AKT-dependent regulation. Human cells carrying non-phosphorylatable mutant TRF1 alleles showed accelerated telomere shortening, demonstrating that AKT-dependent TRF1 phosphorylation regulates telomere maintenance in vivo. In addition, TRF1 mutant cells showed an impaired response to proliferative extracellular signals as well as a decreased tumorigenesis potential (Sánchez-Vázquez et al., PLOS Gen, 2021).
In mouse, we demonstrated that TRF1 is phosphorylated by AKT, ERK, B-Raf and mTOR and identified residue T330 as common phosphorylation site for these kinases (Méndez-Pertuz et al., Nature Comm., 2017; Bejarano et al., EMBO Mol Med, 2019;). Therefore, we hypothesize that mouse TRF1 phosphorylation at T330 is important for telomere biology in vivo. To address the potential role for this TRF1 post-translational modification in vivo, we have generated homozygous knock-in (ki) mice with non- phosphorylatable T330A or phosphomimetic T330D Trf1 mutant alleles. In this meeting, we will report the effects of these mutations on telomere maintenance as well as in cancer and aging.

6-thio-2'-deoxyguanosine drives telomere shortening by impairing telomerase translocation after nucleotide addition

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Telomeres cap chromosome ends and are essential for genome stability and human health, but they shorten in most human somatic cells with each cell division due to the end replication problem. Telomerase is specialized reverse transcriptase that lengthens telomeres by adding GGTTAG repeats to chromosome ends and is upregulated in most human cancers to enable limitless proliferation. Previous reports have demonstrated that a newly developed cancer therapeutic, 6-thio-2'-deoxyguanosine (6-thio-dG), reduces tumor growth in mouse tumor xenografts of lung, glioblastoma, melanoma, and colon cancers. 6-thio-dG treatment can cause telomere shortening and dysfunction, however the mechanism was unknown. We conducted a series of direct telomerase extension assays in the presence of the therapeutic 6-thioguanine metabolite, 6-thio-dGTP, on various telomeric substrates. Telomerase continues elongation after inserting 6-thio-dGTP, but insertion disrupts translocation and inhibits further repeat

addition. Using a single molecule assay which allows direct visualization of translocation of fluorescently labeled telomerase, we recapitulated our biochemistry result that 6-thio-dG inhibits telomerase. The assay also revealed that telomerase stalls after 6-thio-dG consistent with failed translocation along the product DNA for further repeat addition. Furthermore, telomerase processivity factor POT1-TPP1 fails to restore processive elongation in the presence of 6-thio-dGTP. In contrast, when 6-thio-dG is present in the primer DNA, telomerase can binf to and then extend a telomere. In cancer cells, we found evidence that 6-thio-dG inhibits telomerase and promotes telomere losses, and found cancer cells with critically short telomeres are hypersensitive to 6-thio-dG treatment. These results directly demonstrate that 6-thio-dG is a potent telomerase inhibitor.

High resolution long-read telomere sequencing reveals dynamic mechanisms in aging and cancer

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Telomeres are the protective nucleoprotein structures at the end of linear eukaryotic chromosomes. The precise assessment of the sequence and length of entire human telomeres has been traditionally challenging due to telomeres' repetitive nature and length. To resolve individual human telomeres at base pair resolution we have developed Telo-seq. Telo-seq combines telomeric enrichment via telomere end capture with telorette-based adapters and Oxford Nanopore Technologies' native long-read sequencing. We showed that Telo-seq allows to measure bulk, chromosome arm- and allele-specific telomere length. Our analysis highlights the intrasample chromosome arm- and allele-specific telomere length heterogeneity. Telo-seq resolves telomere shortening in primary human cells as close as five population doublings apart.
We assessed telomerase-dependent telomere elongation in induced pluripotent stem cells relative to their parental fibroblasts. Results obtained from telomerase- and ALT-positive cancer cell lines indicate that Telo-seq not only resolves the very long telomeres present in some ALT-positive cells, but also discriminates ALT-positive from normal and telomerase-positive cells. In addition to telomere length measurement, Telo-seq allows to investigate the composition of human telomeres and determine presence and location of telomere sequence variations like telomere variant repeats. Further, Telo-seq reads facilitate the analysis of subtelomeric CpG site methylation closest to telomeres.
In summary, we present Telo-seq as a novel tool to determine telomere sequence and length. Our study illustrates various applications for Telo-seq on primary human cells and cell lines and highlights how telomere long-read sequencing will enable future studies on human telomere biology during development, aging and cancer at unprecedented resolution.

Telomere length inheritance through the budding yeast sexual cycle

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In humans, short and long telomere syndromes are caused by inheritance of mutations affecting telomere length. These syndromes often show genetic anticipation, where disease has earlier onset and a more severe manifestation in each generation. This is presumably because later generations inherit not only the mutation affecting telomere length, but also abnormal length telomeres. If telomere length alters during human generations, then any changes most likely depend on the number of mitotic and meiotic cell divisions in egg or sperm cell lineages.

Many aspects of telomere length homeostasis are conserved between mammals and yeast. Here we asked if yeast, like humans, can transmit abnormal telomeres through the generations. By following the inheritance of a single telomere, rather than bulk telomeres, we find that if haploid yeast with short telomeres mate with wild-type yeast to create diploids, short telomere lengths rapidly normalize (within 30 cell divisions). However, if yeast with long telomeres mate with the wild type, long telomeres can persist for more than 200 mitotic cell divisions and be transmitted through meiosis. Furthermore, long telomeres can be transmitted down the generations (sexual cycles), independently of mutations that cause long telomeres.

Our findings suggest that even in yeast there is a complex relationship between telomere length, mutations that affect telomere length and corresponding phenotypes. These findings may have implications for families affected by telomere syndromes.

S. cerevisiae telomerase RNA flexibly scaffolds the essential Est1-arm Conserved Region (ECR), bound by Est1 and Pop proteins

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The three long "arms" that radiate out away from the catalytic core of *S. cerevisiae* telomerase RNA TLC1 bind to critical holoenzyme-subunit proteins. For example, the essential Est1-arm Conserved Region (ECR) binds both Est1 and Pop1/6/7. The ECR has some established roles in telomerase function *in vivo*: (1) Est1 recruits and activates telomerase by binding the telomere-capping factor Cdc13 and (2) the Pop proteins have a post-recruitment telomerase-activating function, since senescence occurs when their TLC1 binding site is deleted in an Est2-Cdc13 fusion strain (where telomerase recruitment is hardwired). We are testing the hypothesis that the ECR makes the telomeric DNA-Cdc13 complex accessible for telomerase extension. We have previously shown that the telomerase holoenzyme subunits Est1, Ku, and Sm7 can function at very different locations in TLC1 *in vivo*. Here we report that the ECR can also function when repositioned to three diverse positions, showing that not only Est1, but also the Pop proteins can perform their essential actions when repositioned on the RNA flexible scaffold. Senescence can even be rescued in a tlc1Δecr mutant if the ECR is expressed *in trans*. Additionally, we find that the ECR can act from the end of a 157 bp dsRNA (stiff) arm *in vivo*. Overall, our results provide evidence that Est1 and Pop proteins can perform their essential functions when spatially separated from the catalytic core.

Local chromatin profiling with dCas9 by label-free quantitative proteomics

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Chromatin Immunoprecipitation (ChIP) has been a cornerstone for epigenetic research over the last decades, but even coupled to sequencing approaches (ChIP-seq) it is ultimately limited to one protein at a time. While it would be equally informative to comprehensively know the chromatin composition at individual loci (i.e. one locus to many proteins), a widespread application of simple and robust DNA-centric approaches is still lacking. Here, we systematically explored several strategies for chromatin profiling. First, we combined ChIP with label-free quantitative (LFQ) mass spectrometry (ChIP-MS) and we reasoned that as a gold standard, ChIP-MS should be able to enrich multiple proteins that independently bind to the same chromatin fragment. To this end, we first demonstrate our workflow at telomeres as a particularly well-characterized genomic locus and establish that our gold standard expectation requires double cross-linking for stabilization of chromatin-bound complexes. In return, cross-linking is a source of potential false-positives, and we exemplify how to mitigate this challenge in ChIP-MS reactions via loss-of-function controls such as CRISPR knock-outs or degron knock-ins. Next, to address locus-specificity, we adopted the use of endonuclease-deficient Cas9 (dCas9) as an anchor protein and demonstrate specific enrichment of telomeric chromatin. Finally, we compare dCas9 ChIP-MS with dCas9 fusions to proximity labelling enzymes (CasID). Here, again telomeres served as a model locus and allowed us to systematically compare various proximity labelling enzymes such as BirA*, BASU, TurboID and APEX2. Overall, our results provide important insights towards the broad adoption of locus-specific chromatin profiling.

Separation of telomere protection from length regulation by different point mutations at position 492 of mRtel1

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RTEL1 is an essential DNA helicase, playing important roles in genome stability and telomere integrity. Yet, its mechanistic roles at telomeres are not well understood. A germline missense mutation in human RTEL1 (M492I), causing Hoyeraal-Hreidarsson syndrome (HHS), led us to generate two mouse models. In the first, termed 'Telomouse', methionine 492 is changed to lysine (M492K), copying a variation found in the short telomere mouse Mus spretus and reducing the length of the M. musculus telomere to human length1. In the second, termed 'HHS mouse', methionine 492 is changed to isoleucine (M492I). The HHS mouse telomeres gradually shortened in mouse embryonic fibroblasts (MEFs) grown in culture for 250

population doublings, and in the blood and tail of successive generations, though to a lesser extent than the Telomouse telomeres. However, the HHS mouse MEFs displayed increased levels of telomere dysfunction-induced foci (TIFs), telomere fragility, telomere sister chromatid exchange (T-SCE), anaphase bridges, micronuclei, and mitotic catastrophe, as compared to WT and Telomouse MEFs. Thus, altogether, the M492K mutation mostly affected telomeric length regulation, consistent with the natural occurrence of a lysine in this position of M. spretus RTEL1, while the M492I mutation mostly affected telomere protection, consistent with its causative role in HHS. This separation-of-function helps distinguishing specific functions of RTEL1 and study the underlying mechanisms of action. Hopefully, these mouse models will provide important insights into how short telomeres and DNA damage cause telomere biology diseases and how they contribute to cancer and ageing.

1Smoom R., et al. Nature Communications 14(1):670(2023).

Investigating the functional consequences of telomeric heterochromatin formation in ALT vs non-ALT cells

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Recent evidence challenges the longstanding view of telomeres as heterochromatic domains, revealing that in most human cell types, telomeres lack significant enrichment for heterochromatic features. A notable exception are Alternative Lengthening of Telomeres (ALT) cells, which exhibit strong enrichment for heterochromatic features like H3K9me3 and Heterochromatin Protein 1 alpha (HP1a). The functional consequences of telomeric heterochromatin in both ALT and non-ALT contexts remain contentious.
Our recent data indicates that telomeric heterochromatin formation promotes ALT phenotypes in ALT+ cells. Experimental elevation of H3K9me3 or HP1a levels at telomeres results in increased ALT-associated features, such as ALT-associated PML bodies (APBs), c-circles, and Mitosis-Dependent DNA Synthesis (MiDAS). This discovery prompted us to investigate the consequences of increased heterochromatin in non-ALT cells. To increase heterochromatin specifically at telomeres, we expressed a TRF1-HP1a fusion protein into ALT negative cells and found that it led to robust formation of APBs, as well as other features of an ALT-like state. Our ongoing investigations aim to utilize this system of robust induction of APBs in a non-ALT context to uncover mechanistic insight into the role of heterochromatin in the promotion of ALT, potentially revealing a pivotal role for telomeric chromatin in ALT activation and maintenance.

Mechanisms involved in differential telomere length homeostasis.

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Telomeric DNA is highly conserved and composed of short tandem repeats ending in a single-strand extension of the 3′-end. Telomeric repeat length is an important regulator of cell proliferation potential. According to a strong tenet in the field, all telomeres in a cell are of similar or standard length; for example, in the budding yeast Saccharomyces cerevisiae, all telomeres are thought to comprise 300±75 bp of telomeric repeats. However, this theory has only been derived from analyses of Y'-telomeres and a few X-telomeres and was not tested experimentally on all individual chromosomal ends. In this study we analyzed telomere length homeostasis in various ways, including genome wide sequencing approaches, and discovered that the telomere from the left arm of chromosome III (TEL03L) is almost twice as long as the so-called 'standard' telomere length. The results show that the HML locus (a silent copy of the MATα allele 10 kb away of the left telomere) is a key important element involved in the over elongation of TEL03L, and we also observed that deletions of Sir-genes abolish this over elongated phenotype. Furthermore, the accessory interaction for telomerase recruitment between Tlc1, yKu and Sir4 is involved in Tel03L overlengthening, and we propose that boundary proteins that limit Sir-complex spreading are involved in determining individual telomere set-lengths. Given that a single very short telomere is enough to cause cell senescence, this finding raises the remarkable possibility that very special telomeres could be responsible for many of the crucial consequences of telomere failures.

From telomere dysfunction to bone marrow failure: investigating hematopoietic vulnerability in mice upon Trf2 knockout

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Telomeres are associated with the shelterin complex, in which Telomere Repeat Binding Factor 2 (TRF2) is necessary to protect chromosome ends and avoid their repair and signaling. Trf2 gene deletion causes telomere dysfunction through telomere uncapping, activation of DNA damage response (DDR), and chromosomal fusions (Celli Nat Cell Biol. 2005). In vasculature and liver, TRF2 loss can lead to apoptosis or cellular senescence establishment, depending on inactivation extent and cell proliferation status (Lechel EMBO Rep. 2005; Lazzerini Denchi Genes Dev. 2006; Morgan J Mol Cell Cardiol. 2019; Sullivan Aging Cell. 2023). Differently, TRF2 is dispensable for telomere protection in embryonic stem cells (Markiewicz-Potoczny Nature. 2020; Ruis Nature. 2021). However, the effects of TRF2 inactivation on the hematopoietic compartment, including hematopoietic stem and progenitor cells (HSPCs), have received less attention so far.
We observed that systemic knockout of Trf2 in mice is lethal after 7-10 days upon induction of gene deletion. This sudden mortality prompted us to investigate the potential involvement of the hematopoietic system. Our preliminary analyses show that these animals developed bone marrow failure phenotypes, comprising altered structure and severe loss of cellularity in bone marrow, and decreased leukocyte number in blood. Interestingly, while the number of HSPCs did not significantly change upon Trf2 deletion, they displayed a dramatically reduced ability to form colonies in vitro.
These results suggest that the hematopoietic system might be particularly sensitive to telomere

uncapping, and Trf2 knockout mice could represent a model for studying bone marrow failure triggered by telomere dysfunction.

Characterizing the mechanism of end-protection mediated by the TPP1-POT1a/b complex.

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The evolution of two distinct Pot1 genes in mice provides convenient separation of function analyses of Pot1's role in end-protection and telomere end fill-in. In somatic cells, Pot1b is required for telomere end fill-in but is dispensable for telomere protection. In contrast, Pot1a is required for end-protection but dispensable for telomere end fill-in. This study aims to uncover the unique dependence of Pot1 at telomere ends in a system that permits the single loss of each of the Pot1 paralogs. Here we show that due to the elusive nature of mouse embryonic stem cells (mESCs), Pot1a depletion mounts an inconsequential DNA damage response (DDR) that has little effect on cell survival. However, the subsequent loss of Pot1a in Pot1b-/-mESCs incites a rapid, yet transient DDR, coupled with an induction of single-stranded(ss) telomeric DNA. The viability of mESCs deficient in POT1 paralogs allows to further unravel the function of TPP1-mediated recruitment of POT1 paralogs to chromosome ends both for telomere end-protection and telomere end fill-in.

Development of RNA-based therapy for telomere biology disorders

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Human telomerase maturation follows a stepwise 3' end processing pathway with consecutive protein component exchanges. Mutations in genes affecting the telomerase function would lead to telomere biology disorders (TBDs). TBDs are a heterogeneous group of inherited diseases with limited treatment options. The clinical spectrum of TBDs is very broad and currently there is no specific cure. Human telomerase RNA maturation involves 5' end modification, such as the cap structure, and 3' end processing, which requires multiple processing factors. In addition, the mature hTR needs the H/ACA complex and TCAB1 for its maturation, stability, and trafficking. Here we took the advantage of circular RNA structure, in which neither 5' end nor 3' end modification is required. We generated a circular form of hTR (cir-hTR). The telomerase assembled with cir-hTR could bypass the requirements of H/ACA complex and form a functional telomerase. Our data suggest a promising medical intervention for TBDs.

Terc gene duplication in Murinae: evolutionary evidence and functional implication

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The telomerase holoenzyme, responsible for telomere elongation, comprises the catalytic protein TERT and the RNA component TERC. In all bony vertebrates, TERC gene position is

conserved in a syntenic block between MYNN (myoneurin) and ACTRT3 (actin related protein T3). However, in the house mouse (Mus musculus) Terc is located in a different position (on the same chromosome 3, but 65 Mb away from the Mynn-Actrt3 block). Interestingly, in Mus musculus a non-coding RNA is located between Mynn and Actrt3: our analysis revealed 80% identity between this gene (which we named Terc1) and Terc. Analysing all the genome assemblies available from rodents, we identified Terc1 in all species belonging to the subfamily Murinae, excluding those belonging to the Rattini tribe. Viceversa, all other rodent species displayed only one Terc gene. Alignment of Terc and Terc1 genes showed that a gene duplication event took place after the division between Rattini and the other Murinae: Terc is the new gene, which retained a conserved sequence, whereas Terc1, although being placed in the original syntenic position, underwent several sequence modifications. The most important is the loss of the 3'-terminal ACA box sequence, necessary for the interaction with telomeric accessory proteins. Nonetheless, the CAB box and, most importantly, the telomere template sequence are conserved, raising the possibility that Terc1 could be functional in the telomerase holoenzyme. Beside the evolutionary aspect, the study of these genes could elucidate the non-telomeric functions of Terc. Moreover, it raises the need for studying Terc1 functions in Terc-/- mouse strains.

Sensibility and specificity of ALT markers among different cell types

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Beside telomerase activation, a few tumors employ a recombinational mechanism, called Alternative Lengthening of Telomeres (ALT), in order to counteract telomere shortening-induced replicative senescence.
So far, several hallmarks have been used to identify cell lines and tumors as ALT-positive. These markers are: high frequencies of ALT-associated PML bodies (APBs), long telomeres and heterogeneous telomere length, presence of extrachromosomal telomere repeats (C-circles) and high levels of telomere sister chromatid exchange (T-SCE). However, although not widely known, some of these markers are present also in ALT-negative cells, raising doubts as to which marker is more reliable. For this reason, we analyzed a panel of ALT-positive and -negative cell lines (from different tumor types and characterized by different mutations), to investigate the specificity and sensibility of markers that are usually associated with ALT.
We found that the most specific and sensible markers are C-circles and telomere length heterogenicity. Telomere length, instead, is sensible, but not specific. Other markers are more difficult to interpret. Interestingly, we found that some ALT markers were present also in senescent fibroblasts and that their levels were influenced by presence or absence of p53 and ATRX.

Relative Telomere Length measurement of Colorectal cancer, adenoma and healthy controls from Austria - a CORSA study

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Telomeres are repetitive DNA (TTAGGG)n tracts located at the ends of eukaryotic chromosomes. They play a crucial role in the chromosome end protection and maintenance of the genomic stability. It is well known that any disruptions in the telomere length (TL) and its integrity result in human malignancies, including colorectal cancer (CRC). The main aim of the current study is to correlate the observed dynamics of TL measurements for adenomas and various tumor stages (i.e., carcinomas with adjacent mucosa pairs in comparison to the healthy controls) with dietary, lifestyle habits; effect of other comorbidites on disease progression, impact of the identified SNPs on the risk and survival of CRC patients. TL was measured as relative in PBMCs of 5371 subjects using a modified monochrome qPCR method in which the telomere and albumin products were amplified simultaneously at different annealing temperatures in a single reaction. The signal for telomere was collected first, followed by albumin at a higher melting temperature than telomere. In our study, it was observed that the TL was significantly decreased in adenomas and tumors in comparison to the controls. Additionally, GWAS was performed in ~2400 individuals, to determine the SNPs associated with high risk CRC. Further, a detailed statistical analysis of the TL measurements with respect to CRC progression and the determination of the statistically significant SNPs that effect the TL are still in progress. Acknowledgements: Research study support: Grant No. NU22J-03-00028; AU supported by Grant No. LX22NPO5102 and GACR 22-05942S; PV and LV by GACR 21-27902S.

Functional whole genome CRISPR screen identifies putative regulators of genome stability upon telomere uncapping

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Understanding the mechanisms that maintain genome stability is essential given the clinically relevant use of chemotherapeutics compounds that target DNA. Telomeres are a special case of a DNA lesion where the end of the human chromosome resembles a double strand break. This "break" is capped by shelterin, which prevents aberrant activation of DNA damage signalling. Loss of TRF2 elicits an ATM-dependent response and leads to irreversible cell cycle arrest or death as a consequence of telomere fusion formation. This process has been studied using targeted approaches or biased shRNA libraries leaving an opportunity to take an unbiased approach. We have thus performed a functional genetic CRISPR screen in cells expressing iCas9 and hygro-sgTRF2 transduced with the pooled Brunello library. The inducible deletion of TRF2 leads to cell death and telomere fusions which could be rescued by loss of factors that affect DNA damage signalling, repair or cell cycle regulation. The screen has revealed approximately 270 significant hits with the most significant ones being SHLD1, ATM, RNF168, RIF1 and others. Considering that some well characterized proteins such a Rev7, NBN, Lig4 are at the bottom of our candidate list our screen has potential to reveal previously uncharacterized genome stability factors. To examine this, we have performed crRNA based arrayed screen to select candidates affecting cell survival and/or 53BP1 foci formation and confirmed these hits by deconvoluting the sgRNAs from the library used. We are have functionally validated novel hits and found new factors with telomere specific and genome wide roles.

TRF1-dependent repression of human telomere fragility requires fork reversal and telomerase activity

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Telomeres are hard-to-replicate regions where DNA replication is challenged and cannot be rescued by a converging fork. In TRF1-depleted cells, telomeres experience replication stress and manifest as fragile telomeres. While it is established that TRF1 plays a major role in facilitating telomere replication by recruiting helicases for secondary structures resolution, a recent study unveiled a new factor, TFIIH, involved in mouse telomere replication through a yet undefined mechanism.
Here, we took advantage of two isogenic post-crisis survivor (PCS) cell lines from E6/E7-immortalized human fibroblasts that spontaneously activated telomerase to further investigate TRF1 function in repressing replication stress at human telomeres. PCSLT cells, with long telomeres, displayed fragile telomeres on both lagging and leading strands that were not detected in PCSST cells with shorter telomeres. Despite displaying a subset of ALT-like markers, PCSLT cells do not engage into BIR. Telomere fragility in PCSLT cells resulted from TRF1 undersaturation and was rescued by TRF1 level restoration at telomeres. Using this system, we found that the TRF1-dependent repression of lagging strand telomere fragility depends on fork reversal through a mechanism involving telomerase activity. Interestingly, TRF1-dependent repression of telomere fragility was lost upon removal of DNA:RNA hybrids through RNase H1 overexpression, suggesting an unexpected positive role of these hybrids in promoting telomere replication. Together, these observations suggest that TRF1 may not only alleviate telomeric replication fork barriers but, instead, promote efficient fork restart at human telomeres.

Identification and Characterisation of Novel Telomere-binding Proteins in Ascomycota Yeast Species

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Telomeres, the specialised nucleoprotein structures at the linear eukaryotic chromosomes, are essential for genome stability and integrity of DNA termini. These structures ensure intactness by preventing fusion and recombination events at the chromosomal ends, as well as facilitating replication with the help of telomerase. In most organisms, telomeric DNA consists of short repetitive sequences. It is believed that the canonical, TTAGGG, telomeric repeat is one of the ancient ones and is conserved among eukaryotes. However, telomeric repeat sequences in Saccharomycotina subphyla of buddying yeast are exceptionally diverse, TG-degenerated, and vary in overall length of telomeric arrays, gradually showing less resemblance to the TTAGGG in evolutionary young species. Thereby, telomere-binding proteins (TBPs) in these yeasts are

not necessarily conserved and may even vary among different yeast clades. Here, we aimed to inspect the co-evolution of TBPs in the Ascomycota group by performing Telo pull-downs with telomeric repeat sequences of different yeasts, with the prospect of discovering novel telomere binders. As a result, two TBPs, the paralogs TDA9 and RSF2, were found to bind telomere sequences of several yeasts used in this study in vitro, and in vivo in S. cerevisiae. Additionally, the deletion of both of the proteins in S. cerevisiae leads to the postponed formation of survivors that overcome replicative senescence crisis. We will show how these proteins affect telomere length, rates of senescence and the telomere position effect and speculate about when they become functionally important.

Genomic instability, microenvironment and telomere homeostasis in solid malignancies.

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Both impaired DNA repair mechanisms and disrupted telomere length homeostasis represent key culprits in cancer initiation, progression and prognosis. Understanding the mechanisms and dynamics of tumor genomic diversification, where DNA damage response and telomere homeostasis are important players, is critical to understand carcinogenesis and overcome the drug resistance. Telomere shortening has a dual role in tumorigenesis. It promotes cancer initiation by inducing CIN, while TL maintenance characterized by telomerase expression is required for cancer cell proliferation and tumour growth. We undertook a comparison of telomere homeostasis genetics (based on GWAS study) with telomere length (TL) in 7,000 patients with sporadic CRC. We also studied TL as a biomarker for cancer risk, patient therapy response and/or survival.
The mitochondrial dysfunction is linked with DNA repair capacity and compensate for damage by increasing the mitochondrial DNA copy number (mtDNA-CN). We investigated mtDNA-CN in CRC tissues and adjacent mucosa in relation with TL. Moreover, gene expressions within DNA repair pathways were related to the damage of mtDNA. The association of mtDNA damage and gene expressions has been found among 48 genes representing all putative repair pathways in mitochondria. In comparison with adjacent mucosa CRC tumors exhibited lower extent of mtDNA damage (P=0.047), but there was higher expression of the majority of DNA repair genes. Out of 48 DNA repair genes 14 with the strongest correlation between the expression and mtDNA damage (LIG3, MUTYH, RAD50, BRCA2, BRCA1, LIG1, PARG, NEIL3, RFC3, POLD3, POLD4, MRE11, NEIL1, UNG) are validated on a larger cohort of patients.

Interstitial telomeric sequences promote gross chromosomal rearrangement via multiple mechanisms in Saccharomyces cerevisiae

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Telomeric DNA sequences are difficult to replicate. Replication forks frequently pause or stall at telomeres, which can lead to telomere truncation and dysfunction. In addition to being at chromosome ends, telomere repeats are also present at internal locations within chromosomes, where they are called interstitial telomeric sequences (ITSs). These sequences are unstable and prone to triggering gross chromosomal rearrangements (GCRs). In this study, we quantitively examined the effect of ITSs on GCR rate in Saccharomyces cerevisiae using a genetic assay. We find that GCR rates increase exponentially with ITS length. This increase can be attributed to the presence of the telomere repeat binding protein Rap1, which is known to impede DNA replication, and a bias of repairing DNA breaks at or distal to the ITS via de novo telomere addition. Additionally, we performed a genome-wide screen for genes that modulate the rate of ITS-induced GCRs. We find that mutation of core components of the DNA replication machinery leads to an increase in GCRs, but many mutants known to increase GCR rate in the absence of an ITS do not significantly affect GCR rate when an ITS is present. We also identified genes that promote the formation of ITS-induced GCRs, including genes with roles in nucleotide excision repair and transcription. Our work uncovers multiple mechanisms by which an ITS promotes GCR.

DNA damage at tandem repeats generates i-loops that can be excised as eccDNA

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IFOM ETS - The AIRC Institute of Molecular Oncology

Extrachromosomal circular DNA (eccDNA) molecules are ubiquitous species found in normal tissues that accumulate in cancer cells. Though affecting several cellular functions, the exact molecular steps enabling their genesis remain elusive. We have recently discovered internal loop structures (i-loops) arising at damaged telomeres that can be excised as eccDNA. We proposed a model defining the molecular steps involved in the formation and excision of i-loops at telomeres. In this work we investigate the i-loop formation and excision model at other repetitive elements. Using genome-wide damage or through targeted CRISPR-Cas9 nickase (nCas9)-induced nicks we show that, apart from telomeres, i-loops are formed as a consequence of DNA damage also at centromeric α-satellite repeats. As in the case of telomeres, i-loop formation at centromeres was associated with an accumulation of eccDNA. I-loop and eccDNA accumulation was confirmed also by electron microscopy analysis of centromere-enriched samples, indicating that the i-loop excision mechanism might be an important mechanism of eccDNA generation and tandem repeat instability. While investigating the possible physiological sources of i-loops, we found a strong accumulation of i-loops in cells undergoing programmed cell death. In these conditions, i-loop accumulation was independent from the original stimulus triggering programmed cell death, but it required caspase activity. Cell-death induced i-loop accumulation was observed both at telomeres and alpha satellite repeats and was associated with the release of both telomeric and centromeric eccDNA. Together these findings reveal an unanticipated link between DNA damage, programmed cell death signaling and genome remodeling through i-loop release.

S. pombe telomerase RNA: secondary structure and flexible-scaffold function

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Little is known about the structure of the telomerase RNA from the 1212 nt fission yeast *Schizosaccharomyces pombe*, TER1. We have derived a robust secondary structure model based on phylogenetics, bioinformatic modeling, as well as genetic and biochemical analyses. We find several conserved regions of the rapidly evolving TER1 RNA are essential based on determining functionality of truncation mutants *in vivo*. Overall, large regions are dispensable without causing senescence. This is similar to budding yeast telomerase RNA, TLC1, and consistent with flexible-scaffold function. We tested if the essential three-way junction (3WJ) works from other locations in TER1, finding that indeed it can, supporting that it is flexibly scaffolded in the RNP. Furthermore, we find that a half-sized Mini-TER1 allele, built from the core of the model, supports catalytic activity with TERT *in vitro*. Overall, we provide an evidence-based secondary structure model for the large fission yeast telomerase RNA and begin to characterize features of its physical organization as they relate to function *in vivo*.

Humanization of the mTert gene reset mouse telomeres to human length

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Telomeres shorten with each cell division, triggering replicative senescence and marking human aging. Mice, with their excessively long telomeres and widespread telomerase activity, limit their suitability for studying human diseases. To address this, we engineered a humanized mouse telomerase reverse transcriptase gene (hmTert) by replacing the 5' intergenic region, introns 2 and 6 of the mTert gene with human counterparts.
The hmTert gene, encoding the wildtype mTert protein, was repressed in most adult tissues and expressed only in gonads and a limited subset of immune cells, mimicking the hTERT gene. It successfully rescued telomere dysfunction in late generations of Tert-/- mice. Through successive intercrosses of Terth/- mice, telomere length progressively decreased and stabilized below 10 kb. Terth/h mice achieved an average ~10 kb telomere length, resembling humans, in contrast to the over 50-kb average in C57BL/6J mice.
Despite short telomeres and no telomerase in most adult tissues, Terth/h mice maintained normal body weight and cellular homeostasis in high-proliferative tissues, such as testes, intestine, and bone marrow. Notably, colonocytes and mouse embryo fibroblasts from Terth/h mice exhibited reduced proliferative potential compared to their counterparts from C57BL/6J mice under ulcerative colitis-like pathology and in vitro culture conditions, indicating limitations on cellular renewal due to short telomeres.
Our findings demonstrate that humanizing the mTert gene resets mouse telomeres to human lengths, highlighting the genetic determination of telomere homeostasis by the TERT/Tert locus. This transformation provides mice with humanized telomeres, offering a promising model for exploring essential aspects of human aging and cancer.

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