Universidade de Lisboa

Faculdade de Farmácia

The roles of telR-loops in the stability of telomeres in primary and telomerase-positive cancer cells

Leonardo Amaro Gaio

Dissertation supervised by Doctor Claus Azzalin and co-supervised by Professor Susana Solá

Master course in Biopharmaceutical Sciences

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Summary

Telomeres are heterochromatic nucleoprotein structures that protect the ends of eukaryotic linear chromosomes. In mammals, their core complex comprises tandem arrays of DNA duplex repeats (5'-TTAGGG-3'), from which the G-rich strand extends beyond its complement to form an overhang (G overhang), and a multiprotein complex known as "shelterin" that ensures proper telomere protection, integrity and length regulation. Telomeres are also transcribed into long noncoding RNA molecules known as TElomeric Repeat containing RNA (TERRA). Although a full characterization of TERRA functions is lacking, accumulating data show that it stimulates telomere protection/replication and heterochromatin formation. Additionally, TERRA is seen as a fine regulator of telomerase, since it promotes telomerase-mediated telomere elongation *in vivo*, but telomerase inhibition *in vitro*. TERRA can hybridize with its template substrate in both yeast and humans and form a three-stranded structure composed of an RNA-DNA hybrid and a displaced DNA strand, which is defined as a telomeric R-loop (telR-loop). We recently proved that TERRA functions might be partly mediated by the formation of telR-loops stimulated by TRF2. Furthermore, additional observations in telomerase-negative, pre-senescent yeast cells and alternative lengthening of telomeres (ALT)-positive tumors have implicated telR-loops in the promotion of homology-directed repair (HDR)-dependent telomere elongation to partially compensate for the erosion of chromosome ends in the absence of telomerase. TelR-loops are restricted by the RNase H1 and RNase H2 enzymes, which specifically hydrolyze the RNA moiety of RNA-DNA hybrids. While the accumulation of telR-loops in yeast cells lacking both enzymes delays senescence onset by promoting telomeric HDR, telR-loop depletion by RNase H1 overexpression leads to weakened recombination and, consequently, to telomere shortening and premature senescence.

This study aims to better understand the importance of telR-loops in maintaining telomere stability in telomerase-positive and negative human cells and characterize how telR-loops support TERRA-mediated telomere length regulation. For this purpose, we established a cellular system based on telomere targeting of the catalytic domain of RNase H1 via fusion to the shelterin protein POT1. This system was employed for the specific removal of telR-loops in a telomerase-positive fibrosarcoma cell line (HT1080), primary human lung fibroblasts (HLF), and in HLF cells immortalized by overexpressing the telomerase reverse transcriptase subunit of human telomerase (HLF-hTERT). We discovered that telR-loop degradation, as a consequence of the exogenous expression of RNase H1 from our system, compromises telomere integrity in all the tested cell lines,

as shown by the accumulation of DNA damage markers at telomeres 48 hours after telR-loop removal. Moreover, RNase H1 targeting to telomeres causes telomere loss in telomerase-negative HLF cells, but not in telomerase-positive HT1080 and HLF-hTERT cells.

Altogether, these results suggest that telR-loops have a protective function against telomere instability, most prominently in primary human fibroblasts. Given the previous work detailing the recombinogenic nature of ALT and telomerase-negative yeast telomeres, we propose that telR-loops stimulate telomere elongation in an HDR-dependent manner at shortened telomeres to delay premature senescence and uncontrolled cell death.

Keywords: Telomeres; Telomere Stability; TERRA; TelR-loops; RNase H1

Resumo

Os telómeros são estruturas nucleoproteicas compostas por heterocromatina que protegem as regiões terminais dos cromossomas lineares eucariotas. Em mamíferos, a estrutura telomérica é essencialmente formada por repetições consecutivas de sequências de DNA em cadeia dupla (5'-TTAGGG-3'), da qual a cadeia rica em guanosina estende-se para além do seu complemento para formar uma protrusão conhecida como *G overhang*, e por um complexo multiproteico denominado de *"shelterin"* que assegura a proteção, integridade e regulação dos telómeros. A *shelterin* humana inclui os seis fatores TRF1, TRF2, TIN2, TPP1, POT1 e Rap1. Em células terminalmente diferenciadas, os telómeros são encurtados a cada ronda de replicação semiconservativa de DNA devido ao seu processamento nucleolítico e à incapacidade da maquinaria convencional de replicação de DNA de processar por completo as regiões 3' distais de moléculas lineares de DNA durante a síntese da cadeia atrasada. Esta limitação ficou conhecida como *end-replication problem*. A ausência de um mecanismo de manutenção telomérica resulta na acumulação de telómeros excessivamente curtos e disfuncionais, os quais são reconhecidos como quebras de cadeia dupla (DSBs, do inglês *Double-Stranded Breaks*). Estes, por sua vez, levam à ativação de uma resposta contra o dano de DNA (DDR, do inglês *DNA Damage Response*). A estimulação prolongada de DDR induz uma paragem irreversível do ciclo celular conhecida como senescência celular (ou replicativa), podendo progredir para a morte celular. Embora a senescência celular estabeleça um tempo de vida útil a células somáticas incapazes de conservar os seus telómeros, esta é também considerada como um mecanismo decisivo de supressão tumoral. O potencial imortal das células cancerígenas depende da capacidade das mesmas em impedir o desgaste progressivo dos telómeros. Este processo está dependente da ação da enzima telomerase, a transcriptase reversa que permite a adição de novas repetições teloméricas às extremidades dos cromossomas, ou numa menor fração da via alternativa de alongamento de telómeros (ALT, do inglês *Alternative Lenghtening of Telomeres*), que induz uma reparação dirigida por homologia (HDR, do inglês *Homology-Directed Repair*). Apesar dos telómeros de mamíferos terem sido considerados transcricionalmente inativos devido à sua constituição heterocromática e à ausência de unidades génicas, estudos realizados pelo nosso grupo revelaram que estes são de facto transcritos em moléculas longas de RNA não-codificante conhecidas como *TElomeric Repeat containing RNA* (TERRA). Na verdade, não existe ainda uma caracterização completa das funções de TERRA, mas um número crescente de dados

sustenta o seu papel na proteção e replicação dos telómeros e na formação de heterocromatina. Adicionalmente, TERRA pode funcionar como um regulador da telomerase, promovendo a elongação telomérica mediada por telomerase *in vivo*, apesar de inibir a atividade da mesma *in vitro*. Adicionalmente, TERRA tem a capacidade de emparelhar com a sua cadeia de DNA molde e formar uma estrutura em tripla cadeia constituída por um híbrido de RNA-DNA e uma cadeia codificante deslocada, denominada como *R-loop* telomérico (*telR-loop*). Recentemente, o nosso grupo demonstrou que as funcionalidades de TERRA são em parte mediadas pela formação de *telR-loops* estimulada por TRF2. Observações adicionais em células de levedura telomerase-negativas e pré-senescentes e em células ALT também implicaram os *telR-loops* na extensão dos telómeros por HDR de modo a compensar parcialmente a erosão das regiões terminais dos cromossomas na ausência de telomerase. Por fim, os *telR-loops* são regulados pelas enzimas RNase H1 e RNase H2, as quais hidrolisam especificamente a fração de RNA de híbridos de RNA-DNA. Enquanto que a acumulação de *telR-loops* em células de levedura sem ambas as enzimas retarda a incidência de senescência ao promover HDR telomérico, a remoção de *telR-loops* através da sobrexpressão de RNase H1 debilita a capacidade de recombinação telomérica e, consequentemente, intensifica o encurtamento dos telómeros e a ocorrência prematura de senescência.

Os mecanismos pelos quais a homeostase telomérica é mantida ainda não são inteiramente conhecidos, mas as descobertas recentes acerca da biogénese de TERRA e as novas abordagens direcionadas à sua função trouxeram algum esclarecimento a este tema. Apesar de ainda não ser claro o mecanismo pelo qual TERRA é regulada em concordância com diferentes dinâmicas e estados teloméricos, postula-se que os *telR-loops* desempenhem um papel central neste processo. Assim, este estudo pretendeu elucidar o papel dos *telR-loops* na estabilidade dos telómeros em células humanas que expressam, ou não, a telomerase, e caracterizar o mecanismo pelo qual os *telR-loops* participam na regulação do comprimento dos telómeros mediada por TERRA.

Para tal, estabelecemos um sistema celular direcionado aos telómeros e baseado na sobrexpressão de RNase H1 com o intuito de remover especificamente os *telR-loops* e avaliar a integridade telomérica após a exclusão destas estruturas. Para promover a associação do transgene aos telómeros, o domínio catalítico de RNase H1 humana foi fundido na sua região N-terminal a myc e na região C-terminal a POT1 humana (myc-POT1-RH1 WT). Adicionalmente, três transgenes foram criados, incluindo uma variante de RNase H1 cataliticamente inativa (myc-POT1-RH1 D145G), uma outra constituída ainda pelo seu domínio de ligação a híbridos (HBD, myc-POT1-HBDRH1

WT), e uma versão contendo apenas o domínio POT1, também marcado por myc na região N-terminal (myc-POT1). Os transgenes foram expressos numa linha celular de fibrossarcoma telomerase-positiva (HT1080), em fibroblastos primários de pulmão humano (HLF), e numa linha celular HLF imortalizada através da sobrexpressão da subunidade de transcriptase reversa da telomerase humana (HLF-hTERT). Excecionalmente, myc-POT1-HBDRH1 WT foi incluído apenas em HLF-hTERT. A expressão ectópica controlada por doxiciclina (dox) dos transgenes foi validada por *Western Blot* e a localização telomérica mediada por POT1 confirmada através de experiências de imunofluorescência com marcação a myc. Os nossos resultados demonstraram que tanto myc-POT1 como myc-POT1-RH1 WT e myc-POT1-RH1 D145G co-localizaram, na sua maioria, com a fração telomérica de HT1080, HLF e HLF-hTERT. Pelo contrário, a deteção de myc-POT1-HBDRH1 WT foi bastante variável e a sua localização também reduzida em telómeros de HLF-hTERT quando comparado com os restantes transgenes. Seguidamente, confirmámos em células HT1080 que a sobrexpressão do domínio catalítico de RNase H1 dirigida aos telómeros induz a degradação de *telR-loops* no cromossoma 10q. Por outro lado, a variante inativa do transgene promoveu a acumulação dos mesmos. Demonstrámos ainda que a ausência de *telR-loops* compromete a integridade dos telómeros em todas as linhas celulares testadas devido a um aumento do número de *foci* induzidos por disfunção telomérica (TIFs, do inglês *Telomere Dysfunction-Induced Foci*). No entanto, o número de TIFs não continuou a aumentar, o que possivelmente ilustra uma ativação adequada dos mecanismos de reparação de DSBs que bloqueiam a progressão contínua dos danos no DNA telomérico. De notar que a formação de TIFs foi significativamente restringida em células de HT1080 e HLF-hTERT que expressavam myc-POT1-RH1 D145G, sugerindo que, quando desprovido da atividade catalítica, RNase H1 pode fisicamente limitar a dissolução de *telR-loops* ao protegê-los de possíveis fatores de degradação de *R-loops*. Finalmente, observámos um aumento da quantidade de cromossomas desprotegidos pela ausência de sinais teloméricos em células HLF que expressavam myc-POT1-RH1 WT, mas não em células HLF-hTERT e HT1080. Por outro lado, a acumulação de *telR-loops* através da sobrexpressão de myc-POT1-RH1 D145G diminuiu os níveis de fragilidade telomérica nestas linhas celulares telomerase-positivas.

Estes resultados sugerem, assim, que os *telR-loops* sustentam a proteção contra a instabilidade telomérica, e que esta função se torna determinante para a manutenção dos telómeros em fibroblastos primários humanos. Nas células que expressam telomerase, a perda de telómeros parece ser compensada pela presença desta enzima, que estabiliza a erosão progressiva das extremidades dos cromossomas lineares e

suporta a homeostase telomérica. Como tal, e de acordo com as descrições associadas à natureza recombinante dos telómeros de células ALT e de levedura telomerase-negativas, propomos que os *telR-loops* também estimulam a extensão telomérica de uma maneira dependente de HDR em telómeros humanos curtos e danificados, de forma a adiar a ocorrência prematura de senescência e morte celular descontrolada. Adicionalmente, os nossos resultados sugerem que a RNase H1 pode funcionar como um fator limitante de recombinação telomérica nestas células ao regular os níveis de *telR-loops* e assegurar um equilíbrio entre senescência, supressão tumoral e estabilidade telomérica ao longo do ciclo de vida celular. Finalmente, os nossos resultados indicam também que o HDR telomérico pode ser exercido através de replicação induzida por quebras (BIR, do inglês *Break-Induced Replication*), tal como já foi verificado na manutenção de telómeros dependentes de HDR em células eucariotas que não expressam telomerase.

Palavras-chave: Telómeros; Estabilidade Telomérica; TERRA; *telR-loops*; RNase H1

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Table of Contents

List of Figures

List of Tables

Abbreviations

AID	Activation-induced cytidine deaminase
ALT	Alternative lengthening of telomeres
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad53-related
ATRX	Alpha-thalassemia/mental retardation X-linked
BIR	Break-induced replication
BLM	Bloom syndrome protein
BSA	Bovine serum albumin
CMV	Cytomegalovirus
CTCF	CCCTC-binding factor
D-loop	Displacement loop
DDR	DNA damage response
dHJ	Double Holliday junction
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
ssDNA	Single-stranded DNA
Dox	Doxycycline
DRIP	DNA-RNA immunoprecipitation
DSB	Double-stranded break
ECTR	Extra-chromosomal telomeric repeat
EV	Empty vector
FANCM	Fanconi anemia, complement group M
FBS	Fetal bovine serum
FISH	Fluorescence *in situ* hybridization

FT	Fragile telomere
G4	G-quadruplex
H3k9m3	Histone H3 trimethylated at lysine 9
HBD	Hybrid-binding domain
HDR	Homology-directed repair
HLF	Human lung fibroblasts
HLF-hTERT	HLF overexpressing hTERT
hnRNPA1 or A1	Heterogenous ribonucleoprotein A1
HP1α	Heterochromatin protein 1 alpha
HSF1	Heat shock factor 1
IF	Indirect immunofluorescence
m^7G	7-methylguanosine
NHEJ	Non-homologous end joining
NMD	Nonsense-mediated decay
NRF1	Nuclear respiratory factor 1
PARP1	Poly(ADP-ribose) polymerase 1
PML	Promyelocytic leukaemia
Polα	Polymerase alpha
POT1	Protection of telomeres 1
PP	Polymerase alpha-primase
qPCR	Real-time PCR
Rap1	Repressor/activator protein 1
RH1 D145G	RNase H1 variant carrying a D145G mutation
RH1 WT	Wild-type variant of RNase H1
RNA	Ribonucleic acid
dsRNA	Double-stranded RNA

lncRNA	Long noncoding RNA
RNAPII	RNA polymerase II
RNase H	Ribonuclease H
RPA	Replication protein A
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SETX	Senataxin
T-loop	Telomeric loop
T-Rec	Telomerase recruitment cluster
T-SCE	Telomeric sister chromatid exchange
telR-loop	Telomeric R-loop
TERC	Telomerase RNA component
hTERC	Human telomerase RNA component
TERRA	Telomeric repeat containing RNA
TERT	Telomerase reverse transcriptase
hTERT	Human telomerase reverse transcriptase
TFE	Telomere-free end
TIF	Telomere dysfunction-induced foci
TIN2	TRF2- and TRF1-interacting nuclear protein 2
TPP1	POT1-interacting protein
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
WB	Western blot or western blotting
WT	Wild-type

1. Introduction

1.1 Telomeres: function and composition

Telomeres are heterochromatic nucleoprotein structures located at the termini of eukaryotic linear chromosomes (Azzalin & Lingner, 2008; de Lange, 2009). Throughout vertebrates, conserved tandem arrays of double-stranded 5'-TTAGGG-3' repeats have been reported at the telomeric tract, with the G-rich strand extending beyond its complement (C-rich strand) to form an overhang, known as the G overhang (**Figure 1**) (Moyzis et al., 1988; McElligott & Wellinger, 1997; Azzalin & Lingner, 2008). This protrusion varies between 30 to 400 nt, being present at both ends of each chromosome, and is generated by the nucleolytic end-processing of telomeres and the inability of the conventional DNA replication machinery to fully duplicate the 3' ends of linear DNA molecules during lagging strand synthesis. This process is called the end-replication problem (Levy et al., 1992; McElligott & Wellinger, 1997; de Lange, 2009), which will be further discussed in Section 1.1.2. Telomeres are essential to ensure cellular and genome stability by securing the physical protection of natural chromosome ends from inappropriate DNA repair activities that may lead to DNA degradation, fusion and erroneous recombination events (see Section 1.1.1) (Counter et al., 1992; van Steensel et al., 1998; Sfeir et al., 2005). In addition, they are also very important tumor suppressors and may contribute to tissue and organismal aging by regulating the lifespan of cells (Harley et al., 1990; Allsopp et al., 1992; Levy et al., 1992; Bodnar et al., 1998). Finally, the heterochromatic nature of telomeres, as shown by the presence of histone H3 trimethylated at lysine 9 (H3K9m3), histone H4 trimethylated at lysine 20 (H4K20m3), histone hypoacetylation, accumulation of various isoforms of heterochromatin protein 1 (HP1) and the hypermethylation state of cytosines from CpG islands at subtelomeric regions (the chromosomal regions adjacent to telomeres), may favor chromosome positioning and movement within the nucleus (Blasco, 2007; Azzalin & Lingner, 2008; Michishita et al., 2008; Ottaviani et al., 2008).

Even though telomere length varies considerably between mammals (human telomere length ranges between 10 and 15 kb at birth, while telomeres of rats and laboratory mice stand at 20-50 kb), they all share the presence of a specialized multiprotein complex dubbed "shelterin" (de Lange, 2005; Palm & de Lange, 2008; de Lange, 2018). The shelterin complex is a telomere-binding structure sufficiently abundant to cover all double-stranded and single-stranded telomeric DNA throughout

the cell cycle (Palm & de Lange, 2008; Takai et al., 2010). Shelterin is essential for telomere integrity, length regulation and protection by avoiding that telomeres are recognized as DNA double-stranded breaks (DSBs) and thus suppressing the activation of DNA Damage Response (DDR) pathways (Azzalin & Lingner, 2008; Palm & de Lange, 2008; Arnoult & Karlseder, 2015; de Lange, 2018).

Human shelterin includes six core proteins, of which Telomeric Repeat binding Factor 1 and 2 (TRF1 and TRF2) directly bind to the duplex telomeric repeats with nanomolar affinity (de Lange, 2005a; Palm & de Lange, 2008, Erdel et al., 2017) and recruit the remaining shelterin components. TRF2- and TRF1-Interacting Nuclear Protein 2 (TIN2) stabilizes the association of TRF1 and TRF2 with chromosome ends, while simultaneously linking both proteins and the POT1-Interacting Protein (TPP1) through distinct domains, therefore creating a bridge between the different shelterin factors (Kim et al., 2004; Ye, et al., 2004; Erdel et al., 2017). In turn, TPP1 recruits to the telomeres and forms a stable heterodimer with Protection Of Telomeres 1 (POT1), which also binds with high specificity to telomeric single-stranded DNA (ssDNA) (Lei et al., 2004; Chen et al., 2007). The TPP1-POT1 interaction is not only vital for the telomeric connection of POT1, but it also regulates its subcellular localization, as POT1 variants lacking the TPP1-binding domain are mainly excluded from the nucleus and TPP1 knockdown decreases the levels of nuclear POT1 (Chen et al., 2007). Depletion of TPP1 or the expression of mutants deficient in POT1 binding ablates the detection of POT1 from telomeres. Additionally, impaired TPP1 function leads to telomere deprotection and telomere length events consistent with POT1 loss (Liu et al., 2004; Ye et al., 2004; Denchi & de Lange, 2007; Hockemeyer et al., 2007; Xin et al., 2007). *In vivo*, POT1 associates with telomeric ssDNA via its two N-terminal oligonucleotide/oligosaccharide-binding (OB) folds, and this binding is weakened upon TRF2 inhibition, which leads to degradation of the G overhang (Baumann & Cech, 2001; Loayza & De Lange, 2003; Lei et al., 2004). Yet, a study has shown that POT1 binding to ssDNA is not required for this process, since a POT1 mutant lacking the DNA-binding domain ($POT1^{\Delta OB}$) could still associate with telomeres through its interaction with the TRF1 complex that was deemed to be essential for telomere length control (more to be discussed at Section 1.2.1) (Loayza & De Lange, 2003). The six-subunit complex is completed by the human ortholog of the yeast Repressor/activator protein 1 (Rap1), which associates with TRF2 to regulate its localization and increase its binding specificity for telomeric sequences (**Figure 1**) (Li et al., 2000; Arat & Griffith, 2012; Janoušková et al., 2015). In the absence of TRF2, most of Rap1 is lost from chromosome ends due to its lack of DNA binding activity, but Rap1 can still bind non-telomeric regions, where it plays a role in gene

expression regulation, more precisely in metabolic pathways associated with fat metabolism (Celli & de Lange, 2005; Martínez et al., 2013; Yeung et al., 2013). Rap1 slightly diminishes the interaction of the TRF2 N-terminal Basic domain with branched DNA, while also preventing it from forming sequence-independent DNA interactions and thus increasing the specificity of TRF2 to TTAGGG repeats (Gaullier et al., 2015; Janoušková et al., 2015; Nečasová et al., 2017). Interestingly, mouse shelterin is composed of seven proteins, as a result of a recent gene duplication that led to the generation of two POT1 paralogs (POT1a and POT1b) (He et al., 2006; Hockemeyer et al., 2006). Also, human and mouse cells contain about ten times more TRF1, TRF2, TIN2 and Rap1 than TPP1 and POT1, therefore reflecting the existence of some complexes devoid of the latter two proteins due to their lower abundance (**Figure 1**). Nevertheless, TPP1-POT1 is in tenfold excess over its single-stranded TTAGGG binding sites, and given that the shelterin core is sufficient to cover all double-stranded telomeric repeats, it is suggested that most of the telomeric DNA is associated with shelterin proteins (Takai et al., 2010; de Lange, 2018). Moreover, even though the specific localization of shelterin to telomeres is strictly dependent on TRF1 and TRF2, since the removal of both proteins completely ablates telomere binding of the remaining elements (Sfeir & De Lange, 2012), POT1-TPP1 can still form a stable complex, as it does not require any interactions with DNA to be established (Palm & de Lange, 2008; Erdel et al., 2017).

Many other different molecular strategies are employed by each shelterin component to counteract pathways that may pose a threat to telomere stability and integrity. These are mostly related to the initiation steps of DNA signaling or repair reactions, which will be further discussed below.

1.1.1 The end-protection problem

The protection of natural chromosome ends by telomere capping is crucial for chromosome stability, since telomeres chemically resemble DSBs and thus have the potential to activate DNA damage signaling and trigger a DDR. When a chromosome breaks, the exposed DNA ends can be subjected to at least seven distinct DDR pathways (de Lange, 2018). These include three DNA damage signaling enzymes activated by DSBs: the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad53-related (ATR) kinases and poly(ADP-ribose) polymerase 1 (PARP1); three main DNA repair pathways that patch broken chromosomes: non-homologous end joining (NHEJ), which divides into classical non-homologous end joining (c-NHEJ) and alternative non-

homologous end joining (alt-NHEJ), and homology-directed repair (HDR); and telomeric hyper-resection (de Lange, 2009, 2018). Telomeres, if not properly protected, can also become substrates for these mechanisms, which can lead to disastrous events that include cell cycle arrest and possibly senescence or apoptosis (via ATM and/or ATR activation), end-to-end chromosome fusions (promoted by NHEJ) and sequence recombination (driven by HDR) between telomeres or a telomere and other parts of the genome (de Lange, 2009). This end-protection problem is solved by the shelterin complex, which employs distinct strategies through different subunits to suppress nucleolytic attacks and DNA repair activities at telomeres (Arnoult & Karlseder, 2015; de Lange, 2018).

TRF2 is essential to repress the downstream activation of the ATM kinase pathway by directing the maintenance of telomere ends via formation and protection of a large lariat DNA structure known as telomeric loop (T-loop) (Griffith et al., 1999; Wu et al., 2012; de Lange, 2018). T-loops are established through intramolecular invasion of the G overhang into the double-stranded telomeric region to anneal with the C-rich strand, consequently displacing the G-rich strand at this site into a displacement loop (D-loop) (**Figure 1**) (Griffith et al., 1999; O'Sullivan & Karlseder, 2010). Thus, by sequestering the telomere terminus and folding it into a higher-order DNA complex, TRF2 provides an architectural solution to conceal the G overhang and avoid the DNA-end binding activity of the Mre11/Rad50/Nbs11 (MRN) complex, which is essential to activate ATM signaling at telomere ends (Karlseder et al., 1999; Celli & de Lange, 2005; Stracker & Petrini, 2011; de Lange, 2018). Since TRF2 is a primary stabilizer of T-loops, being both essential and sufficient for their configuration, not only can T-loop stability and formation be compromised upon its depletion (Doksani et al, 2013), but the cell cycle is also arrested due to up-regulation of p53. Also, other hallmarks of ATM signaling, such as the phosphorylation of ATM and Chk2, and DNA damage foci can be detected at telomeres, which become sites of accumulation of DDR factors, including a phosphorylated variant of histone H2AX (γ-H2AX), the mediator of DNA damage checkpoint protein 1 (MDC1), and the p53-binding protein 1 (53BP1) (Fagagna et al., 2003; Takai et al., 2003). TIN2 deletion also activates ATM, although partly due to the loss of TRF2, which is stabilized by the TIN2-TRF1 bridge at telomeres (Takai et al., 2011).

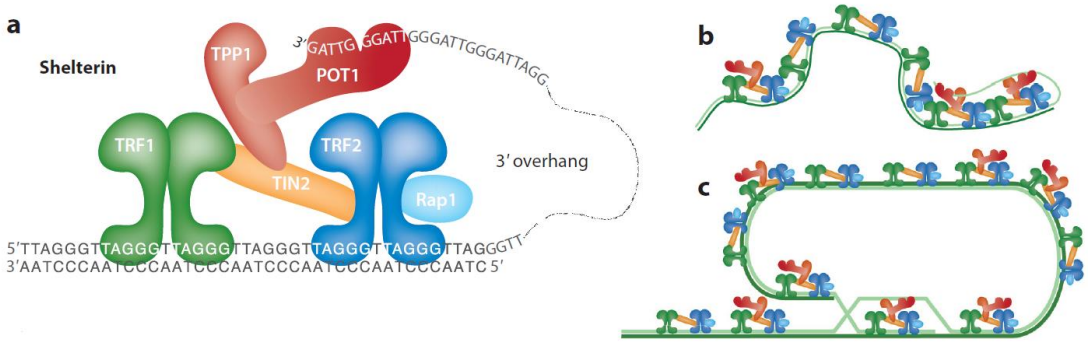

Figure 1 | The shelterin complex and associated telomeric structures

Shelterin is a multiprotein complex that plays a crucial role in telomere length regulation, protection and maintenance. (**a)** The human shelterin comprises six essential factors: TRF1,TRF2,TIN2,TPP1,POT1 and Rap1. TRF1 and TRF2 bind to the duplex telomeric DNA. TIN2 and TPP1 bridge TRF1 and TRF2 with POT1, which interacts with TTAGGG repeats in a single-stranded form. Rap1 binds to TRF2. (**b)** Since TPP1 and POT1 are much less abundant than the remaining shelterin proteins, some complexes may not be provided with them in an open linear configuration. (**c)** TRF2 mediates telomere protection by promoting T-loop formation. T-loops are formed by strand invasion of the G overhang into the homologous dsDNA to pair with the C-rich strand via base complementarity, thus creating a displaced ssDNA region from the G-rich strand known as D-loop. (Adapted from de Lange, 2018)

The ATR kinase pathway can be suppressed by the action of POT1 (Denchi & de Lange, 2007). ATR is stimulated genome-wide by ssDNA formed when the 5' end of a DSB gets resected, which is primarily observed during the S phase of the cell cycle. Nevertheless, telomeric DNA resection does not trigger ATR, thus explaining its presence at telomeres in the G_1 and G_2 phases as well (Gong & de Lange, 2010; de Lange, 2018). ATR upstream signaling relies on the loading of the ssDNA-binding factor Replication Protein A (RPA) (Denchi & De Lange, 2007; de Lange, 2009). Due to the localization of RPA at telomeres, it has been proposed that telomeres avert ATR activation via competition between POT1 and this sensor protein over ssDNA (Denchi & de Lange, 2007). Owing to its tethering to the shelterin core, POT1 has a binding advantage over RPA to accumulate in excess at its specific telomeric single-stranded target sites, which include the G overhang and possibly the displaced DNA strand of the D loop, therefore blocking RPA from gaining access to telomeres (Denchi & de Lange, 2007; Barrientos et al., 2008; de Lange, 2009). However, both proteins have the same degree of affinity towards telomeric ssDNA, with RPA being even more abundant than POT1 at a cellular level (Takai et al., 2011). As a result, a second model based on RPA exclusion proposes that it is first removed by heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1 or A1), to be then replaced by POT1 (Flynn et al., 2011). Nonetheless, RPA is not promptly detectable at mammalian telomeres and becomes available for

telomeric ssDNA binding when POT1 is impaired (Barrientos et al., 2008). Additionally, POT1 can only inhibit the ATR kinase when tethered to the rest of the shelterin core, since inhibition of TPP1 also stimulates this DNA damage signaling pathway (Denchi & de Lange, 2007; Guo et al., 2007; Hockemeyer et al., 2007).

TRF1 can also assist in ATR suppression by supporting the semiconservative replication of telomeres (Sfeir et al., 2009; Zimmermann et al., 2014). Indeed, TRF1 depletion results in the formation of fragile telomeres (FTs), which can be seen in multiple or shredded telomeric FISH signals in metaphase spreads as a sign of compromised replication, activation of ATR signaling and initial development of chromosomes with telomere-free ends (TFEs) (Sfeir et al., 2009; Lee et al., 2018). Single-stranded regions within the telomeric G-rich strand can fold into G-quadruplex (G4) structures that are formed by stacking of square planar alignments of four guanine rings paired via Hoogsten hydrogen bonds and stabilized by hydrogen bonds between neighboring guanines (Paeschke et al., 2005; León-Ortiz et al., 2014). Together with T-loops, G4 complexes can impair telomere replication by promoting replication fork stalling, which may lead to the activation of DDR and consequent chromosome instability (Sfeir et al., 2009; Zimmermann et al., 2014). These events can be prevented by TRF1 and also TRF2, which respectively recruit the Bloom syndrome protein (BLM) helicase and the regulator of telomere elongation helicase 1 (RTEL1) to resolve these structures and enable replication fork progression through telomeric DNA (Sfeir et al., 2009; Vannier et al., 2013; Zimmermann et al., 2014).

T-loops can also form double Holliday junctions (dHJs) if branch migration occurs at their base. PARP1 is a significant stimulator of DNA damage at telomeres due to its ability to promote T-loop cleavage by recruiting Holliday junction (HJ) resolvases that process dHJs, which can lead to the removal of T-loops, activation of NHEJ and large telomere deletions (Wang et al., 2004; Saint-Léger et al., 2014; de Lange, 2018). These harmful events can be prevented by the N-terminal Basic domain of TRF2, which prevents PARP1 recognition of T-loops. However, and since no T-loop cleavage occurs even if PARP1 is activated at TRF2-containing telomeres, a second model suggests that TRF2 can block branch migration at the base of the T-loop, thus preventing dHJ formation. In the absence of the Basic domain, the BLM helicase, which can dissolve dHJs, moderates the levels of T-loop cleavage by possibly branch migrating the dHJ back to the T-loop complex (Schmutz et al., 2017; de Lange, 2018). TIN2 can also repress PARP1 binding to telomeres, although through a mechanism still not elucidated (Schmutz et al., 2017).

Both TRF2 and POT1 subunits play an essential role in the inhibition of the NHEJ and HDR pathways. NHEJ is mediated by the Ku70/80 heterodimer, which is a ring-shaped complex that loads onto accessible DNA extremities and brings them together, thus promoting the ligation of DSBs via DNA ligase IV (Lig4) in complex with other factors (Lieber, 2010; Chiruvella et al., 2013; de Lange, 2018). If two telomeres fuse due to the processing of their single-stranded overhangs, NHEJ directs the formation of dicentric chromosomes, which poses a substantial threat to the genome due to their instability during mitosis (de Lange, 2009). NHEJ is prevalent in the G_1 phase of the cell cycle, where it is mainly suppressed at telomeres by TRF2, while during the G_2 phase (i.e., after DNA replication), both POT1 and TRF2 contribute to the inhibition of this pathway (Celli & de Lange, 2005; Hockemeyer et al., 2006; Wu et al., 2006; Konishi & De Lange, 2008; Lieber, 2010). TRF2 primarily blocks NHEJ activation and subsequent telomere fusions by promoting T-loop formation, since the presence of these lasso-like structures at telomere ends blocks the loading of the Ku70/80 ring onto the G overhang. Depletion of TRF2 but not of other shelterin components (except for TIN2, which results in TRF2 destabilization) not only hinders T-loop formation but consequently provides the substrate needed for Ku70/80 loading and NHEJ stimulation (Takai et al., 2011; Doksani et al., 2013). Still, it has been shown that in the absence of TRF2, Rap1 limits the fusion of telomeres (Sarthy et al., 2009; Benarroch-Popivker et al., 2016). On the other hand, POT1 can most likely repress NHEJ in the absence of T-loops by blocking the efficient loading of Ku70/80 or by preventing cleavage when bound to the G overhang (Hockemeyer et al., 2006). Nonetheless, Ku70/80 still binds to chromosome ends, probably not by binding to their terminal regions but rather by interacting with the shelterin complex (Hsu et al., 2000). POT1 and TRF2, as well as Rap1, inhibit HDR at telomeres, and loss of these proteins leads to an increased frequency of telomeric sister chromatid exchanges (T-SCEs) observed in metaphase spreads, reflecting recombination events in S/G_2 (Wang et al., 2004; Celli & de Lange, 2005; Wu et al., 2006; Palm et al., 2009; de Lange, 2018). The ability of POT1 to do so may rely on its inherent competition towards telomeric ssDNA with other ssDNA-binding proteins, which includes not only RPA but also the HDR factor Rad51 (de Lange, 2009). The Ku70/80 heterodimer also suppresses the HDR pathway at DSBs and telomeres (Celli et al., 2006). This protective ability contrasts with the role of Ku70/80 as a component of the NHEJ pathway and its subsequent necessity of being blocked from telomeres, which is believed to be balanced in a way that secures the repression of HDR without being able to threaten telomere stability by initiating NHEJ (de Lange, 2009).

1.1.2 The end-replication problem

As mentioned above, the end-replication problem induces telomere attrition at each cell division as a result of the inability of the canonical DNA replication machinery to fully duplicate the distal ends of linear double-stranded DNA (dsDNA) molecules (Olovnikov, 1973; Levy et al., 1992; Hug & Lingner, 2006). While the eukaryotic DNA polymerases are able to consistently replicate the G-rich strand through leading strand synthesis, lagging strand formation of the C-rich strand implies a discontinuous process that results in the successive loss of genetic material at each replication round **(Figure 2)** (Olovnikov, 1973; Levy et al., 1992; Osterhage & Friedman, 2009). During DNA replication, DNA polymerase alpha (Polα) initially requires short RNA primers, which are synthesized by the primase activity of the Polα-primase (PP) multiprotein complex. The RNA oligomers provide the free 3'-OH group Polα needs to bind and promote primer extension with deoxyribonucleotides (Pellegrini, 2012; Zhang et al., 2014). In circular genomes, the subsequent removal of these primers does not disrupt replication, since the 8-12 nt gaps created can be filled in by extending a preceding one (Smogorzewska & de Lange, 2004). In linear chromosomes, although replication on the leading strand is complete, it creates a transient blunt-ended molecule that must be processed to generate a maturely sized single-stranded overhang (Lingner et al., 1995; Chow et al., 2012). Furthermore, and even though 5' end processing may also occur at the lagging strand (Sfeir et al., 2005), the removal of the terminal RNA primer leaves a non-replicated gap that cannot be filled in by the polymerase activity of Polα, resulting in the loss of terminal sequences and the recreation of an almost mature G overhang very soon after a replication round **(Figure 2)** (Osterhage & Friedman, 2009; Chow et al., 2012; Pellegrini, 2012). Thus, incomplete lagging strand synthesis, together with pos-replicative resection of the C-rich strand, are essential to generate the G overhangs at both newly synthesized telomeres, while also contributing to telomere shortening with every cell cycle (Lingner et al., 1995; Huffman et al., 2000).

In the absence of a telomere maintenance mechanism, the rate by which telomeres of many eukaryotes (including fungi, trypanosomes, flies, and mosquitos) shorten is at 3-5 bp per cell division, just as expected from the inability of the eukaryotic DNA replication machinery to fully replicate linear DNA molecules (Levis, 1989; Lundblad & Szostak, 1989; Johnson et al., 2001; Walter et al., 2001). However, human and mouse telomeres erode much faster (50-150 bp per cell division), suggesting an active degradation of chromosome ends in these organisms (Harley et al., 1990; Blasco et al., 1997; Niida et al., 1998). Telomere length has been shown to positively correlate with

longevity and healthy life in humans (Njajou et al., 2009; Atzmon et al., 2010). Accordingly, unceasing erosion of the telomeric tract with no counter-balance strategies sets a finite lifespan to human somatic tissues, as critically short telomeres lose their protective function due to reduced shelterin binding and T-loop stability, become dysfunctional and are recognized as DSBs, thus leading to the induction of DNA damage signaling pathways (Harley, 1991; Hug & Lingner, 2006; Palm & de Lange, 2008). As a consequence, accumulation of critically short telomeres and persistent stimulation of the DDR through p53 and Rb signaling triggers the activation of an irreversible cell cycle arrest known as cellular (or replicative) senescence that eventually leads to cell death (**Figure 2**) (Harley et al., 1990; Shay et al., 1991; Lindsey et al., 1991; Allsopp et al., 1992; Fagagna et al., 2003; Zglinicki et al., 2005). Interestingly, four to five critically short telomeres are sufficient to induce senescence in human cells, while in yeast only one is enough (Kaul et al., 2012). Cells may bypass this barrier, leading to continued proliferation and telomere shortening until they undergo a distinct process known as replicative crisis. Unlike cellular senescence, the proliferative capacity of post-senescent cells is not affected by halted division but instead counteracted by extensive death as a result of increased telomere deprotection, leading to the mitotic delay of critically short telomere fusions (Wei & Sedivy, 1999; Hayashi et al., 2015; Nassour et al., 2019). Replicative crisis displays a finely programmed mechanism at telomeres, as it is controlled by telomeric DNA damage signaling (Hayashi et al., 2015). Recent data have shown that telomere dysfunction explicitly stimulates autophagy in epithelial cells and fibroblasts during crisis, thus avoiding further proliferation and oncogenic transformation of pre-cancerous cells (Nassour et al., 2019).

The limited proliferative capacity of somatic cells may seem detrimental to humans and other long-lasting animals due to its role as a genetic “aging time-clock” system that regulates cellular and individual lifespan. However, it also represents one of the most potent molecular barriers against cancer development and progression (Stewart & Weinberg, 2006; Finkel et al., 2007). Indeed, replicative senescence suppresses unlimited cell growth, thus limiting tumorigenesis that could lead to the accumulation of oncogenic mutations that can, over time, progress towards malignancy (Harley, 1991; Kim et al., 1994; Shay & Bacchetti, 1997). In agreement with this, studies show that telomeres are often shorter in tumor samples than in the related healthy tissues (de Lange et al., 1990; Harley et al., 1990). Since cancer cells rely on their immortal potential to maintain aberrant growth, they must overcome this programmed senescence by activating at least one telomere maintenance mechanism to counteract telomere sequence loss (Arora et al., 2014).

1.2 Telomere maintenance mechanisms in cancer

1.2.1 Telomerase

Most cancer cells overcome replicative senescence and divide indefinitely by re-expressing the enzyme telomerase in order to re-elongate or maintain telomeres long enough to avoid DDR (Kim et al., 1994). Telomerase is a specialized ribonucleoprotein complex composed of a highly conserved catalytic subunit with reverse transcriptase activity known as telomerase reverse transcriptase (TERT), and an associated RNA moiety, the telomerase RNA component (TERC, also known as TR or TER). Together, they are capable of compensating telomere erosion by adding TTAGGG-repeat arrays to the 3' ends of each chromosome (Greider, 1990; Smogorzewska & de Lange, 2004; Schmidt & Cech, 2015). To do so, the template sequence of TERC (AAUCCCAAUC in mammals) anneals to the G overhang and TERT promotes reverse transcription, with the PP complex copying the extended telomere terminus to fill in the C-rich strand (**Figure 2**) (Reveal et al., 1997; Palm & de Lange, 2008; Schmidt & Cech, 2015). However, while G-strand extension coincides with telomere replication throughout S phase, C-strand fill-in is delayed until late S phase in a step-wise process different from the standard Okazaki fragment synthesis (Zhao et al., 2009). In *Saccharomyces cerevisiae* (*S. cerevisiae* - budding yeast), it is believed that this, together with the termination of telomerase-mediated telomere extension, is stimulated by the TTAGGG repeat-binding CST complex, which increases at chromosome ends at this stage (Chen, et al., 2012).

Telomerase expression seems to correlate with lifespan, as commonly short-lived beings express telomerase, while long-lived ones only for a limited period of time, which is thought to be a tumor-suppressor mechanism, given their higher vulnerability towards cancer growth (Kim et al., 1994). Consistently, in most unicellular organisms, like yeast, telomerase has a housekeeping function, hence being constitutively expressed (Smogorzewska & de Lange, 2004; Wellinger & Zakian, 2012). By contrast, in humans, while telomerase is present in all cells during the first weeks of fetal development, it is suppressed in terminally differentiated cells of somatic tissues that become susceptible to telomere shortening with age. Nevertheless, telomerase activity can still be found post-development in the germline and high proliferative tissues, such as embryonic stem cells (ESCs) and other stem cell compartments (Allsopp et al., 1992; Kim et al., 1994; Wright et al., 1996; Martínez & Blasco, 2015). This regulation primarily occurs at the transcriptional level of human *TERT* (*hTERT)*, which is expressed at relatively low levels

(even in cancer cells), whereas human TERC (hTERC) exists in an excessive amount and is ubiquitously expressed (Yi et al., 2001; Zhao et al., 2009; Xi & Cech, 2014).

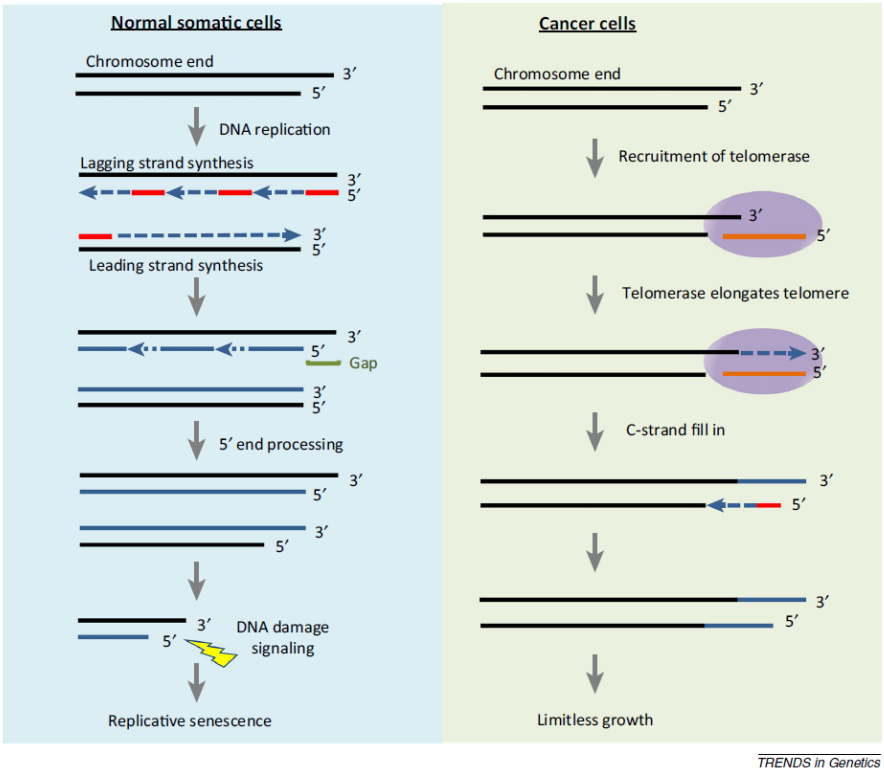

Figure 2 | The end-replication problem in telomerase-negative cells vs telomerase-positive cancer cells

Left panel: In normal somatic cells, telomeres shorten with each cell cycle division due to the inability of the canonical DNA replication machinery to fully duplicate the terminal regions of linear dsDNA molecules. Indeed, the polymerase activity of Polα cannot replace the terminal RNA primer (in red) by newly synthesized DNA during lagging strand synthesis, leading to continuous erosion of the C-rich strand. Furthermore, the blunt-ended duplex DNA molecule generated during replication of the leading strand must undergo through nucleolytic processing to create a mature G overhang, while also contributing to telomere erosion. In the absence of a telomere maintenance mechanism, telomeres become critically short upon successive replication rounds, lose their protective function and start getting recognized as DSBs, thus exposing the chromosome ends to constant DNA damage signaling that eventually leads to cellular senescence and subsequent cell death. Right panel: To obtain their immortal phenotype, cancer cells counteract the end-replication problem by expressing the enzyme telomerase. Telomerase is composed by the reverse transcriptase TERT subunit and the RNA substrate TERC. Together, they are capable of compensating telomere erosion by adding TTAGGG repeats to the G overhang. C strand fill-in is then completed by the PP complex to promote telomere homeostasis and aberrant cancer growth/proliferation. (Adapted from Wong et al., 2014)

Although telomerase is subjected to transcriptional, post-transcriptional and epigenetic control, it is still quite unclear which regulatory pathways are responsible for telomerase suppression during its development and re-expression in carcinogenesis (Yi et al., 2001; Wong et al., 2014). It is suggested that the heterochromatin status of

telomeres regulates the access of telomerase to the chromosome ends, thus acting as a modulator of telomere length homeostasis and structural integrity (Bianchi & Shore, 2008; Schoeftner & Blasco, 2010; Canudas et al., 2011). Indeed, while telomerase does not act on every telomere, being preferentially recruited to the shorter ones in healthy cells, it extends most telomeres at every replication cycle in cancer cells, suggesting differential length regulation, most likely associated to variations in the epigenetic status and regulation of telomere maintenance pathways between the two cell types (Bianchi & Shore, 2008; Zhao et al., 2009; Martínez & Blasco, 2015).

Additionally, telomerase activity is regulated at individual chromosome ends by the shelterin complex to mediate telomere length homeostasis (de Lange, 2005a). The initial model suggested that shelterin (more specifically the direct telomeric DNA-binding proteins TRF1, TRF2, and POT1) mediated a *cis*-regulatory negative feedback loop that counteracted excessive telomere elongation. This negative regulation is based on the ability to count telomeric repeats according to the amount of telomere-bound shelterin factors, which proportionally inhibits telomerase activity and the subsequent extension of chromosome ends (van Steensel & de Lange, 1997; Smogorzewska et al., 2000; Loayza & De Lange, 2003). Consistent with this, several telomerase-positive cell lines have stable telomere lengths that are maintained within a restricted range (Counter et al., 1992; van Steensel & de Lange, 1997). Therefore, telomere length would be assessed by the occupancy of TRF1 and TRF2 at the telomeric dsDNA, although it is quite unclear how it could affect the gradual elongation of chromosome ends, given the considerable distance of both proteins from telomerase and its active substrate, the G overhang. For this, POT1 has been considered a key regulator of telomere length, as it is the only shelterin factor able to bind to the 3' ends of telomeres and thus directly inhibit telomerase activity (Smogorzewska & de Lange, 2004; Palm & de Lange, 2008). Consistently, reduced loading of POT1 or substitution by the POT1$^{\Delta OB}$ mutant lacking the DNA-binding domain was shown to promote telomerase-mediated telomere elongation (Loayza & De Lange, 2003). Additionally, TRF1 depletion led to POT1 removal from the chromosome ends, but the increment in the presence of both of these components was associated with longer telomeres. This suggested that POT1 not only is recruited by the TRF1 complex, but the variation of telomere-bound POT1 was also proportional to the existing amount of TTAGGG repeats, which could be used as a measurement mechanism of telomere length (Smogorzewska & de Lange, 2004; Palm & de Lange, 2008). In conclusion, TRF1 and TRF2-associated control of telomere length could regulate POT1 loading at the telomeric ssDNA, which would then act as a terminal

transducer that limited unwarranted telomere extension by directly restricting telomerase access to the 3' ends of long telomeres (Loayza & De Lange, 2003).

There are two current models to explain the inhibitory role of POT1 towards telomerase engagement to the G overhang. Firstly, it was observed *in vitro* that POT1 could directly compete with telomerase for the G overhang and thereby bind with affinity to this region, blocking the access to the substrate for telomerase-mediated telomere elongation (Kelleher et al., 2005; Lei et al., 2005). Secondly, telomerase access to the telomere terminus can also be blocked through the rescue of the G overhang by the structural configuration of T-loops, which can be further stabilized by POT1 via potential binding to the displaced ssDNA moiety of the D-loop (Loayza et al., 2004). Either way, both models could justify the inability of POT1 to control telomere length when overexpressing the $POT1^{\Delta OB}$ mutant. Even though this truncated variant can still be recruited to telomeres, it cannot inhibit telomerase due to its severely compromised single-stranded DNA-binding activity (Loayza & De Lange, 2003). Furthermore, POT1 depletion in telomerase-positive tumor cells results in rapid telomere elongation (Ye et al., 2004).

Additional data indicate that the influence of shelterin over telomerase-dependent telomere length homeostasis extends over the ability to block telomerase access to the G overhang. Besides POT1 and TRF1, other studies have also detailed the roles of TIN2 and TPP1 as negative regulators of telomerase-mediated telomere elongation (Kim et al., 1999; Liu et al., 2004; Ye, et al., 2004). For instance, TPP1, while tethered to TIN2, is involved in the recruitment and processivity of telomerase when interacting with POT1 via the surface of its telomerase-interacting OB fold, named the TEL patch (Abreu et al., 2010; Nandakumar et al., 2012; Pike et al., 2019). This suggests that TPP1 binding of telomerase, alongside POT1, can also be a limiting factor of telomerase activity and thus a potential effector of the cis-regulatory negative feedback loop on telomere length. However, other than the impact of the TPP1 TEL patch, the contributions of the shelterin complex to telomerase recruitment and engagement are still not fully understood. It is believed that the shelterin complex could facilitate telomerase targeting to the G overhang over the internal telomeric repeats. In contrast, telomerase could engage the chromosome 3' ends after being recruited internally into the telomeres, but only activated once the substrate 3'-OH is found, possibly through translocation of TPP1-POT1 along the telomeric ssDNA (Hwang et al., 2012; Hockemeyer & Collins, 2015).

Because telomerase is a commonly found feature in cancer cells, it is acknowledged as a promising target for the development of highly specific anti-cancer therapies. This

concept relies on the typically short telomere length of primary cancers and cancer cell lines due to their continued proliferation, which could be targeted via telomerase inhibition to promote cancer cell death while not critically harming other telomerase-dependent tissues with longer telomeres, like germ and stem cells (Wong et al., 2014). Several strategies have been designed to achieve telomerase activity suppression, including antisense oligonucleotides, ribozymes, G-quadruplex stabilizers, natural compounds, small-molecule inhibitors (BIBR1532) and RNA interference (RNAi), and while they demonstrate promising effects *in vitro*, they hardly ever succeed into clinical trials due to lack of potency, low specificity and/or high toxicity (Wright et al., 1996; White et al., 2001; Wong et al., 2014). Additionally, the fact that telomerase is expressed at a remarkably low abundance even in cancer cells has proven to be a substantial obstacle against therapeutic approaches for telomerase inhibition (Wong et al., 2014).

1.2.2 Alternative Lengthening of Telomeres (ALT)

While the majority of human cancers reactivate telomerase to promote telomere length homeostasis, the remaining 10-15% resort to a telomerase-independent Alternative Lengthening of Telomeres (ALT) mechanism (Kim et al., 1994; Henson & Reddel, 2010; Arora et al., 2014). ALT relies on HDR to maintain telomeres, and several HDR proteins have been identified at ALT chromosome ends, with their functional inactivation leading to telomere shortening and eventual cell cycle arrest and death (Muntoni & Reddel, 2005; Conomos et al., 2013; Draskovic & Londono Vallejo, 2013; Apte & Cooper, 2017). It is suggested that the HR-based telomeric maintenance is mediated by break-induced replication (BIR), which is a conservative repair pathway that relies on a homologous donor template to synthesize new DNA stretches starting from one-ended DSBs and stalled replication forks (Kramara et al., 2018). Additionally, HDR may be induced in ALT telomeres via annealing of the ssDNA from eroded telomeres to extra-chromosomal telomeric repeats (ECTRs) that are partially single-stranded (Kramara et al., 2018).

ALT is found in an increasing number of aggressive cancers, being most common in specific subtypes of soft tissue sarcomas with complex karyotypes, central nervous system tumors (including astrocytomas), as well as in neuroblastomas, gastric and bladder carcinomas and in a group of *in vitro* immortalized cell lines (Henson & Reddel, 2010; Heaphy et al., 2011). ALT has also been detected in a small subset of breast carcinomas, although it is rarely reported in other epithelial cancers (Henson & Reddel, 2010). Moreover, ALT-positive malignancies occur with higher frequency at the

mesenchyme, which is suggested to happen due to stricter repression of telomerase at these tissues (Henson et al., 2002). In agreement with this, treatment of telomerase-positive tumors with telomerase inhibitors has been shown to lead to the development of an ALT secondary phenotype in several model systems, including cultured human cancer cells, mice and plants (Růčková et al., 2008; Chen et al., 2010; Hu et al., 2012). The prevalence of ALT varies significantly among these cancers types, and although they are less likely to metastasize, they are also characterized by a poor prognosis when treated within currently existing therapies, probably due to chemoresistance (Onitake et al., 2009; Henson & Reddel, 2010; Venturini et al., 2010; Heaphy et al., 2011). Telomeres in ALT cells display higher levels of DNA damage and replication stress than ALT-negative telomeres (Cesare et al., 2009; Arora et al., 2014; Min et al., 2017), with the epigenetic state of the chromosome ends being a major regulator of this mechanism, since compromised telomeric heterochromatin features may facilitate recombination events and thus ALT progression (Gonzalo et al., 2006; Episkopou et al., 2014). This would explain why most ALT-positive tumors are often found to be inactivated for one or both of the ATP-dependent chromatin remodelers ATRX (Alpha-Thalassemia/mental Retardation X-linked) and DAXX (Death domain-Associated protein-6), which promote heterochromatin formation by establishing a complex that incorporates the histone variant H3.3 into heterochromatic loci, including in telomeres (Heaphy et al., 2011; Lovejoy et al., 2012; Schwartzentruber et al., 2012; Episkopou et al., 2014).

The presence of ALT in cancers has been determined by association with phenotypical markers consistently found in ALT-positive cell lines and tumors. These include: prominent telomere length heterogeneity (from very long to very shorts telomeres), as well as increased chromosome instability; accumulation of ECTRs in the form of double-stranded telomeric circles (t-circles), partially single-strand circular C- and G-rich structures (C- and G-circles) and linear dsDNA, which generate telomeric signals detected outside the telomeres in fluorescence *in situ* hybridization (FISH) on metaphase spreads; extensive telomeric recombination mediated by T-SCEs and telomere elongation through BIR; and clustering of telomeres in ALT-associated promyelocytic leukaemia (PML) nuclear bodies (APBs), detected by telomeric DNA FISH and immunofluorescence (IF) staining for the PML protein, which has been a widely validated method for classification of ALT-positive cancers in fixed human tissues (Tokutake et al., 1998; Henson & Reddel, 2010; Heaphy et al., 2011; Arora et al., 2014). Nevertheless, the C-circle assay has been considered the most convenient and versatile of the ALT assays for clinical use, given the specificity of C-circles as a feature of ALT activity (Henson et al., 2009; Henson & Reddel, 2010). Lastly, ALT cancers have also been

associated with elevated levels of TElomeric Repeat containing RNA molecules, also known as TERRA (see Section 1.3) (Azzalin et al., 2007; Arora et al., 2014).

1.3 TERRA features and functions

Even though mammalian telomeres were thought to be transcriptionally silent due to their heterochromatic state and gene-less nature, studies from our group unveiled that they are in fact transcribed into long noncoding RNA (lncRNA) molecules dubbed as TERRA (Azzalin et al., 2007). TERRA is a nuclear G-rich telomeric RNA primarily transcribed by RNA polymerase II (RNAPII) from the telomeric C-rich strand (**Figure 3**) (Azzalin et al., 2007; Schoeftner & Blasco, 2008; Luke & Lingner, 2009). Although components of the RNAPII holoenzyme have been detected in association with human and yeast telomeres, TERRA synthesis is not entirely abolished upon RNAPII specific inhibition, suggesting that other polymerases may contribute to TERRA transcription (Schoeftner & Blasco, 2008; Azzalin & Lingner, 2015). TERRA is transcribed starting from subtelomeric regions towards chromosome ends, to which it can remain associated (**Figure 3**). For this reason, TERRA harbors an initial subtelomeric RNA sequence followed by a variable number of G-rich telomeric repeats (5'-UUAGGG-3' in vertebrates) (Azzalin et al., 2007; Luke et al., 2008; Azzalin & Lingner, 2015).

So far, TERRA has been characterized as a widely conserved molecule in eukaryotes, with significant heterogeneity in both abundance and length (Azzalin et al., 2007; Schoeftner & Blasco, 2008; Luke & Lingner, 2009; Azzalin & Lingner, 2015). In human cells, TERRA length ranges from 100 bp to 9 kb (Azzalin et al., 2007; Schoeftner & Blasco, 2008), as shown in telomerase-positive HeLa (human cervical carcinoma) cells and primary human lung fibroblasts (HLF) (Porro et al., 2010). Meanwhile, ALT-positive cells comprise much longer TERRA transcripts that most likely include longer tracts of UUAGGG repeats (Arora et al., 2014). Human TERRA molecules are 7-methylguanosine (m^7G) capped at their 5' ends, and around 10% presents 3' end polyadenylation, similarly to *Schizosaccharomyces pombe* (*S. pombe* - fission yeast) (Azzalin et al., 2007; Luke et al., 2008; Porro et al., 2010; Bah et al., 2012). Even though the mechanisms behind TERRA polyadenylation remain to be clarified since a canonical polyadenylation signal is not found at telomeres, it is believed to contribute towards TERRA stability and localization (Porro et al., 2010; Bettin et al., 2019). In agreement with this, polyadenylated TERRA displays a longer half-life (8 hours) and is primarily found diffused in the nucleoplasm, while non-polyadenylated TERRA has a shorter half-

life (3 hours) and is mainly chromatin-associated (**Figure 3**) (Schoeftner & Blasco, 2008; Porro et al., 2010). Even though TERRA is believed to be exclusively nuclear, short species of TERRA transcripts (approximately 200 nt) can also be found in extracellular exosomes from human cell cultures (Wang et al., 2015).

Several studies using telomeric repeat-specific reverse transcription and subtelomere-specific PCR show that TERRA is expressed from multiple telomeres in human cells (Nergadze et al., 2009; Porro et al., 2010; Farnung et al., 2012; Feretzaki & Lingner, 2017; Feretzaki et al., 2019). Also, about half of human chromosome ends comprise CpG island-containing TERRA promoters in tandem repeats of 29 and 37 base pairs (**Figure 3**), located in the proximity of telomeric sequences and at variable distances from the telomeric tract (Nergadze et al., 2009). Approximately half of subtelomeres have also been shown to comprise binding motifs for the chromatin organizing factor CTCF (CCCTC-binding factor), the nuclear respiratory factor 1 (NRF1) and the heat shock factor 1 (HSF1), which play essential regulatory roles in TERRA transcription (discussed at Section 1.3.1). While most CTCF and NRF1 binding sites colocalize with the CpG dinucleotide-rich TERRA promoters, the HSF1 counterpart is found at about 1 to 2 kb upstream of these regions (Deng et al., 2012; Diman et al., 2016; Koskas et al., 2017). More specifically, CTCF, together with the cohesin subunit Rad21 (radiation-sensitive 21), promote TERRA expression (**Figure 3**) (Deng et al., 2012), and have been implicated in the regulation of genome folding, which thereby facilitates telomere integrity, transcription, and replication (Merkenschlager & Nora, 2016; Beishline et al., 2017). CTFC depletion not only leads to decreased Rad21 and RNAPII binding to TERRA promoters and diminished TERRA levels but also stimulates the activation of DDR at chromosome ends and consequent formation of “Telomere dysfunction-induced foci” (TIFs) (Deng et al., 2012). For that reason, CTCF and Rad21 may contribute towards chromosome end-protection by recruiting RNAPII to subtelomeres and promoting TERRA transcription (Deng et al., 2012; Porro et al., 2014).

The finding of telomere transcripts at chromosome ends creates a new perspective on how telomeres may be established and regulated. Although a full characterization of TERRA functions is lacking, rising data shows that it sustains telomere protection and replication, heterochromatin formation, and regulation of telomerase activity (Luke & Lingner, 2009; Balk et al., 2013; Porro et al., 2014; Montero et al., 2016).

1.3.1 TERRA and telomere stability

An increasing amount of evidence shows that TERRA actively contributes towards telomere function and homeostasis through associated regulatory mechanisms. Indeed, deregulation of TERRA expression has been observed during DDR-mediated establishment of TIFs and dysfunctional telomeric DNA replication (Bettin et al., 2019). Additionally, TERRA expression is controlled by tumor suppressor genes, such as p53 and Rb, thus suggesting that its transcription can be a central trigger of tumorigenesis (Tutton et al., 2016; Gonzalez-Vasconcellos et al., 2017). Therefore, the physical bond of TERRA with chromosome ends and its close relation with telomere stability suggests a fundamental and specialized regulation of this lncRNA in avoiding detrimental effects against chromosomes and the telomeric complex (Azzalin & Lingner, 2015).

TERRA expression is regulated in a cell cycle-dependent manner. Human cells, including telomerase-positive ones, exhibit the highest TERRA levels in G_1, from where they begin progressively decreasing until reaching their lowest state at the transition between late S and G_2 phases, to then start recovering through G_2/M (Porro et al., 2010; Arnoult et al., 2012). This effect is not observed in ALT-positive cells, possibly due to a lack of ATRX activity (Porro et al., 2010; Flynn et al., 2015). This regulation is associated with the chromatin status of telomeres and subtelomeric tracts, given that TERRA transcriptional activity is repressed when the DNA methyltransferases DNMT1 and DNMT3b methylate the CpG islands at TERRA promoters, thus stimulating heterochromatin formation at these regions (**Figure 3**). Derepression, alongside telomere shortening, is achieved upon the simultaneous depletion of both enzymes (Yehezkel et al., 2008; Nergadze et al., 2009). On the other hand, TERRA expression is stimulated by histone acetylation; consistently, TERRA levels increase with treatment with Trichostatin A, which is a histone deacetylase inhibitor (Azzalin & Lingner, 2008). TERRA transcription is also negatively regulated by the H3K9 histone methyltransferase SUV39H1 and recruitment of the H3K9me3 binding protein HP1α (heterochromatin protein 1 alpha), thus suggesting that it is directly influenced by DNA methylation and histone modifications (**Figure 3**) (Arnoult et al., 2012). Interestingly, TERRA has been proposed to be a regulator of heterochromatin formation since it not only interacts with both the above-mentioned proteins but also with several other heterochromatic marks and chromatin modulators; this implies the existence of a regulated negative feedback loop through which TERRA suppresses its own transcription (Arnoult et al., 2012; Porro et al., 2014).

Additional evidence indicates that TERRA accumulation at human telomeric heterochromatin is also controlled by the nonsense-mediated RNA decay (NMD) machinery, which is a highly conserved eukaryotic RNA quality control pathway that targets abnormal mRNA molecules for degradation (Azzalin et al., 2007; Behm-Ansmant et al., 2007). NMD is mediated by SMG (Suppressors with Morphogenic defects in Genitalia) proteins, of which the RNA and DNA helicase and ATPase UPF1 (or SMG2), the RNA endonuclease hEST1A (or SMG6), and the protein kinase SMG1 had been previously indicated to promote genome integrity via NMD-independent mechanisms (Brumbaugh et al., 2004; Reichenbach et al., 2003; Azzalin & Lingner, 2006). The direct association of these NMD effectors with telomeres *in vivo* physically displaces TERRA from telomeric heterochromatin, thereby assuring telomere integrity (Reichenbach et al., 2003; Brumbaugh et al., 2004; Azzalin & Lingner, 2006). Consistent with this, further analysis of their depletion in human cells led to the stochastic loss of entire telomeric tracts (**Figure 3**) (Azzalin et al., 2007).

A role of the shelterin complex in TERRA regulation has also been demonstrated. Specifically, TRF1 and TRF2 physically interact with TERRA molecules, with TRF2 possibly anchoring them to chromosome ends (**Figure 3**) (Deng et al., 2009). A recent study from our group has demonstrated that TRF2, through its N-terminal B domain, also facilitates intramolecular invasion of TERRA into telomeric dsDNA *in vitro*, which is counteracted by TRF1 (Lee et al., 2018). Consistently, the expression of a TRF2 variant lacking the B domain (TRF2ΔB) impairs TERRA localization at telomeres in U2OS cells (Deng et al., 2009). Also, *in vivo* TRF1 depletion or replacement with a variant lacking its N-terminal A domain (TRF1ΔA) leads to TRF2-induced formation of TERRA-telomeric DNA hybrids, which in turn causes telomere loss (Lee et al., 2018). TERRA may favor telomere protection by averting the activation of DDR through the proper assembly of telomere-binding proteins at chromosome ends or by telomere capping (Bettin et al., 2019). Indeed, *in vitro* evidence suggests that TERRA may associate with hnRNPA1 (A1), which retains a high affinity towards both single-stranded TTAGGG DNA and UUAGGG RNA repeats, to promote POT1-TPP1 binding to telomeric ssDNA after displacing its natural competitor RPA from the same regions. This way, the TERRA-hnRNPA1 complex coordinates RPA-to-POT1 switch after DNA replication (Nandakumar et al., 2010; Flynn et al., 2011; Redon et al., 2013).

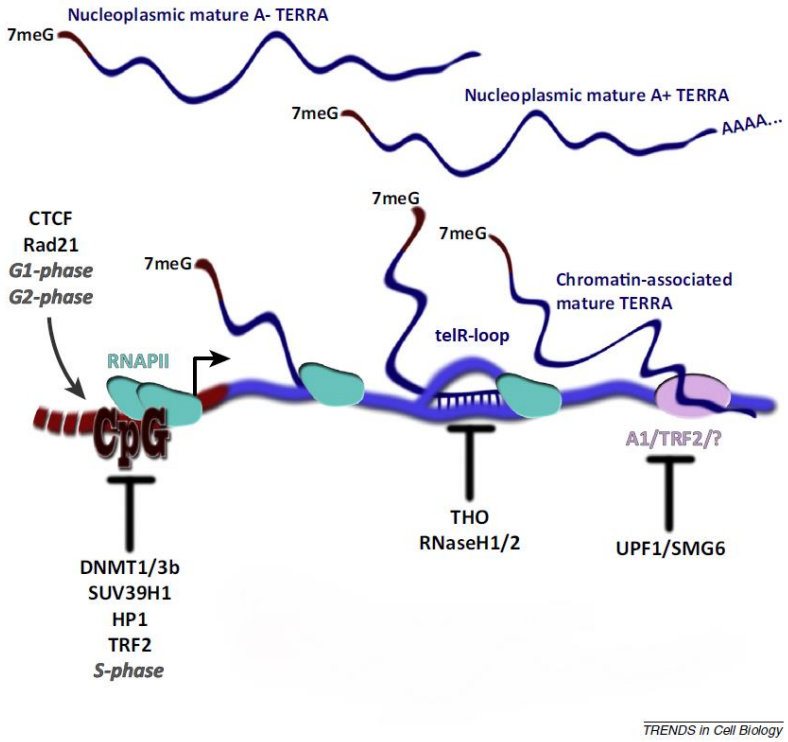

Figure 3 | TERRA as a promoter of telR-loop formation and associated regulatory factors

TERRA is a nuclear G-rich telomeric RNA transcript primarily transcribed by RNAPII from CpG dinucleotide-rich promoters located at subtelomeric regions and towards the C-rich strand of telomeres. TERRA is transcribed from multiple chromosome ends in human cells, being highly heterogenous in abundance and length of UUAGGG repeats. It also comprises an N-terminal m^7G cap, as well as a 3' polyadenylation tail in a very small percentage of total TERRA transcripts, which tend to be more chemically stable and found diffused in the nucleoplasm, contrary to the non-polyadenilated majority, which is normally chromatin-associated. Human TERRA expression peaks at G_1 and slowly decreases during S phase, until reaching its lowest level at G_2/M. CTCF and Rad21 stimulate TERRA transcription at its promoters, while DNMT1/3b, SUV39H1, HP1α, and TRF2 inhibit it. HnRNPA1 (A1), TRF2 or other unknown components may promote TERRA association with telomeres, which is efficiently suppressed by the NMD factors UPF1 and SMG6. Studies have described the ability of TERRA to invade the telomeric dsDNA to form a three-stranded structure known as telR-loop. Formation of telR-loops may be resolved through the hydrolytic activity of both Rnases H1 and H2, and by the THO multiprotein complex. (Adapted from Azzalin & Lingner, 2015)

Finally, new data uncovered novel pathways by which TERRA transcription may sustain telomere integrity under different sources of cellular stress. On one hand, the HSF1 protein directly upregulates TERRA expression when bound to subtelomeres during stress conditions (e.g., heat shock) in human cells. HSF1 knockout cells do not present increased levels of TERRA transcripts during heat shock events but do manifest enhanced DNA damage at telomeres and TIF formation when compared to wild-type (WT) cells cultured in the same growing conditions (Koskas et al., 2017). On the other side, TERRA transcription and telomeric localization are enhanced in human muscle tissues during endurance exercise by the antioxidant transcription factor NRF1, together

with the peroxisome proliferator-activated receptor coactivator 1α (PGC-1a), which is activated during exercise through the upregulation of the 5'-monophosphate (AMP)-activated protein kinase (AMPK) (Diman et al., 2016). Since telomeric DNA is particularly sensitive to oxidative damage due to its high-content guanine composition that can be oxidized to 8-oxo guanines (8-oxoG) and hinder telomere stability (Oikawa & Kawanishi, 1999), TERRA may safeguard chromosome ends by positively influencing an antioxidant response in skeletal muscles during endurance exercise (Diman et al., 2016). In support of this hypothesis, a recent study has detailed the role of TERRA in containing telomeric DNA damage induced by reactive oxygen species (ROS) in a BIR-mediated manner (Tan et al., 2019).

1.3.2 The role of TERRA in the regulation of telomerase activity

To date, all studies focusing on TERRA in telomerase-positive human cancer cells have stated the presence of TERRA expression at several chromosome ends (Nergadze et al., 2009; Arnoult et al., 2012; Farnung et al., 2012; Arora et al., 2014; Porro et al., 2014). Nevertheless, relatively limited and somewhat conflicting data have been described concerning TERRA and its role in the regulation of telomerase activity and recruitment to telomeres in humans. Since the UUAGGG repeats of the TERRA sequence are complementary to the RNA template region of telomerase TERC, TERRA transcripts can associate with it through base-pairing while also interacting with the TERT subunit without actively extending TERRA (Redon et al, 2010). Thus, TERRA was shown to be a natural ligand and direct inhibitor of human telomerase (Schoeftner & Blasco, 2008; Redon et al., 2010). However, *in vivo* evidence demonstrates that the upregulation of TERRA does not restrict telomerase-mediated telomere extension. Overexpression of TERRA in cells deficient for the two DNA methyltransferases DNMT1 and DNMT3b, as well as experimentally stimulated telomere transcription from transcriptionally inducible telomeres (tiTELs), did not interfere with telomerase activity (Farnung et al., 2012). It has been suggested that TERRA association with TERRA-binding proteins (e.g., hnRNPA1) could obstruct the interaction with telomerase and therefore its inhibition (de Silanes et al., 2010; Redon et al., 2013). Yet, no correlation was found between the variable telomere lengths of clones from different human cancer cell lines and TERRA levels (Smirnova et al., 2013).

Pioneering studies in budding and fission yeast show that short telomeres induce TERRA expression to mediate the recruitment and activity of telomerase for *in cis*

telomere extension (Cusanelli et al., 2013; Moravec et al., 2016). These high levels of transcription coincide with the early S phase of the cell cycle, where TERRA nucleates the RNA component of telomerase TLC1, thus generating TERRA-telomerase clusters known as T-Recs (telomerase recruitment clusters) that then associate with telomeres in late S phase to possibly promote their elongation (Gallardo et al., 2011; Cusanelli et al., 2013). Since the formation of these clusters requires telomerase-dependent factors involved in telomere lengthening, it is believed that T-Recs represent the active form of telomerase (Cusanelli et al., 2013). Curiously, they also seem to colocalize with the shortened telomeres from where TERRA was initially originated, suggesting that TERRA plays a role in the localization of telomerase activity by guiding it to its transcription origin (Cusanelli et al., 2013; Azzalin & Lingner, 2015). Conversely, other reports propose that TERRA accumulation at critically short telomeres of the budding yeast is not a direct result of increased telomere transcription, but rather a consequence of the deregulation of TERRA degradation throughout the cell cycle due to the inactivation of the 5'-3' exonuclease Rat1, which actively degrades TERRA molecules (Luke et al., 2008; Iglesias et al., 2011; Graf et al., 2017). Either way, it remains to be uncovered how TERRA dissociates from telomerase within T-Recs to allow telomerase association and activity at telomeres.

As mentioned before, the subsequent accumulation of TERRA promotes telomerase recruitment and activity and coordinates telomerase-mediated elongation of the telomeres from which the TERRA molecules originate (Gallardo et al., 2011; Cusanelli et al., 2013; Moravec et al., 2016). Given that telomerase was only found to associate with polyadenylated TERRA transcripts (Cusanelli et al., 2013; Moravec et al., 2016), it was important to assess the basis of this molecular interaction, since most of the TERRA fraction in *S. pombe* is non-polyadenylated. As such, we have proposed that manipulating the 3' ends of TERRA transcripts by transcription termination may regulate telomerase activity in this model, and possibly also in human cells. Indeed, continued telomere erosion and release of Rap1 induced the transcription of G-rich TERRA, which exerted an inhibitory effect on telomerase by competing with telomeres over TERC. However, the cleavage and polyadenylation of these molecules would generate polyadenylated TERRA with few to no telomeric sequences, which interacted with TERT and promoted telomerase recruitment and nucleation at the transcribed short telomere to specifically elongate it (Moravec et al., 2016).

1.4 R-loops

R-loops are three-stranded byproducts of transcription that are formed when an RNA molecule anneals co- or post-transcriptionally to its antisense or template DNA strand, thereby generating an RNA-DNA hybrid and a displaced non-template ssDNA (Aguilera & García-Muse, 2012). R-loops occur naturally along the genome and have been classified as potent regulators of gene expression and genome stability, but their regulation is still a poorly understood feature of molecular biology (Skourti-Stathaki & Proudfoot, 2014; Costantino & Koshland, 2015). The most prevalent model of R-loop formation, known as the thread back model, suggests that the ability of the transcribing RNA polymerase to generate negatively supercoiled dsDNA behind itself facilitates DNA unwinding, thus allowing RNA invasion and the possible formation of an R-loop with its template strand (Liu & Wang, 1987; Roy et al., 2008). Although this model explains high levels of RNA-DNA hybrids in mutants defective in transcription elongation, termination, splicing, and relaxation of supercoiled DNA, it does not directly explain the accumulation of R-loops in mutants affecting the post-transcriptional processing of RNA. Yet, it has been proposed that such mutations could lead to a longer retention of RNA transcripts in the nucleus and therefore turn them more available to possibly hybridize with a complementary DNA sequence, even after transcription (Costantino & Koshland, 2015).

Besides supercoiled DNA, there are other features that favor RNA-DNA hybrids formation and stability *in vitro*. For instance, R-loops have higher thermodynamic stability than naturally occurring dsDNA, mainly due to their increased GC content and unique conformation that is neither the one present exclusively at DNA nor RNA molecules (Roberts & Crothers, 1992; Roy et al., 2010). Although recent reports in yeast genome-wide profiling of hybrids have shown a weak correlation between GC content and hybrid formation, further studies *in vivo* are still required to effectively address the importance of the nucleic acid composition at these structures (Chan et al., 2014; El Hage et al., 2014; Costantino & Koshland, 2015). Additionally, the high GC skew, particularly with guanosine enrichment in the non-template DNA and RNA strands (Roy & Lieber, 2009), the presence of DNA nicks (Roy et al., 2010), and the propensity of the displaced ssDNA strand to generate G4 structures may also contribute towards R-loop formation (Duquette et al., 2004).

R-loops can also be formed *in trans* since an RNA transcript can localize to another locus, where it hybridizes with a homologous DNA sequence. This process was shown to be mediated by the HDR factors Rad51 and Rad52. However, it is still unclear if R-loops promote standard recombination via RNA-strand invasion into a duplex DNA

structure (Wahba et al., 2013; Costantino & Koshland, 2015). Even so, the ability of R-loops to target several other loci with sequence similarity to its transcription origin further supports an important function across the genome (Costantino & Koshland, 2015).

1.4.1 The impact of R-loops on genome structure and function

For years, the negative role of R-loops has been emphasized due to their hazardous potential against genomic stability. Studies in both yeast and mammalian cells demonstrated that RNA-DNA hybrids can trigger cell cycle checkpoint activation, DNA damage and chromosome rearrangements through a considerable range of different mechanisms (**Figure 4, upper half**) (Li & Manley, 2005). The chemical instability of the displaced ssDNA stretches formed at these structures is associated with increased transcription-associated mutagenesis (TAM) and recombination (TAR), and DNA damage, which eventually leads to DSBs (Li & Manley, 2005; Muers, 2011; Wimberly et al., 2013). Indeed, most of these mutations are primarily targeted towards the non-complementary strand rather than the DNA template annealing with RNA by a variety of DNA and RNA modifying enzymes (Beletskii & Bhagwat, 1996). In mammals, these include the activation-induced cytidine deaminase (AID) and members of the ApolipoproteinB mRNA-editing polypeptide (APOBEC), which can promote deamination and generate base substitutions or ssDNA nicks that then progress into DBSs upon encountering the replication fork (Dickerson et al., 2003; Nabel et al., 2014; Skourti-Stathaki & Proudfoot, 2014). Further studies have described the role of replication-coupled nucleotide-excision repair (NER) endonucleases in processing R-loops into DSBs in human and yeast cells (Sollier et al., 2014). Several studies also suggest that R-loop formation promotes genome instability through replication fork stalling, which causes a direct and inevitable conflict between the DNA replication and transcription machineries, thus leading to fork collapse and subsequently to DSBs (Tuduri et al., 2009; Gan et al., 2011; Alzu et al., 2012; Hamperl et al., 2017). Finally, the RNA segment of R-loops can function as a primer to promote unscheduled replication or possible re-replication. In agreement with this, bacteria have been shown to use them as sites of replication initiation (Kogoma, 1997).

Despite the detrimental effects of R-loops, they also exert multiple positive physiological outcomes that have already been described in different settings (**Figure 4, bottom half**). The most highlighted function is associated with immunoglobulin class switch recombination (CSR) in activated B-cells of mammals. Transcription-mediated R-loop formation and accumulation throughout the switch immunoglobulin locus makes the

ssDNA substrate available for the AID enzyme, from which deoxyuridine residues are processed into DSBs, leading to the genomic rearrangements responsible for the production of the different classes of Ig isotypes (Roy et al., 2008). R-loops are also described as genome-wide transcription regulators since they can promote gene expression through modulation of DNA methylation at human CpG island-containing promoters. Indeed, RNA-DNA hybrids have been reported within a subset of these regions as a way of protection against the methyltransferase DNMT3B in order to sustain gene transcriptional activity and their unmethylated state (Ginno et al., 2012; Grunseich et al., 2018). Additionally, R-loops regulate the chromatin status of chromosomes, as they have been implicated in the recruitment at G-rich transcription-termination sites of numerous epigenetic marks associated with H3K9me2 heterochromatin formation *in cis* via transcription of antisense RNA, which in turn assists towards efficient transcription termination (Skourti-Stathaki et al., 2014). Moreover, R-loops promote RNAPII pausing at these regions, which in turn recruits Senataxin (SETX) to induce Xrn1-mediated transcription termination. Thus, the formation of RNA-DNA hybrids might be involved in chromatin modulation at the promoter, gene body, and terminator sites as an extensive regulator of gene expression levels (Skourti-Stathaki et al., 2011, 2014). Further proof of R-loops association with the chromatin status comes from the correlation between their accumulation and the presence of the H3S10P heterochromatin mark in both yeast and humans, which is linked to chromatin condensation (Castellano-Pozo et al., 2013). Finally, RNA-DNA hybrids can counteract DNA damage by mediating DNA repair through BIR and HDR (Amon & Koshland, 2016). R-loops have also been shown to stimulate ATR activation at centromeres during mitosis to activate Aurora B kinase and promote faithful chromosome segregation (Kabeche et al., 2018).

1.4.2 R-loop regulation

The dual role inherently involved in the biological activity of R-loops towards genome integrity is expected to be tightly regulated at the cellular level by many distinct factors. It is then crucial that these regulatory processes create a balance between R-loop dissolution when negatively affecting genome structure and function, and their protection to conceivably enhance the associated positive effects.

Topoisomerases are one of the strategies employed by cells to inhibit R-loop formation due to their ability to alleviate the torsional stress applied by the DNA transcription machinery, which may lead to RNA strand invasion into the dsDNA. In yeast, Top1 and Top2 depletion results in R-loop accumulation in the ribosomal DNA

(rDNA), which can cause RNA polymerase I (RNAPI) stalling and defective pre-ribosomal RNA (rRNA) synthesis (El Hage et al., 2010). In human cells, defects in TOP1 increase DNA breaks at highly transcribed genes (Tuduri et al., 2009). TOP3B is also able to selectively relax negatively supercoiled DNA and therefore repress R-loop formation and chromosomal translocations in both humans and mice (Yang et al., 2014).

Regulators of messenger ribonucleoprotein particle (mRNP) formation are also involved in R-loop expression blockage, possibly by preventing the annealing of RNA transcripts to the template DNA strands. These include the THO/TREX complex (**Figure 3**) and ASF/SF2 (alternative splicing factor/splicing factor 2), also known as SRSF1 (serine/arginine-rich splicing factor 1), which have also been shown to control co-transcriptional splicing (Huertas & Aguilera, 2003; Li & Manley, 2005; Domínguez-Sánchez et al., 2011).

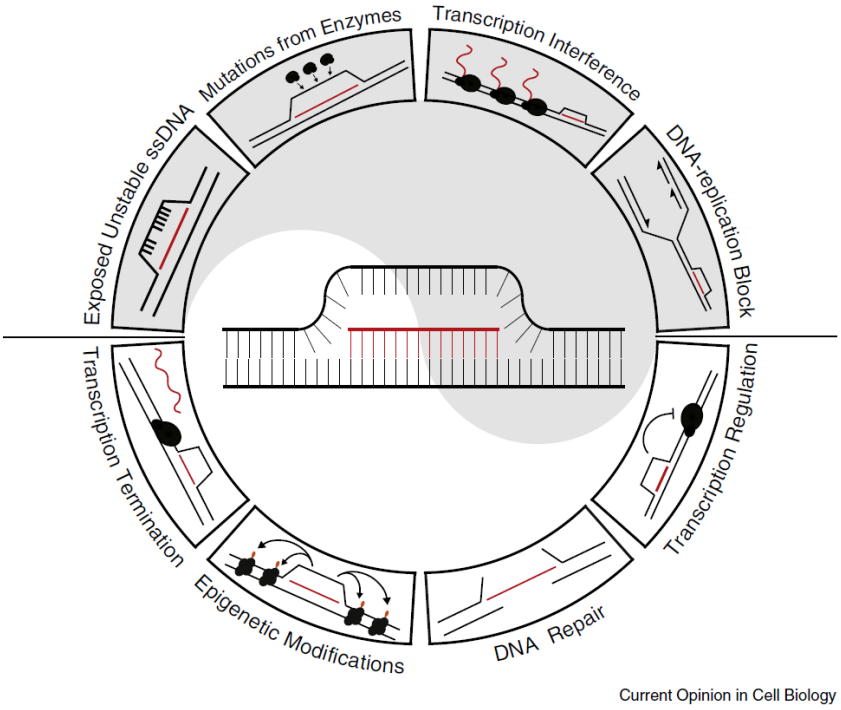

Figure 4 | The positive and negative impact of R-loops in the genome

R-loops are three-stranded byproducts of transcription comprising an RNA-DNA hybrid and a displaced ssDNA strand. Upper half: R-loop formation may exert detrimental effect against genome structure and function. The displacement of the non-template DNA strand is highly unstable and prone to exposure to mutagenic and recombination factors; the R-loop structure naturally interferes with the replication and transcription machineries, leading to replication fork stalling and possible collapse. Lower half: the presence of R-loops may also present multiple and positive physiological roles, which includes the promotion of efficient transcription regulation and termination, modulation of the chromatin status and subsequently gene expression, and stimulation of DNA repair of DSBs. (Adapted from Constantino & Koshland, 2015).

Many other factors have since been identified that can suppress R-loops, thus reflecting the diversity in strategies that cells may employ to regulate these structures. They include RNA helicases capable of unwinding these structures, like SETX (see Section 1.4.1), DDX23 (DEAD box protein 23), which has been suggested to resolve R-loops at transcription pause sites, and AQR (Aquarius), which processes R-loops in a still undetermined manner (Skourti-Stathaki et al., 2011; Sollier et al., 2014; Sridhara et al., 2017). Furthermore, the ATPase/translocase activity of FANCM (Fanconi anemia, complementation group M) has been shown to resolve RNA-DNA hybrids genome-wide, and R-loops accumulate in FANCM-deficient cells (Schwab et al., 2015). Lastly, R-loop suppression may also be achieved by a class of Ribonuclease H (RNase H) enzymes.

1.4.3 Ribonucleases H and their impact on R-loops

RNase H is a class of endonucleases that specifically hydrolyze the RNA moiety of an RNA-DNA hybrid with limited sequence preference (Nowotny et al., 2008; Cerritelli & Crouch, 2009). Two distinct, evolutionary conserved RNase H proteins have been identified from bacteria to humans - RNases H1 and H2. Both present similar overall structure but are distinguishable in specific biochemical properties, including different active site configurations and substrate preference (Ohtani et al., 1999; Nowotny et al., 2008).

In all eukaryotes and some bacteria, RNase H1 is a monomeric enzyme which, in humans, is composed by an N-terminal double-stranded RNA (dsRNA) and RNA/DNA hybrid-binding domain (HBD) of approximately 50 amino acids, which enhances by 25-fold RNase H1 processivity and affinity for RNA-DNA hybrids over dsRNA (Cerritelli & Crouch, 1995; Gaidamakov et al., 2005; Nowotny et al., 2008). Importantly, it can only recognize hybrids of at least four ribonucleotides (Cerritelli & Crouch, 2009). At the C-terminal region, RNase H1 contains an approximately 150 residue-long catalytic domain responsible for catalyzing the cleavage of RNA, which is tethered to HBD via a flexible polylinker of variable length (Cerritelli & Crouch, 1995). The active site was predicted to be formed by four conserved amino acids - D145, E186, D210, and D274 -, of which the former three were confirmed by mutagenesis studies to be vital for the enzymatic activity (Wu et al., 2001). In unicellular organisms, RNase H1 depletion only slows cell growth rate with no lethal effects, most likely because the RNA counterpart of RNA-DNA hybrids may be processed by other nucleases (Nowotny et al., 2008). However, in higher eukaryotes, it has been deemed essential for mitochondrial DNA replication, since RNase H1 knockout mice die during embryogenesis due to the inability of amplifying

mitochondrial DNA (Cerritelli et al., 2003). Furthermore, RNaseH1 has been found to associate with ALT telomeres and regulate the levels of telomeric RNA-DNA hybrids as a way to control their recombinogenic ability (Arora et al., 2014).

In contrast to bacteria, RNase H2 is composed of three subunits, Rnh201 (catalytic subunit), Rnh202 and Rnh203 in *S. cerevisiae*, and RNASEH2A, RNASEH2B and RNASEH2C in humans (Lockhart et al., 2019). Besides being able to degrade long RNA-DNA hybrids, RNaseH2 can remove RNA primers from Okazaki fragments during lagging strand synthesis and perform ribonucleotide excision repair (RER), whereby single ribonucleoside monophosphates (rNMPs) misincorporated into DNA during replication are removed and the remaining nick gets repaired (Eder et al., 1993; Qiu et al., 1999; Hiller et al., 2012; Williams et al., 2016). The loss of RNase H2 results in a stronger genome instability than RNaseH1, as it accounts for most of the RNase H activity in the cell. In agreement with this, RNASEH2B and Rnh202 may be partially involved in DNA replication by interacting with the DNA polymerases clamp loader PCNA via their PIP-box domain located at the C-terminal region (Chon et al., 2009; Nguyen et al., 2011; Lockhart et al., 2019).

Both RNases contribute to the suppression of R-loops in the genome, thus reflecting the non-redundant functions of these enzymes (Nguyen et al., 2017a). Consistently, an increase in the levels of RNA-DNA hybrids is observed upon their concomitant depletion, suggesting that they can replace each other's catalytic activity (Wahba et al., 2011). RNase H1 overexpression is sufficient to reduce R-loops and associated genomic instability (Paulsen et al., 2009; Stirling et al., 2012). This is due to the fact that while RNase H2 is only able to process R-loops post-replicatively (i.e. during the G_2 phase), RNase H1 activity is not dependent on cell cycle regulation, although it requires R-loop-induced stress to trigger its accumulation onto chromatin (Lockhart et al., 2019). Besides, RNase H enzymes are not essential in yeast, but recent evidence correlates their activity with the efficient repair of DNA damage originated from R-loops, in a process that most likely requires the removal of these hybrids to avoid the highly mutagenic BIR-mediated repair pathway (Amon & Koshland, 2016).

1.4.4 Telomeric R-loops (telR-loops)

Both yeast and humans have been proposed to hold RNA-DNA hybrids at telomeres, known as telomeric R-loops (telR-loops), and thus containing TERRA (Balk et al., 2013; Arora & Azzalin, 2015; Rippe & Luke, 2015; Sagie et al., 2017). The first evidence came from the fact that TERRA colocalized with Rap1 in human cells, suggesting that it could associate with telomeres (Azzalin et al., 2007), and that its levels were reduced via RNase H2 overexpression in *rat1-1* yeast mutants (Luke et al., 2008). R-loops can be directly detected at telomeres by resorting to the S9.6 antibody, which specifically recognizes RNA-DNA hybrids of at least 6 to 8 bp (Phillips et al., 2013). More recently, and as mentioned before, our group has unveiled the role of TRF2 in stimulating *in vitro* TERRA invasion into the telomeric duplex DNA by directly binding its N-terminal basic domain to these transcripts, which raised the possibility that TRF2 promotes telR-loop formation independently of ongoing transcription (Lee et al., 2018).

Formation of telR-loops is implicated in telomere maintenance and genome stability through multiple mechanisms, which include the promotion of HDR (Balk et al., 2013; Arora et al., 2014; Lombraña et al., 2015; Ohle et al., 2016), chromatin regulation (Castellano-Pozo et al., 2013; Skourti-Stathaki et al., 2014; Grunseich et al., 2018), and telomeric DNA replication (Lombraña et al., 2015). They may also stimulate homologous recombination between telomeres, which can keep DNA replication fork from collapsing and becoming dysfunctional (Lombraña et al., 2015; Ohle et al., 2016; Ait Saada et al., 2018).

In yeast, telR-loops have been extensively studied in telomerase-negative pre-senescent cells, where their accumulation at critically short telomeres has been shown to activate DDR and subsequently promote telomere elongation via Rad51-mediated HDR, therefore compensating the progressive loss of chromosome ends and preventing early senescence onset (Balk et al., 2013; Graf et al., 2017). How exactly R-loops promote recombination at telomeres remains to be described, but it has been proposed to depend on a regulatory response stimulated by replicative stress (Balk et al., 2013). Moreover, only a small amount of yeast TERRA transcripts are engaged in R-loops, which are negatively regulated by the THO complex and both RNases H1 and H2 (Balk et al., 2013; Pfeiffer et al., 2013).

TelR-loops are detected at high levels in human ALT cells, where they have been linked to telomere homeostasis and the recombinogenic activity associated with this pathway. Indeed, telR-loops seem to correlate with sustained physiological damage from replicative stress, which is necessary to promote ALT-mediated telomere elongation via

HDR. However, these levels must be tightly kept within a specific limit, since excessive replication stress may heavily compromise telomere integrity and lead to cell death. RNase H1 is a fine regulator of telR-loops and telomere maintenance in ALT tumor cells. Since it can specifically interact with ALT telomeres, overexpression of RNase H1 leads to weakened recombination and telomere shortening most likely due to inefficient *de novo* synthesis of telomeric DNA, while RNase H1 depletion causes telR-loop accumulation and telomere loss due to abrupt telomere excision (Arora et al., 2014). Furthermore, ATRX upregulation in ALT cancer cells decreases telR-loop formation, which probably explains why most of these cells lose ATRX expression (Nguyen et al., 2017b). Very recently, our group has also reported on the ability of the ATPase/translocase FANCM to control ALT activity and cell proliferation, partly by unwinding telR-loops. Consistently, FANCM depletion leads to sustained replicative stress, exacerbated ALT activity and death specifically in ALT cells (Silva et al., 2019).

1.5 Hypothesis and aims

How telomere stability is maintained remains to be fully understood. The discovery of TERRA has shed some light on this matter, and even though a full characterization of TERRA connections with different telomeric functions and states is still lacking, recent data from several laboratories have suggested the centrality of telR-loops in mediating these processes. Human ALT cancer cells, as well as their yeast counterparts (type II survivors), escape replicative senescence by upregulating telR-loops when telomeres reach critically short lengths as a necessary mechanism to promote recombination-mediated telomere elongation in the absence of telomerase (Balk et al., 2013; Arora et al., 2014; Yu et al., 2014). However, the biological significance of telR-loops is not yet fully understood in both telomerase-positive and negative human cells.

With the ever-growing incidence of human malignancies, predominantly associated with the reactivation of telomerase, and the incessant, yet unsuccessful search for highly-specific telomerase-based anti-cancer therapies (Wright et al., 1996; White et al., 2001; Wong et al., 2014), the rising emphasis on telR-loops opens a new window of opportunity in telomere biology.

Given the above-described roles of telR-loops and their expanding array of functionalities, we hypothesize that they may serve telomere maintenance not only in ALT and yeast cells but also in telomerase-positive cancer and/or primary human cells.

The main question addressed in this study is how and to what extent telR-loops intersect with telomere stability in these cells.

These questions were addressed by specifically:

- Establishing a cellular system for the specific removal of R-loops at telomeres
- Evaluating telomere integrity upon telR-loop removal

With this, we aimed to characterize how telR-loops promote telomere stability and possibly coordinate TERRA-mediated regulation of telomere length at short telomeres. It is expected that this project will not only expand the knowledge behind chromosome stability mechanisms but also reveal novel circuits associated with TERRA transcription.

2. Materials and Methods

2.1 Cell lines, culture conditions and plasmid generation

Tumor-derived HT1080 fibrosarcoma cells and HEK 293T embryonic kidney cells stably expressing SV40 large T antigen were purchased from ATCC. Primary HLF cells were kindly offered by J. Lingner (CHUV, Lausanne, Switzerland). Immortalization of HLF was established by overexpressing the human telomerase catalytic subunit through retroviral infection with the pBABE-puro-hTERT plasmid (Addgene plasmid #1771) (Counter et al., 1998), followed by selection in 1 µg/ml of puromycin (Merck Millipore). Viruses were produced in HEK 293T cells according to standard procedures described in Section 2.2. All cell lines were cultured in high-glucose DMEM, GlutaMAX (Thermo Fisher Scientific), and grown in a humidified atmosphere containing 5% CO_2 at 37°C. HT1080 and HEK 293T were supplemented with 5% tetracycline-free fetal bovine serum (FBS) (Pan BioTech) and 100 U/ml penicillin-streptomycin (Thermo Fisher Scientific). HLF and HLF-hTERT were supplemented with 10% tetracycline-free FBS (Pan BioTech) and 100 U/ml penicillin-streptomycin (Thermo Fisher Scientific). A gBlock comprising the N-terminally myc-tagged human HBD fused to the catalytic domain of human RNaseH1 via a flexible polylinker (HBDRH1 WT) was purchased from Integrated DNA Technologies and cloned into a pLPC retroviral vector containing the full-length human POT1 gene and a myc epitope tag (pLPC myc hPOT1 - Addgene plasmid #12387) (Loayza & De Lange, 2003). The cloning process was accomplished by double digestion with the restriction enzymes BamHI and SnaBI (New England Biolabs), followed by digested pLPC myc hPOT1 isolation and ligation with the target transgene via T4 DNA Ligase (New England Biolabs). The final cloning product was amplified by bacterial transformation in DH5α competent cells, purified with the NZYMiniprep kit (NZYTech), and validated through a digestion-based screening assay with HindIII (New England Biolabs) and by automated DNA sequencing (Eurofins Genomics).

2.2 Ectopic protein expression

The remaining constructs used in this study were previously generated and provided by the lab. These included the catalytic domain of human RNase H1 (RH1 WT) or a catalytically dead variant, tagged with a myc epitope at the N-terminal region and fused to full-length human POT1 at the C-terminal region; or full-length, N-terminally myc-

tagged human POT1 alone, all cloned into the lentiviral vector pLVX-TetOne^{TM}-Puro (Clontech, #631849). Catalytical inactivation of RH1 WT was obtained by changing the aspartic acid residue at position 145 from full-length RNase H1 into glycine (D145G, referred to as RH1 D145G), based on previous descriptions (Wu et al., 2001; Arora et al., 2014). 1x10^6 HEK 293T cells were cultured per 6 cm dish at previously mentioned conditions and transfected with 3 µg of the expression plasmids or with an empty vector (EV) control, together with 750 ng of pMD2.G, 1250 ng of pMDLg/pRRE and 625 ng of Rev expression plasmids for lentiviral production. For retroviral production, the cells were transfected with 2 µg of the expression plasmid or with an empty vector (EV) control, together with 600 ng of VSVG and 1920 ng of GAG/POL expression plasmids. Further descriptions of all the plasmids used are stated in **Table 1**. Transfections were performed based on a calcium phosphate transfection protocol. Thirty-one microliters of $CaCl_2$, the required amounts of the plasmids, and an equalizing volume of H_2O for a total of 250 µl were mixed in tubes. 2x HBS (50 mM HEPES, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM Na_2PO_4 pH=7.05) was added 1:1 into each tube drop-wise while aerating the solution using a pipet. The resulting complexes were slowly added to the cells at a 1:4 ratio. Transfection medium was replaced by fresh culture medium 18 and 26 hours post-transfection. Lentiviral and retroviral supernatants were collected 40 and 64 hours following transfection, passed through 0.45 µm filters and added to HT1080, HLF, and HLF-hTERT cells for transient infection in a 1:1 ratio with fresh media. Infected cells were selected with 1 µg/ml puromycin (Merck Milipore) 24 hours after infection and remained under selective pressure for the following 48 hours. Ectopic protein expression was induced by culturing the infected cells with 1 µg/ml doxycycline (dox) (Sigma-Aldrich) for 48 or 72 hours.

Table 1: Plasmids used in this study.

Plasmids	Description
pLPC myc hPOT1	Constitutive retroviral expression vector including a N-terminally myc-tagged full-length human POT1 gene. Used for cloning purposes (Addgene plasmid #12387) (Loayza & De Lange, 2003)
pLPC myc-POT1-HBDRH1 WT	Constitutive retroviral expression plasmid generated by inserting an N-terminally myc-tagged human HBD fused to the catalytic domain of human RH1 WT via a flexible polylinker into a pLPC myc POT1 backbone

pMD-VSVG	Vector expressing the *trans-acting* VSVG envelope protein for retroviral production (a kind gift from J. Lingner, ISREC, Lausanne, Switzerland)
pMD-GAG/POL	Vector encoding the *trans-acting* Gag and Pol proteins for retroviral production (a kind gift from J. Lingner, ISREC, Lausanne, Switzerland)
pLVX-TetOne™-Puro	Lentiviral vector with a Tet-One Inducible Expression System (TetOne-Puro) including the Tet-On® 3G transactivator, capable of mediating transcription activation from the TRE3GS inducible promoter in the presence of dox (Clontech, #631849)
pLVX EV	Empty vector control based on the pLVX-TEOne-Puro lentiviral vector
pLVX myc-POT1	Inducible lentiviral expression plasmid based on a pLVX-TetOne-Puro backbone with an N-terminally myc-tagged and full-length human POT1
pLVX myc-POT1-RH1 WT	Inducible lentiviral expression plasmid encoding N-terminally myc-tagged and full-length human POT1 fused to the catalytic domain of human RH1 WT, inserted into a pLVX-TetOne-Puro backbone
pLVX myc-POT1-RH1 D145G	Inducible lentiviral expression plasmid encoding N-terminally myc-tagged and full-length human POT1 fused to the catalytic domain of human RH1 D145G, inserted into a pLVX-TetOne-Puro backbone
pMD2.G	Vector expressing the *trans-acting* HIV-1-derived VSVG envelope protein for lentiviral production (a kind gift from D. Trono, EPFL, Lausanne, Switzerland)
pMDLg/pRRE	Vector expressing the *trans-acting* HIV-1-derived Gag and Pol proteins for lentiviral production (a kind gift from D. Trono, EPFL, Lausanne, Switzerland)
pRSV-Rev	Vector expressing the *trans-acting* HIV-1-derived Rev protein for lentiviral production (a kind gift from D. Trono, EPFL, Lausanne, Switzerland)

2.3 Protein extraction and Western Blotting (WB)

Cells were washed once with ice-cold 1x PBS and lysed in 2x Laemmli buffer (4% SDS, 20% Glycerol, 120 mM Tris pH=6.8) before harvesting, followed by scraping and homogenization by repeated syringing. Protein was then centrifuged at 13,000 rpm for 5 minutes at room temperature, followed by boiling at 95°C for 5 minutes. Protein concentrations were measured in a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific). 20-40 µg of proteins were supplemented with 0.004% Bromophenol blue and 1% β-Mercaptoethanol (Sigma-Aldrich) and equalized with 2x Laemmli Buffer to the final volume. After incubation at 95°C for 5 minutes, protein samples were separated in 8 or 10% polyacrylamide gels at constant 120 V in 1x running buffer (25 mM Tris-Cl, 192 mM glycine, 0.1% SDS), and then transferred to nitrocellulose membranes (Maine Manufacturing, LLC) in 1x transfer buffer (50 mM Tris-Cl, 40 mM glycine, 0.04% SDS, 20% methanol) using a Trans-Blot SD Semi-Dry Transfer Cell apparatus (Bio-Rad) at constant 23 V for 1 hour. Membranes were blocked in freshly-made 5% milk in 1x PBS containing 0.1% Tween-20 (Sigma) (PBS-T) for 1 hour and later incubated overnight at 4°C with primary antibodies diluted in 5% milk/PBS-T. Membranes were then washed in PBS-T three times for 10 minutes each on a shaker, incubated with HRP-conjugated secondary antibodies diluted in 5% milk/PBS-T for 1 hour at room temperature, and washed the same way as before. Chemiluminescent signal detection was performed in an Amersham Imager 680 (GE Healthcare) after signal development with a 1:1 mixture of the two ECL detection reagents (GE Healthcare).

When needed, membranes were stripped off previous antibody stainings by incubation in 60 mM Tris-Cl pH=6.8, 2% SDS, 0.7% 2-mercaptoethanol (Sigma-Aldrich) for 10 minutes at 55°C in a water bath. Stripped membranes were repeatedly washed with dH_2O. For new antibody probing, the process was repeated starting from the membrane blocking step.

Secondary antibodies were HRP-conjugated goat anti-mouse and goat anti-rabbit IgGs (Bethyl Laboratories, A90-116P and A120-101P, respectively, 1:3000 dilution). Further information on the primary antibodies used is described in **Table 2**.

2.4 Indirect immunofluorescence and telomere analysis by fluorescence *in situ* hybridization

2.4.1 Indirect Immunofluorescence (IF)

Cells were grown on coverslips and initially handled on ice. All solutions used for IF experiments were filtered through 0.2 µm vacuum pump filters. Cells were gently washed with ice-cold 1x PBS, and a pre-extraction was performed by incubating the samples in CSK buffer (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES pH=7, 0.5% Triton-X) for 7 minutes, followed by two washes with ice-cold 1x PBS. All subsequent steps were performed at room temperature. 3.6% formaldehyde (Sigma-Aldrich) in 1x PBS was used to fix the cells for 10 minutes, followed by two 1x PBS washes for 5 minutes each and re-permeabilization in CSK buffer for 7 minutes. After two more washes in 1x PBS for 5 minutes each, cells were incubated with blocking solution (0.5% BSA, 0.1% Tween-20 in 1x PBS) for 45 minutes. Primary antibody dilutions were prepared in blocking solution and incubations were done for 1 hour in a humid chamber, followed by three washes in 1x PBS-T for 5 minutes each on a rocking platform. Fluorochrome-conjugated secondary antibody incubations were performed in blocking solution for 40 minutes, also in a humid chamber, followed by three washes as before on a shaking platform and protected from exposure to light. Coverslips were washed once in 1x PBS, and DNA was counterstained with 100 ng/ml DAPI (Sigma-Aldrich) in 1x PBS. Lastly, coverslips were mounted on microscope slides in Vectashield (Vectorlabs) and sealed with nail polish.

Secondary antibodies were Alexa Fluor 488-conjugated donkey anti-mouse and anti-rabbit IgGs (Thermo Fisher Scientific, A21202 and A-21206, respectively, 1:1000 dilution) and Alexa Fluor 568-conjugated donkey anti-mouse and anti-rabbit IgGs (Thermo Fisher Scientific, A10037 and A10042, respectively, 1:1000 dilution). Further information on the primary antibodies used is described in **Table 2**.

Table 2: Primary antibodies used in this study. WB = Western Blot; IF = Indirect Immunofluorescence

ProteinTarget	Host, Clonality	Source	Application	Dilution
β-Actin	Mouse, Monoclonal	Santa Cruz Biotechnology, #sc-47778	WB	1:5000
Lamin B1	Rabbit, Polyclonal	GeneTex, GTX103292	WB	1:5000
Myc-Tag	Mouse, Monoclonal	Cell Signalling Technology, #2276	IF	1:1000
Phospho-histone H2AX (pSer139)	Mouse, Monoclonal	Upstate (Millipore), 05-636	IF	1:1000
POT1	Rabbit, Monoclonal	Abcam, ab124784	WB	1:2000
Rap1	Rabbit, Polyclonal	Bethyl Laboratories, A300-306A	IF	1:300

2.4.2 Metaphase spreads

Metaphase spreads were prepared by incubating cells with 200 ng/ml colchicine (Sigma-Aldrich) for a variable time, depending on the cell cycle length, so that a sufficient number of cells arrested in mitosis could be observed under the microscope. HT1080, as highly proliferative cells, were treated for approximately 2 hours and a half, while HLF and HLF-hTERT, with longer cell cycles, were incubated for approximately 5 hours. Mitotic cells were collected into tubes by shake-off and pelleted by centrifugation at 400 ×*g* for 4 minutes at 10°C. Most of the supernatant was aspirated, but a small amount was left to resuspend the pellet by flicking the tubes. Pre-warmed hypotonic solution (0.075 M KCl) was slowly added into the tubes, followed by incubation for 8 minutes at 37°C in a water bath. Nuclei were pelleted and resuspended as before, and ice-cold fixative solution (methanol:acetic acid, 3:1) was slowly added against the side of the tubes on a vortex mixer, followed by incubation at room temperature for 20 minutes. Nuclei were then centrifuged and resuspended as before. New and ice-cold fixative solution was added to the tubes, and nuclei were again incubated and pelleted with the same previous parameters. Nuclei were finally resuspended in a small volume (between 100 to 500 µl) of fixative solution and spread onto microscope slides (previously cleaned

with 70% ethanol) by releasing 1-2 drops from a considerable vertical distance with the help of a pipette. Slides were air-dried and observed under the microscope to confirm successful metaphase chromosome spreads.

2.4.3 Fluorescence *in situ* hybridization (FISH)

All solutions used for FISH experiments were filtered through 0.2 µm vacuum pump filters. All incubations with microscope slides were performed in Coplin Jars unless said otherwise. Metaphase spreads slides were re-hydrated in 1x PBS for 5 minutes and then treated with 20 µg/ml Rnase A (Sigma-Aldrich) in 1x PBS for 30 minutes at 37°C in a humid chamber. Slides were dipped into 1x PBS and fixated in 4% formaldehyde in 1x PBS for 2 minutes. After being dipped three more times in 1x PBS, slides were treated with 70 µg/ml pepsin (Sigma-Aldrich) in pre-warmed 60 ml of 2 mM glycine pH=2 for 5 minutes at 37°C in a water bath. Slides were dipped again in 1x PBS, incubated in 4% formaldehyde in 1x PBS for 2 minutes, and washed twice in 1x PBS for 5 minutes each on a shaking platform. Dehydration series of 70%, 90% and 100% ethanol were performed for 5 minutes each on a shaking platform, followed by air-drying. Slides were applied with approximately 50 µl drops of a Cy3-conjugated C-rich telomeric PNA probe (TelC-Cy3; 5'-Cy3-OO-CCCTAACCCTAACCCTAA-3'; Panagene) diluted 1:1000 in hybridization solution (10 mM Tris-Cl pH=7.2, 70% formamide, 0.5% blocking solution (Roche #11096176001)), covered with coverslips to spread the probe while avoiding bubble formation, and incubated firstly on a heating plate at 80°C for 3 minutes to allow DNA denaturation, and secondly at room temperature for 2 hours in a humid chamber. Slides were washed twice in 10 mM Tris-Cl ph=7.2, 70% formamide, 0.1% BSA and three times in 0.1 M Tris-Cl pH=7.2, 0.15 M NaCl, 0.08% Tween-20 at room temperature for 10 minutes each on a shaking platform, with DNA being counterstained with 100 ng/ml DAPI. After dehydration in ethanol series and air-drying as before, slides were mounted with Vectashield and sealed with nail polish.

2.4.4 Image acquisition and analysis

All images were acquired with a widefield fluorescence Zeiss Cell Observer microscope using a 63x/1.4NA/0.19WD (mm) oil DIC M27 Plan-Apochromat objective, which was kindly provided by the Bioimaging Facility of the Instituto de Medicina

Molecular João Lobo Antunes (iMM), Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal. Image analysis was performed using ImageJ software.

2.5 DNA-RNA immunoprecipitation (DRIP)

Cells were collected with less than 70% confluency from 15 cm dishes by scraping on ice and pelleted by centrifugation at 500 g for 5 minutes at 4°C. Supernatants were discarded and pellets lysed in 1 ml of RA1 lysis buffer (Macherey-Nagel) containing 1% v/v β-Mercaptoethanol and 100 mM NaCl, with immediate resuspension by vortexing. Samples were supplemented with 250 µl of chloroform:isoamyl alcohol (24:1, Sigma-Aldrich) and 250 µl of phenol equilibrated with 10 mM Tris-Cl pH=8.0, 1 mM EDTA (Sigma-Aldrich), and mixed on a vortex in intervals of 10 seconds followed by cooling on ice for a total of 30 seconds. Phases were separated by centrifugation at 13,000 rpm for 15 minutes at 4°C, and the upper phase transferred into new tubes using a pipette with cut tips. Nucleic acids were precipitated with an equal volume of isopropanol (Merck) by inverting the tubes several times, followed by centrifugation at 13,000 rpm for 20 minutes at 4°C. Supernatants were discarded, and pellets were washed with ice-cold 70% ethanol and centrifuged at 13,000 rpm for 10 minutes at 4°C. After removal of the supernatant and air-drying, nucleic acids were resuspended in 200 µl Tris-EDTA buffer containing 100 mM NaCl and re-hydrated by incubating in a thermomixer at 800 rpm for 30 minutes at 22°C. The quality of nucleic acids, especially on the presence of ribosomal RNA, was verified by running a 1% agarose gel. Samples were then sonicated with a Bioruptor apparatus (Diagenode) at 4°C for 5 minutes (settings: 30 s “ON” / 30 seconds “OFF”; power: “High”) to yield DNA fragments between 100 to 500 bp, which were verified by separation in a 1% agarose gel. This process was repeated until the required degree of sonication was obtained. Five micrograms of sheared nucleic acids were incubated with 2.5 µg of S9.6 antibody (a kind gift from B. Luke, IMB, Mainz, Germany) in 1 ml of FA1 buffer (0.1% SDS, 1% Triton X-100, 10 mM HEPES pH=7.2, 0.1% sodium deoxycholate, 275 mM NaCl) at 4°C for 5 hours on a rotating wheel. Simultaneously, protein G Sepharose beads (GE Healthcare) were washed twice in FA1 buffer by spinning down on a centrifuge at 13,000 rpm for 30 seconds and then blocked with 250 ng/µl sheared *Escherichia coli* DNA and 1.5 µg/µl bovine serum albumin (BSA) for 5 hours at 4°C on a rotating wheel. Blocked beads were washed two times in 1 ml of FA1 buffer. 20 µl of beads were added to each nucleic acids sample and incubated for 2 hours at 4°C on a rotating wheel. Beads were again washed five times with 1.4 ml of FA1 buffer

by centrifuging at 13,000 rpm for 2 minutes at 4°C and then incubated with 400 µl of elution buffer (50 mM Tris-Cl pH=8.0, 10 mM EDTA, 0.5% SDS) supplemented with 10 µg/ml proteinase K (Sigma-Aldrich) at 50°C, 800 rpm shaking for 30 minutes on a thermomixer. Beads were pelleted on a centrifuge at 13,000 rpm for 2 minutes at 4°C and the eluted supernatants were transferred into new tubes. Nucleic acids were precipitated with 400 µl of isopropanol and 20 mg/ml of glycogen, inverted several times, and pelleted by centrifuging at 13,000 rpm for 20 minutes at 4°C. Supernatants were discarded and 1 ml of ice-cold 70% ethanol was added, followed by washing via repeated inversions of the tubes. Nucleic acids were pelleted by centrifuging at 13,000 rpm for 10 minutes at 4°C, supernatants were removed, and pellets were air-dried for 10 minutes. Samples were re-hydrated in 100 µl H_2O for 1 hour at 4°C.

Real-time PCR (qPCR) reactions were prepared for a total of 20 µl containing iTaq™ Universal SYBR ® Green Supermix (Bio-Rad), and with either 50 ng of S9.6-precipitated or non-precipitated DNA, or with 0.5 ng of total DNA input for sample normalization. DNA was analyzed for the presence of telR-loops at 10q subtelomeres, as previously described (Azzalin et al., 2007; Nergadze et al., 2009). TelR-loop signal was detected and quantified in a qPCR Rotor-Gene 6000 (Corbett), following DNA amplification for 5 minutes at 95°C, 15 seconds at 95°C and 30 seconds at 60°C for 45 cycles, and an incremental temperature increase of 1°C from 55°C to 99°C for each cycle. 10q primers used were as follows: Fwd → 5'-GAATCCTGCGCACCGAGAT-3'; Rev → 5'-CTGCACTTGAACCCTGCAATAC-3'.

2.6 Statistical Analysis

Two-tailed Student's t-test was performed in Microsoft Excel for a direct comparison of two groups. One-way analysis of variance (one-way ANOVA) was employed in GraphPad Prism 8 for comparison of more than two groups, followed by Tukey's HSD treatment to adjust the pairwise statistical significance. *P* values are indicated as: **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001. Error bars represent SD.

3. Results and Discussion

3.1 Validation of the cellular system targeting telR-loops

3.1.1 The catalytic domain of RNase H1 colocalizes with telomeres when fused to POT1

With the intention of establishing a cellular system for the specific removal of telR-loops, a strategy focused around RNase H1 was developed since its ectopic expression has already been shown to efficiently eliminate RNA-DNA hybrids throughout the genome via cleavage of their RNA moiety (Nowotny et al., 2008; Cerritelli & Crouch, 2009; Arora et al., 2014). Previous data from our group revealed a lower association of endogenous RNase H1 with telomeres in telomerase-positive cells when compared to ALT cells (Arora et al., 2014). In order to bring RNase H activity to the telomeres of telomerase-positive cells, the catalytic domain of human RNase H1 was fused to human POT1. A catalytically dead version of RNase H1 was also generated by mutating one of the previously described and essential conserved amino acids within the active site of RNase H1 from an aspartic acid to a glycine residue at position 145 (D145G) (Wu et al., 2001; Arora et al., 2014). Overexpression of POT1 alone was used to control for effects on telomere stability deriving from altered POT1 levels. All of the following transgenes were fused to a myc epitope at their N-terminal region, and are here presented as myc-POT1-RH1 WT, myc-POT1-RH1 D145G, and myc-POT1, respectively (**Figure 5a**). The transgenes were cloned into an inducible pLVX-TetOne^{TM}-Puro plasmid backbone for experimentally dox-controlled ectopic expression (**Figure 5b**). The pLVX-TetOne^{TM}-Puro empty vector (EV) was also included as a control.

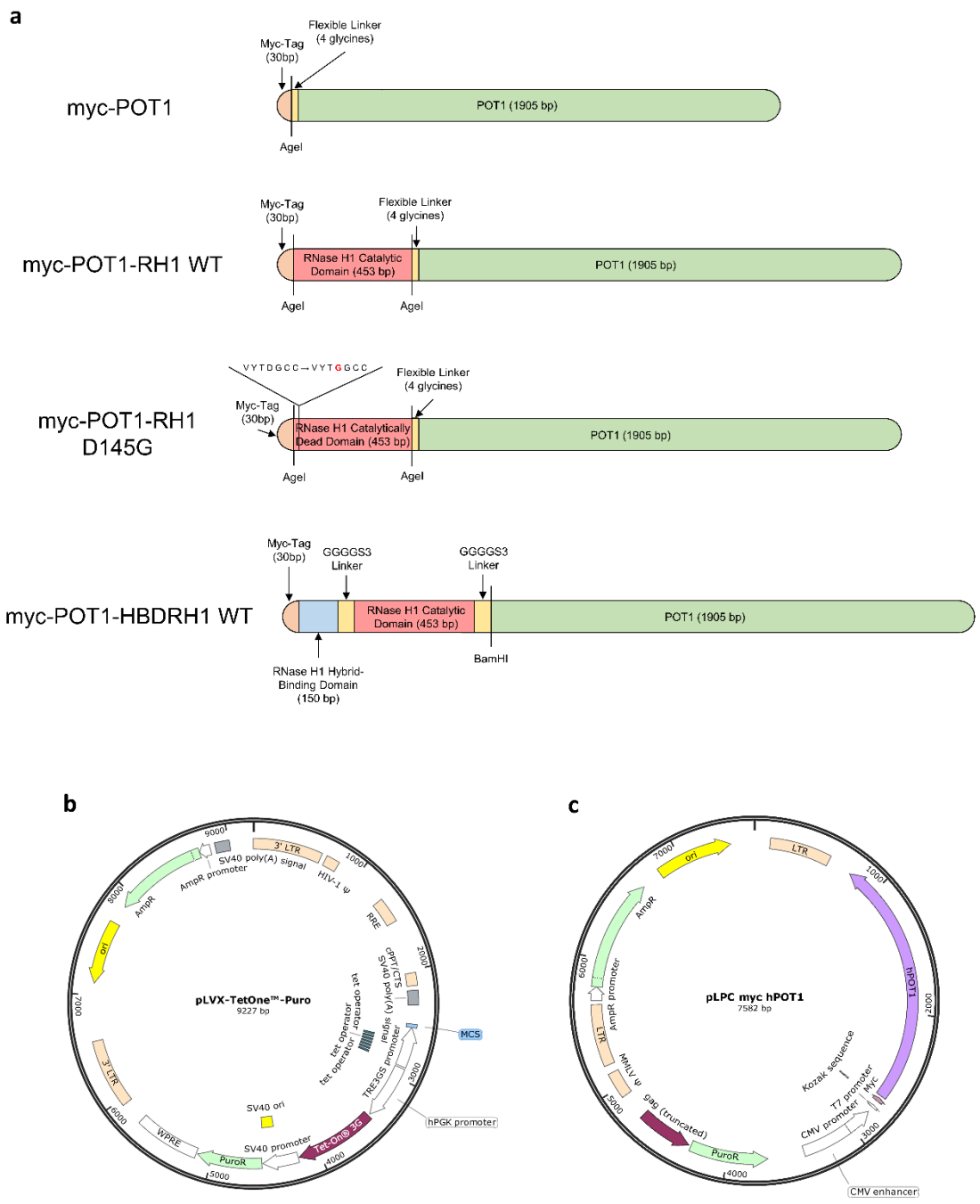

Figure 5 | Schematic representation of the cellular systems used in this study

(a) HLF, HLF-hTERT and HT1080 cells were stably infected with constructs designed to include N-terminally myc-tagged human POT1 (myc-POT1), either tethered to the catalytic domain of human RNase H1 (myc-POT1-RH1 WT), to an inactivated variant via an amino acid substitution from an aspartic acid into a glycine at position 145 of the full-length enzyme (myc-POT1-RH1 D145G), or an additional form containing the HBD region separated by a GGGGS3 flexible polylinker (myc-POT1-HBDRH1 WT). **(b)** myc-POT1, myc-POT1-RH1 WT and myc-POT1 RH1 D145G were cloned into a pLVX-TetOne^{TM}-Puro lentiviral vector, comprised of a Tet-On® 3G transactivation system for dox-controlled inducible expression. **(c)** myc-POT1-HBDRH1 WT was cloned into a pLPC myc hPOT1 retroviral vector of constitutive expression with a CMV promoter.

All constructs were used to produce lentiviruses and stably infect three different human cell types - a telomerase-positive fibrosarcoma cell line (HT1080), primary human lung fibroblasts (HLF), and an immortalized cell line derived from HLF cells by the ectopic expression of hTERT (HLF-hTERT). While hTERT overexpression is sufficient to reconstitute telomerase activity and counteract telomere erosion (Bodnar et al., 1998; Vaziri & Benchimol, 1998; Ramirez et al., 2001), it does not induce tumorigenesis, since *hTERT* is not an oncogene (Hahn et al., 1999; Morales et al., 1999). The three cell types were chosen to better understand the functional interaction between telomerase, telR-loops and telomere stability.

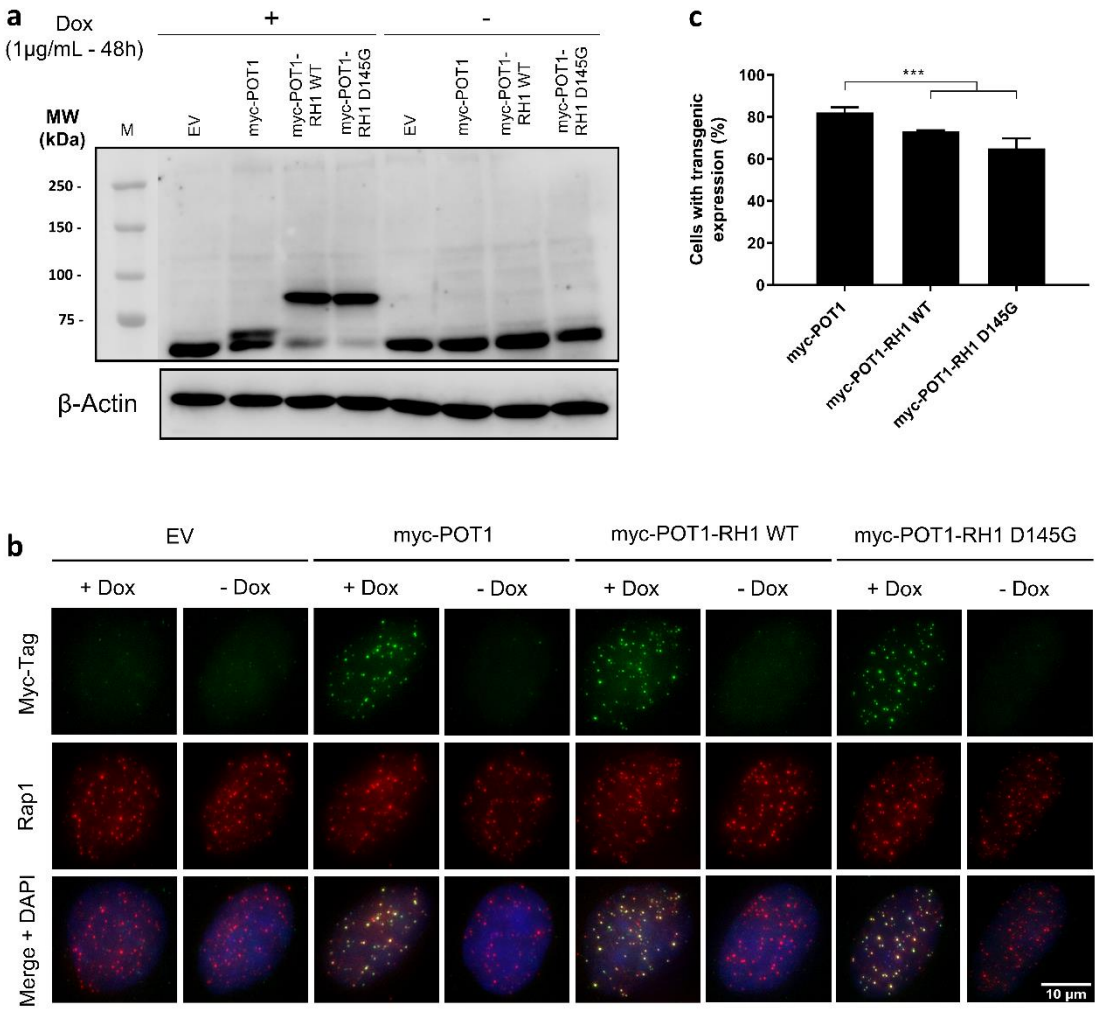

Figure 6 | Transgenic POT1-RH1 fusions efficiently colocalize with the telomere fraction of HLF cells

(a) WB analysis was performed in protein extracts of HLF cells stably expressing the target transgenes 48 hours after dox induction (1μg/ml). Antibodies used were against human POT1 and β-actin as a loading control. EV cells and the remaining not treated with dox were used as expression controls for the pLVX-TetOne^{TM}-Puro-based inducible system. **(b)** Examples of myc-tag immunostaining (green) combined with Rap1 immunostaining (red; telomeres) from the same cellular conditions as in **(a)**. DNA was counterstained with DAPI (blue). EV-infected cells and the remaining not treated with dox were used as expression controls for the pLVX-TetOne^{TM}-Puro-based inducible system. Scale bar: 10 μm. **(c)** Quantifications of HLF cells expressing the target transgenes via detection of myc-tag signal efficiently colocalizing with Rap1-stained telomeres. Bars and error bars represent means and SDs from three independent experiments with a total of 209 (myc-POT1), 239 (myc-POT1-RH1 WT) and 245 (myc-POT1 D145G) nuclei. *P* values were calculated with a two-tailed student's t-test. ***$P \leq 0.001$.

Ectopic expression of the transgenes was confirmed by WB 48 hours after dox administration in all the tested cell lines (**Figures 6a,7a,8a**). Using an anti-POT1 antibody, myc-POT1-RH1 WT and myc-POT1-RH1 D145G were detected as protein bands of similar molecular weight (approximately 90 kDa), while myc-POT1 was only slightly heavier than the endogenous POT1 due to the presence of the myc epitope. Additionally, and as expected, no transgene myc-associated signal was detected either in EV-infected cells or in non-treated dox conditions, with the latter observation ruling out a leakage from the pLVX-TetOne^{TM}-Puro inducible system. Overexpression of the transgenes diminished the levels of endogenous POT1 in HLF (**Figure 6a**) and HT1080 cells (**Figure 8a**). Comparable results have been described in previous studies (Loayza & De Lange, 2003; Chen et al., 2017), showing that POT1 overexpression at telomeres is highly dependent on telomere length. Indeed, longer telomeres provide more substrate for POT1 binding (Lei et al., 2002; Loayza & De Lange, 2003). Since the ability of POT1 to bind is essential to promote its stability, the overexpression of exogenous POT1 may lead to the degradation or no expression at all of the endogenous variant. Consistently, this crosstalk is particularly evident in HT1080 cells, which carry short telomere tracts (Lee et al., 2014). Furthermore, this explains the differences detected between HLF and HLF-hTERT cells (**Figures 6a,7a**) since HLF-hTERT have much longer telomeres than HLF.

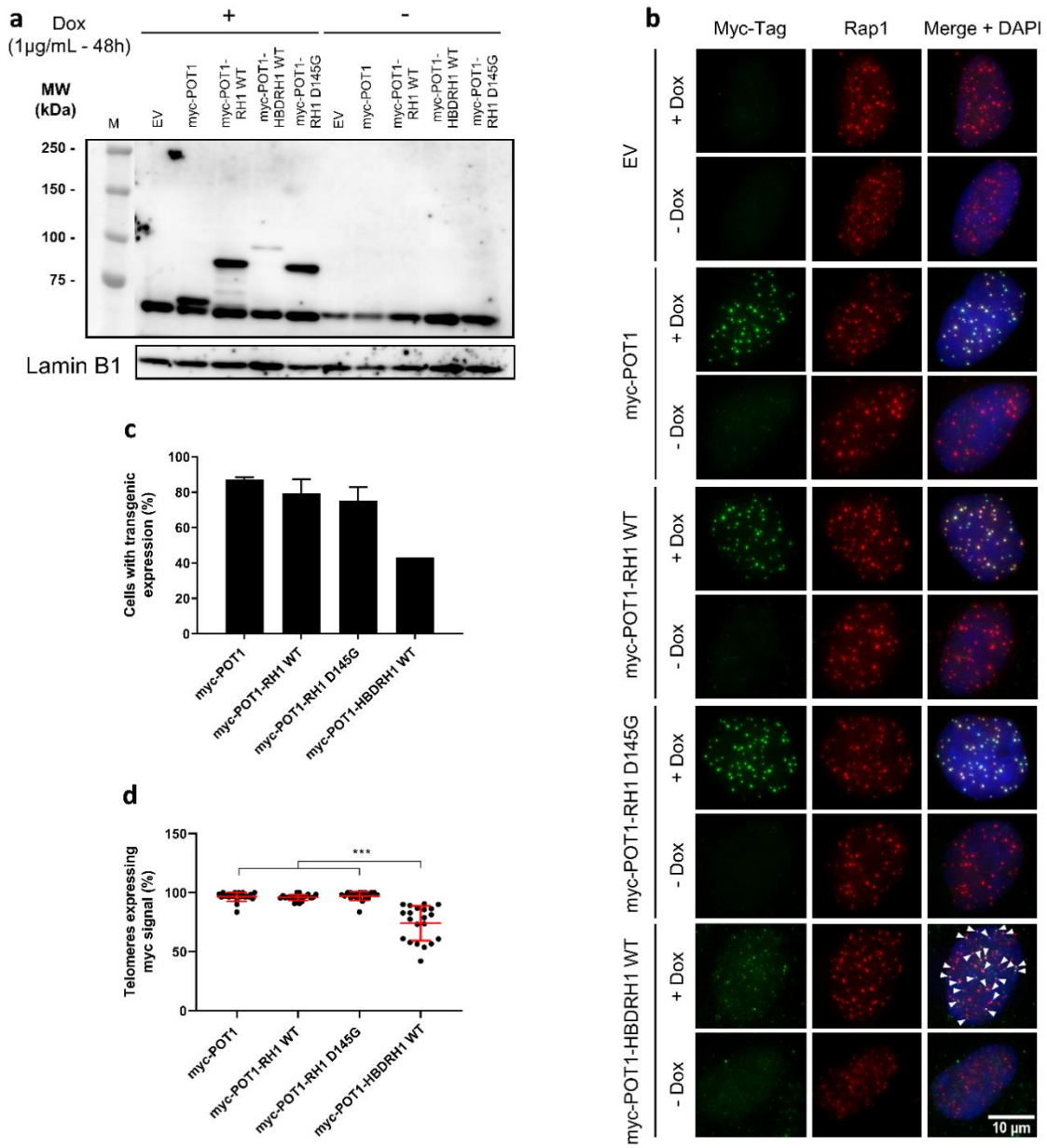

Figure 7 | Transgenic POT1-RH1 fusions efficiently colocalize with the telomere fraction of HLF-hTERT cells in the absence of HBD

(a) WB analysis was performed in protein extracts of HLF-hTERT cells stably expressing the target transgenes 48 hours after dox induction (1µg/ml). Antibodies used were against human POT1 and Lamin B1 as a loading control. EV-infected cells and the remaining not treated with dox were used as expression controls for the pLVX-TetOne^{TM}-Puro-based inducible system. **(b)** Examples of myc-tag immunostaining (green) combined with Rap1 immunostaining (red; telomeres) from the same cellular conditions as in **(a)**. DNA was counterstained with DAPI (blue). EV-infected cells and the remaining not treated with dox were used as expression controls for the pLVX-TetOne^{TM}-Puro-based inducible system. In the merge panels, white arrowheads point to myc-tag foci colocalizing with Rap1-stained telomeres. Scale bar: 10 µm. **(c)** Quantifications of HLF-hTERT cells expressing the target transgenes via detection of myc-tag signal efficiently colocalizing with Rap1-stained telomeres. Bars and error bars represent means and SDs from three independent replicates with a total of 211 (myc-POT1), 182 (myc-POT1-RH1 WT) and 207 (myc-POT1 D145G) nuclei. Data from myc-POT1-HBDRH1 WT expression represents one experiment including 74 nuclei. **(d)** Quantifications of the telomere fraction with detectable myc-tag signal from the target transgenes. Each dot corresponds to an individual nucleus. Bars and error bars represent means and SDs from one experiment with a total of 21 nuclei. *P* values were calculated with a two-tailed student's t-test. ***$P \leq 0.001$.

Telomeric localization of the transgenes was validated by IF. All the transgenes were intentionally myc-tagged to provide an efficient detection tool without relying on POT1 staining. The absence of myc signal in non-treated dox controls was consistent with what was previously described in the immunoblotting assays (**Figures 6b,7b,8b**). Indeed, not only were most of the cells from the three chosen cell lines ectopically expressing the transgenic proteins (**Figures 6c,7c,8c**) but also almost all detected telomere foci in HLF-hTERT were coupled with myc-associated transgene signal **(Figure 7d)**. With these results, we were able to confirm that the transgenes were being efficiently directed towards the chromosome ends in a POT1-dependent manner.

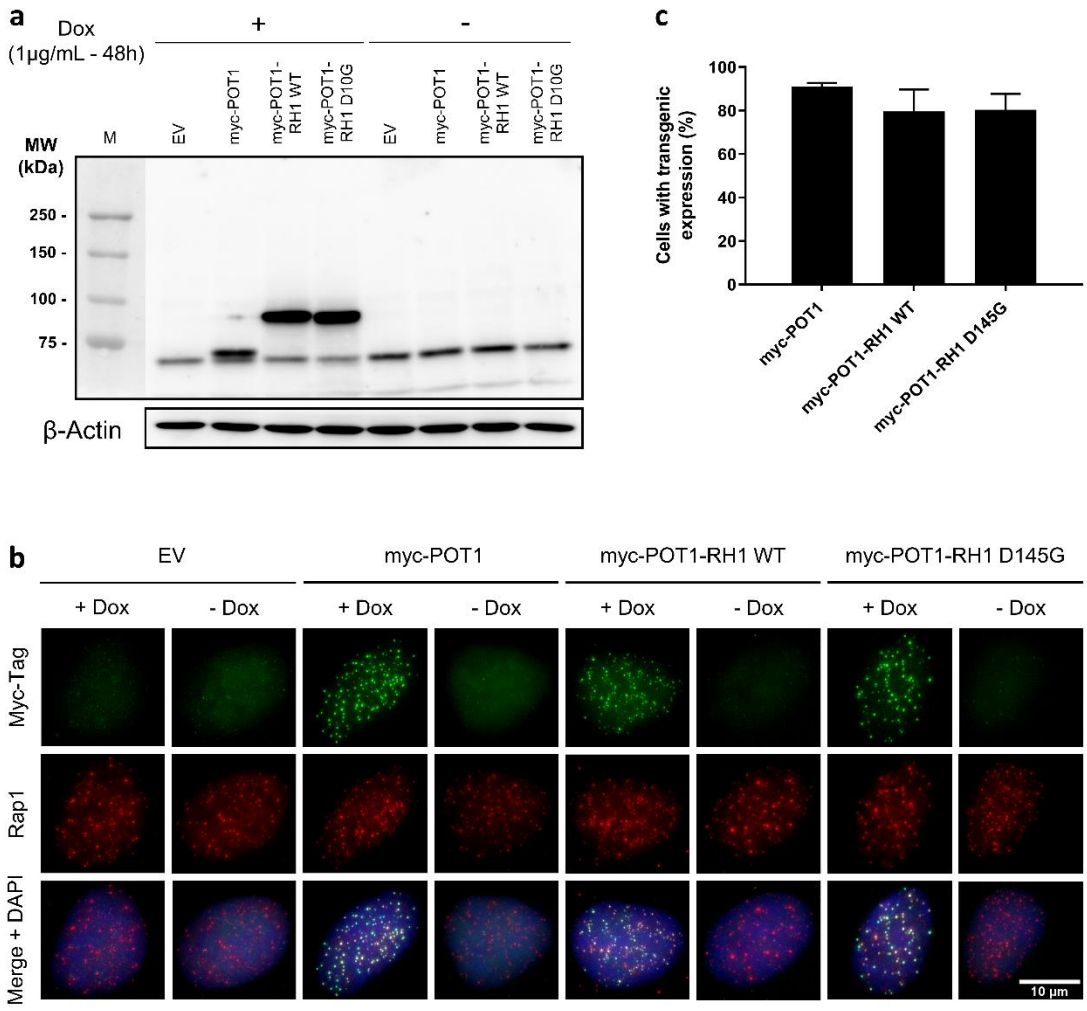

Figure 8 | Transgenic POT1-RH1 fusions efficiently colocalize with the telomere fraction of HT1080 cells telomeres and restricts telR-loops in HT1080 cells

(a) WB analysis was performed in protein extracts of HT1080 cells stably expressing the target transgenes 48 hours after dox induction (1µg/ml). Antibodies used were against human POT1 and β-actin as a loading control. EV-infected cells and the remaining not treated with dox were used as expression controls for the pLVX-TetOne^{TM}-Puro-based inducible system. **(b)** Examples of myc-tag immunostaining (green) combined with Rap1 immunostaining (red; telomeres) from the same cellular conditions as in **(a)**. DNA was counterstained with DAPI (blue). Cells not treated with dox were used as expression controls for the pLVX-TetOne^{TM}-Puro-based inducible system. Scale bar: 10 µm. **(c)** Quantifications of HT1080 cells expressing the target transgenes via detection of myc-tag signal efficiently colocalizing with Rap1-stained telomeres. Bars and error bars represent means and SDs from three independent experiments with a total of 301 (myc-POT1), 336 (myc-POT1-RH1 WT) and 354 (myc-POT1 D145G) nuclei. Bars and error bars represent means and SDs from three independent experiments. *P* values were calculated with a two-tailed student's t-test.

3.1.2 The presence of the hybrid-binding domain compromises telomere colocalization of POT1-RNase H1

Since we initially did not resort to a full-length variant of human RNase H1, we decided to test whether other regions of the endonuclease could interact with the catalytic domain and enhance its activity towards telR-loops. Seeing that HBD has been shown to improve the processivity and affinity of RNase H1 towards RNA-DNA hybrids (Gaidamakov et al., 2005; Nowotny et al., 2008), HLF-hTERT cells were stably infected with retroviruses from a newly designed construct. This was composed of a pLPC retroviral vector for constitutive expression from a cytomegalovirus (CMV) promoter, to which a transgene similar to myc-POT-RH1 WT but also containing the HBD region of RNase H1 was included (**Figure 5c**), and was thus named myc-POT1-HBDRH1 WT.

As before, ectopic protein expression and colocalization with telomeres were examined by WB and IF experiments, respectively (**Figures 7a,b**). When compared to the previous conditions, myc-POT1-HBDRH1 WT expression was highly variable in between cells (**Figure 7c**) and, if detected, revealed lower myc signal intensity across the entire nuclei (**Figure 7b**). Besides, the signal was not consistently detected at telomeres but instead found spread throughout the nucleus and even outside of it (**Figure 7b,d**). While the former observation could be somehow reasoned by the diminished ectopic expression of the transgene from the 91 kDa protein band shown in the immunoblotting assay (**Figure 7a**), the latter could be explained by the described interaction between RPA70 and HBD (at its R32, R33, and R57 conserved amino acids), which has been shown to stimulate the activity and association of RNase H1 with RNA-DNA hybrids (Nguyen et al., 2017a). Because RPA is a non-

telomere specific ssDNA-binding protein, it promotes genome-wide targeting of RNase H1 to R-loops, therefore competing with the POT1-mediated localization of the transgenes to telomeres. Nevertheless, the importance of the HBD in the functional activity of RNase H1 specifically directed at telR-loops requires further testing. For this, a new strategy could be designed by disrupting one of the RPA-HBD binding sites that may be compromising the telomere localization of RNase H1 from our transgenes.

3.1.3 The POT1-RH1 WT fusion suppresses telR-loops

TelR-loops were measured in HT1080 cells via a DRIP assay with the RNA-DNA hybrid-specific, sequence-independent S9.6 antibody (Boguslawski et al., 1986), followed by qPCR (**Figure 9**). Ectopic expression of myc-POT1-RH1 WT diminished the levels of telR-loops at the 10q chromosome. In contrast, HT1080 cells expressing myc-POT1-RH1 D145G accumulated telR-loops at the same telomeres. Because POT1 overexpression (via myc-POT1) did not significantly deviate from the basal number of telR-loops detected in EV control samples, we determined that telR-loop levels were not being affected by the presence of exogenous POT1, but were instead suppressed by the transgenic activity of RNase H1 from our cellular system. Nevertheless, these results could not be entirely conclusive due to the lack of significance between conditions and the high variability between replicates (especially in myc-POT1-RH1 D145G). Further efforts should be made to repeat this experiment and/or design new approaches to confirm the promising trend. This could include evaluating the levels of telR-loops in different telomerase-positive cell lines in a broader set of chromosome ends.

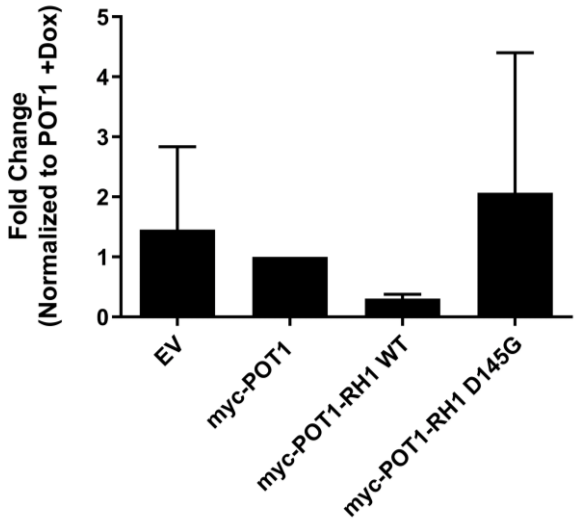

Figure 9 | Transgenic POT1-RH1 WT restricts telR-loops in HT1080 cells

qPCR quantifications of telR-loops at the 10q chromosome in HT1080 cells stably expressing the target transgenes, measured by DRIP and using the S9.6 antibody. All values were normalized to myc-POT1. EV-infected cells were used as a control. Bars and error bars represent means and SDs from three independent experiments. *P* values were calculated with a two-tailed student's t-test.

3.2 telR-loops can be stabilized by POT1-RH1 D145G and sustain telomere integrity in telomerase-positive and primary cells

Previous studies have already described telR-loops as sources of telomere instability in telomerase-negative ALT cells since they are required to stimulate their recombinogenic phenotype and regulate telomere length maintenance (Arora et al., 2014; Lee et al., 2018). Here we evaluated the impact of telR-loops in telomere stability, both in the presence or absence of telomerase. This was first done via IF assays to assess the levels of TIFs containing the DSB-induced DDR marker histone H2AX phosphorylated at serine 139 (γ-H2AX) (Takai et al., 2003; Kuo & Yang, 2008) (**Figures 10a,11a,12a**).

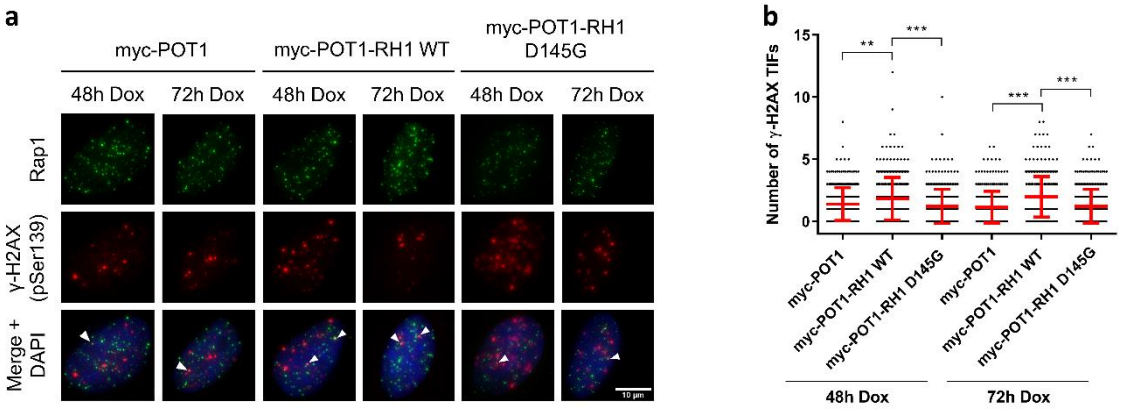

Figure 10 | telR-loop depletion by the catalytic activity of RNase H1 increases the levels of DNA damage-associated TIFs in HLF cells

(a) Examples of Rap1 immunostaining (green; to visualize telomeres) combined with γ-H2AX immunostaining (red) from HLF cells stably expressing the target transgenes, harvested 48 hours and 72 hours after dox induction. DNA was counterstained with DAPI (blue). In the merge panels, white arrowheads point to γ-H2AX foci colocalizing with Rap1-stained telomeres (TIFs). Scale bar: 10 μm. **(b)** Quantifications of the number of TIFs for each experimental condition shown in **(a)**. Each dot corresponds to an individual nucleus. Bars and error bars represent means and SDs from three independent experiments with a total of 300 nuclei for each condition. *P* values were calculated with a two-tailed student's t-test. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

Both HLF and HLF-hTERT cells showed increased TIF incidence when telR-loops were degraded for 48 hours. This effect was nevertheless not detected when the POT1-RH1 D145G variant was used. A similar variation was observed at 72 hours of transgene induction, suggesting that more extended periods of RNase H1 exposure do not increase the levels of TIFs, possibly due to the appropriate activation of DSB repair mechanisms, which counteract DNA damage build-up at telomeres. It could be interesting to include additional time points to test a possible chronological progression of DDR in these cells. It is conceivable that in phases earlier than 48 hours after dox administration an increment in DNA damage signaling and subsequently of TIF formation could be perceived. Given that the number of TIFs stabilizes at 72 hours in most conditions, the canonical DDR activity could start limiting the association of telomeric damage markers and loss of telomere function at gradually later stages due to efficient DSB repair. Furthermore, no major differences could be seen in the overall amount of TIFs between HLF and HLF-hTERT (**Figures 10b,11b**), suggesting that telomerase does not suppress the recruitment of DDR factors at telomeres.

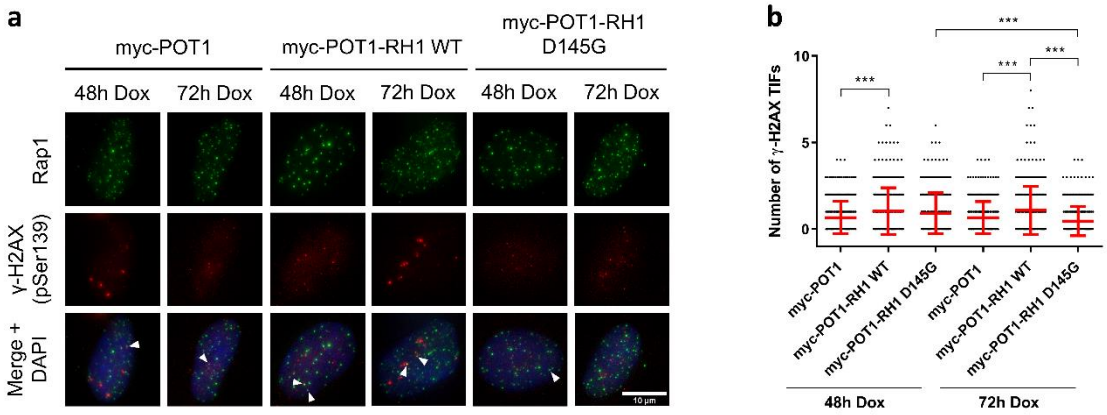

Figure 11 | telR-loop depletion by the catalytic activity of RNase H1 increases the levels of DNA damage-associated TIFs in HLF-hTERT cells

(a) Examples of Rap1 immunostaining (green; to visualize telomeres) combined with γ-H2AX immunostaining (red) from HLF-hTERT cells stably expressing the target transgenes, harvested 48 hours and 72 hours after dox induction. DNA was counterstained with DAPI (blue). In the merge panels, white arrowheads point to γ-H2AX foci colocalizing with Rap1-stained telomeres (TIFs). Scale bar: 10 μm. **(b)** Quantifications of the number of TIFs for each experimental condition shown in **(a)**. Each dot corresponds to an individual nucleus. Bars and error bars represent means and SDs from three independent experiments with a total of 300 nuclei for each condition. *P* values were calculated with a two-tailed student's t-test. ****P* ≤ 0.001.

A comparable pattern was also seen in HT1080 cells (**Figure 12b**), although both the basal levels (in cells stably expressing myc-POT1) and overall variation in TIFs were higher. This was somehow expected, given that oncogenic transformation has long been

associated with telomere dysfunction as a result of persistent DNA damage and inappropriate repair (Maser & DePinho, 2002; Sharpless & DePinho, 2004; de Lange, 2005b). HT1080 cells are positive for the N-*ras* oncogene (Hall et al., 1983; Paterson et al., 1987), and are aberrantly upregulated for the enhancer of zeste homolog 2 (EZH2), which is a histone methyltransferase that may function as a critical element of cancer development and progression by altering the expression of several tumor suppressor genes (Tang et al., 2004; Yamagishi & Uchimaru, 2017; Gan et al., 2018).

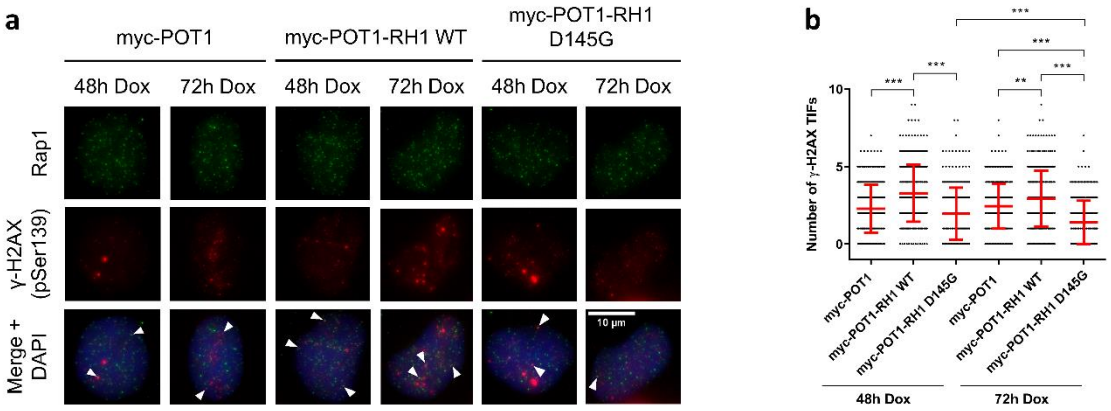

Figure 12 | telR-loop depletion by the catalytic activity of RNase H1 increases the levels of DNA damage-associated TIFs in HT1080 cells

(a) Examples of Rap1 immunostaining (green; to visualize telomeres) combined with γ-H2AX immunostaining (red) from HT1080 cells stably expressing the target transgenes, harvested 48 hours and 72 hours after dox induction. DNA was counterstained with DAPI (blue). In the merge panels, white arrowheads point to γ-H2AX foci colocalizing with Rap1-stained telomeres (TIFs). Scale bar: 10 µm. **(b)** Quantifications of the number of TIF of each experimental condition shown in **(a)**. Each dot symbolizes an individual nucleus. Bars and error bars represent means and SDs from three independent experiments with a total of 300 nuclei for each condition. *P* values were calculated with a two-tailed student's t-test. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

Altogether these results indicate that telR-loops are mediators of telomere integrity and that their unscheduled removal may stimulate telomere instability. Given that R-loops are described as well-established sources of replication stress and genome-wide instability (Groh & Gromak, 2014; Santos-Pereira & Aguilera, 2015), this suggests a probable additional role in ALT-negative human cells that may sustain telomere homeostasis, possibly by promoting DNA repair. It is also important to note that all cell lines presented a high number of telomeres with no associated TIFs. This suggests that a significant fraction is not protected by telR-loops. Reasonably, the reduction in telR-loop levels, which is characteristic of the myc-POT1-RH1 WT cell variants, inversely correlates with the overall increase in TIF incidence **(Figures 9,10b,11b,12b)**. In parallel, a significant mitigation of TIF formation was noticed in HT1080 and HLF-hTERT cells

stably expressing myc-POT1-RH1 D145G, where it was shown for telR-loops to accumulate at the 10q chromosome **(Figure 9)**. While this reduction was noticed and intensified from 48 to 72 hours of dox induction in HT1080 cells, lower levels of TIFs were only detected in HLF-hTERT at 72 hours **(Figures 11b,12b)**. Even so, this further suggests that RNase H1, when catalytically inactive, can act as a dominant-negative stability effector of these structures by blocking the access of additional R-loop dissolution factors into telomeres.

3.3 POT1-RH1 WT causes telomere loss in the absence of telomerase

To further clarify the role of telR-loops in telomere stability in telomerase-positive and negative cells, FISH experiments on metaphase telomeres were performed after 72 hours of dox induction **(Figures 13a,14a,15a)**. We analyzed and quantified the number of abnormally structured telomeres, classified either as fragile sites caused by compromised DNA replication that resembled shredded or multiple telomeric signals (FTs), or to a further extent as in the absence of a clear signal, associated with complete telomere loss (TFEs) (Sfeir et al., 2009; Lee et al., 2018).

While the frequency of FTs did not vary significantly among the tested conditions in HLF cells **(Figure 13b)**, a clear increase of TFEs was noticeable when overexpressing RNase H1 **(Figure 13c)**. Interestingly, the accumulation of deprotected chromosome ends in the myc-POT1-RH1 WT-expressing variant could lead to underestimations in our analysis of the DDR-mediated cellular response to telomere dysfunction, since the formation of TIFs cannot be taken into account when there are no detectable telomeres. This effect was however not seen in the same condition in HLF-hTERT cells, where both the levels of FTs and TFEs remained relatively stable **(Figures 14b,c)**, especially when compared to primary HLF **(Figures 14d,e)**. In contrast, the myc-POT1-RH1 D145G variants of both cell lines presented a subtle decrease in FTs. This may be related to the possible protective role of telR-loops on telomere integrity, which is further stabilized by the association of catalytically inactive RNase H1 to the telomeric RNA-DNA hybrid, as mentioned in Section 3.2.

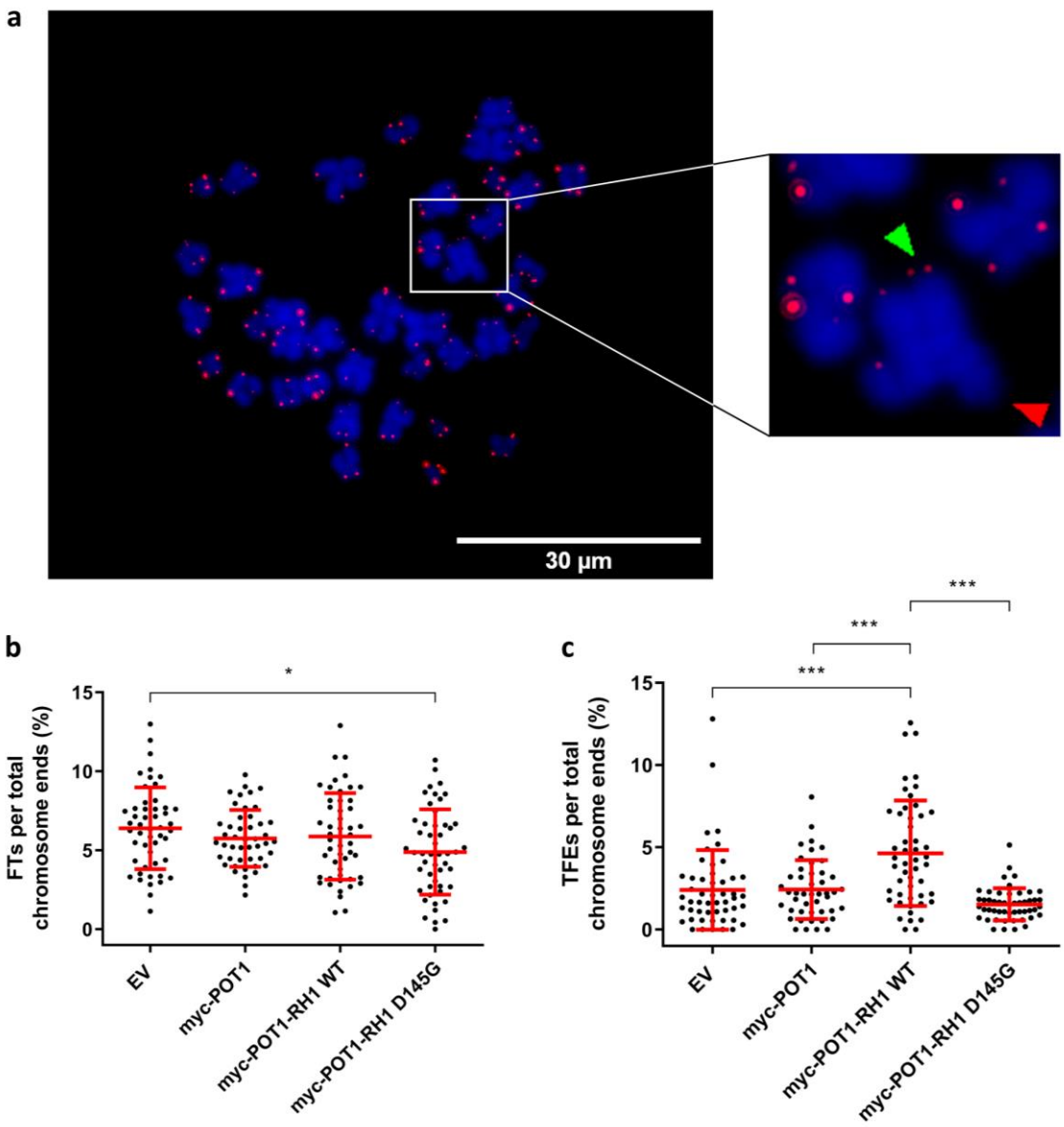

Figure 13 | POT1-RH1 WT-mediated degradation of telR-loops compromises telomere capping of HLF chromosome ends

(a) Example from FISH experiments performed on metaphase spreads of HLF cells stably expressing the target transgenes. Telomeric DNA is shown in red and DAPI-stained total DNA is in blue. Arrowheads from the magnification framing point to FTs (green) and TFEs (red). Scale bar: 30 µm. Quantification of **(b)** FTs and **(c)** TFEs from the FISH experiments indicated in **(a)**. Each dot symbolizes a fraction of FTs and TFEs per chromosome end in one metaphase. Bars and error bars represent means and SDs from two independent experiments with at least 46 metaphase nuclei for each condition. *P* values were calculated with a one-way ANOVA test. ***P* ≤ 0.01, ****P* ≤ 0.001.

In HT1080, the first point of notice stood with the confirmation of altered chromosome numbers **(Figure 15a)**, which is a common outcome of the genome instability that is found in cancer cell lines (Rasheed et al., 1974). While this is attributed to different point mutations and more substantial chromosome segmental changes established during

cancer progression (Eitan & Shamir, 2017), it can also be associated with telomere dysfunction, as damaged telomeres can induce endoreduplication (de Lange, 2005b). No significant variations in both the levels of FTs and TFEs were detected in these cells **(Figures 15c,d)**, which is consistent with what was observed in one of our previous studies (Arora et al., 2014).

As such, these results suggest a protective role of TERRA and telR-loops in telomerase-negative human cells. The progressive loss of telomeric sequences in human cells lacking a telomere maintenance mechanism can be limited by the action of TERRA-mediated telR-loops, thus avoiding a premature onset of cellular senescence that could lead to unscheduled cell cycle arrest and subsequent loss of telomere integrity when reaching critical lengths. This evidence could be used to create a parallelism with previous data obtained from telomerase-negative budding yeast models. As shown before, the accumulation of telR-loops via TERRA transcription stimulates HDR-dependent telomere elongation in recombination-competent telomerase mutants at a pre-senescent stage (Balk et al., 2013; Fallet et al., 2014; Yu et al., 2014; Graf et al., 2017). The compensatory mechanism of these yeast survivors has been attributed to the persistent DDR at critically short telomeres, possibly originated as a response to replicative stress (Balk et al., 2013).

The presence of telR-loops may represent a major source of telomere instability due to their ability to hinder the progression of replication forks and even cause their collapse at chromosome ends, thus leading to replicative stress and DSB formation (Gan et al., 2011; Gómez-González et al., 2011; Aguilera & García-Muse, 2012). In agreement with this, telR-loop build-up in HDR-deficient yeast cells has a detrimental effect, leading to sudden telomere loss and accelerated rates of senescence (Balk et al., 2013). It is only when telR-loops functionally interplay with the HDR pathway that certain cell types can maintain telomeres at a stable length. This has been shown not only in telomerase-negative cells but also in ALT-positive cancer cell lines, which rely on balanced recombination mediated by TERRA-DNA hybrids to counteract telomere erosion in a similar way to what has been described in yeast type II survivors (Arora et al., 2014; Yu et al., 2014; Misino et al., 2018). The combined results of these studies suggest that TERRA R-loops are essential, but also dependent on a tight regulation of telomere length dynamics to achieve a harmonious balance between senescence, tumor suppression, and telomere integrity.

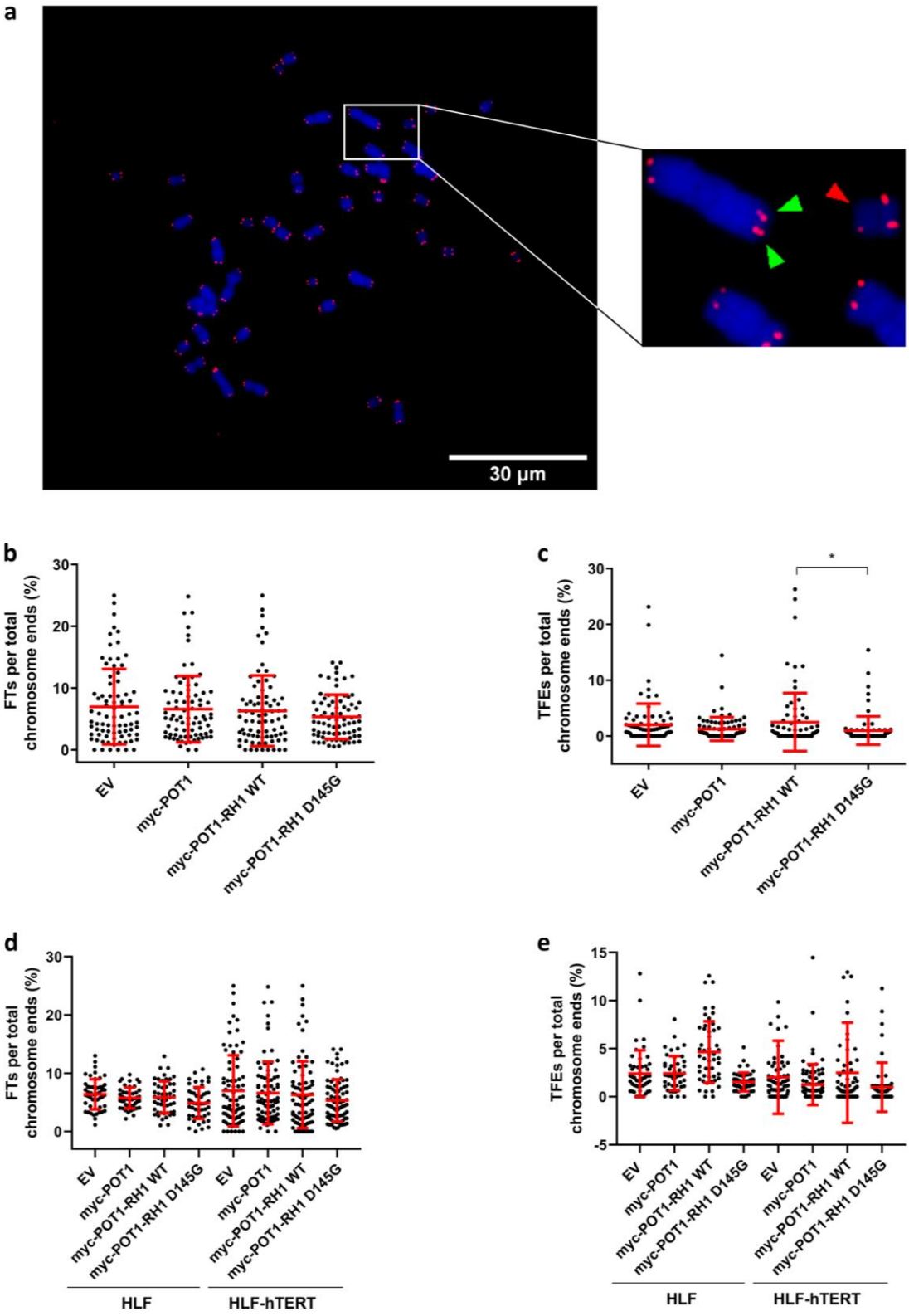
a
30 µm
b
FTs per total chromosome ends (%)
EV
myc-POT1
myc-POT1-RH1 WT
myc-POT1-RH1 D145G
c
TFEs per total chromosome ends (%)
*
d
HLF
HLF-hTERT
e

Figure 14 | Transgenic POT1-RH1 WT does not affect telomere stability in HLF-hTERT cells

(a) Example from FISH experiments performed on metaphase spreads of HLF-hTERT cells stably expressing the target transgenes. Telomeric DNA is shown in red and DAPI-stained total DNA is in blue. Arrowheads from the magnification framing point to FTs (green) and TFEs (red). Scale bar: 30 µm. Quantification of **(b)** FTs and **(c)** TFEs from the FISH experiments indicated in **(a)**. Each dot symbolizes a fraction of FTs and TFEs per chromosome end in one metaphase. Bars and error bars represent means and SDs from three independent experiments with a total of 90 metaphase nuclei for each condition. *P* values were calculated with a one-way ANOVA test. **P* ≤ 0.05. Closer comparison on the levels of **(d)** FTs and **(e)** TFEs from HLF and HLF-hTERT cells shown in **(Figures 13b,c)** and **(b,c)**, respectively.

Therefore, we propose that HLF cells (and most likely other telomerase-negative human primary cells) require telR-loop-mediated HDR to preserve critically short, but not normal length chromosome ends, and decrease the rate of replicative senescence in the absence of a telomere maintenance mechanism. In addition, RNase H1 presents itself as a limiting factor of HDR-dependent telomere elongation by maintaining the levels of telR-loop formation in check, as it has been shown in human ALT cells, yeast survivors and pre-senescent yeast cells (Balk et al., 2013; Arora et al., 2014; Yu et al., 2014). On the other hand, neither the removal nor accumulation of telR-loops has an evident effect on telomere length in telomerase-positive yeast cells (Balk et al., 2013; Yu et al., 2014). This is in agreement with what we have previously stated **(Figures 11,12)**, showing that telomerase activity, even though it may be recruited through TERRA, does not seem to depend on telR-loop-mediated HDR, since its activity is sufficient enough to promote telomere maintenance. Indeed, not only can telomerase elongate telomeres, but it has also been shown to assist chromosome end stabilization via a capping function (Blackburn, 2000). Given that we have previously demonstrated that RNase H1 specifically associates with ALT telomeres but does not perturb telomere homeostasis in telomerase-positive cells (Arora et al., 2014), it could be pertinent to assess possible mechanisms by which RNase H1 may be employed to telomeres in the presence of unresolved telR-loops, and if this is transversal to a telomerase-negative and ALT-negative scenario. To do so, further replication of the observations detailed in this study should be applied to several other primary cell lines, preferentially in a pre-senescent setting, where this effect should be intensified.

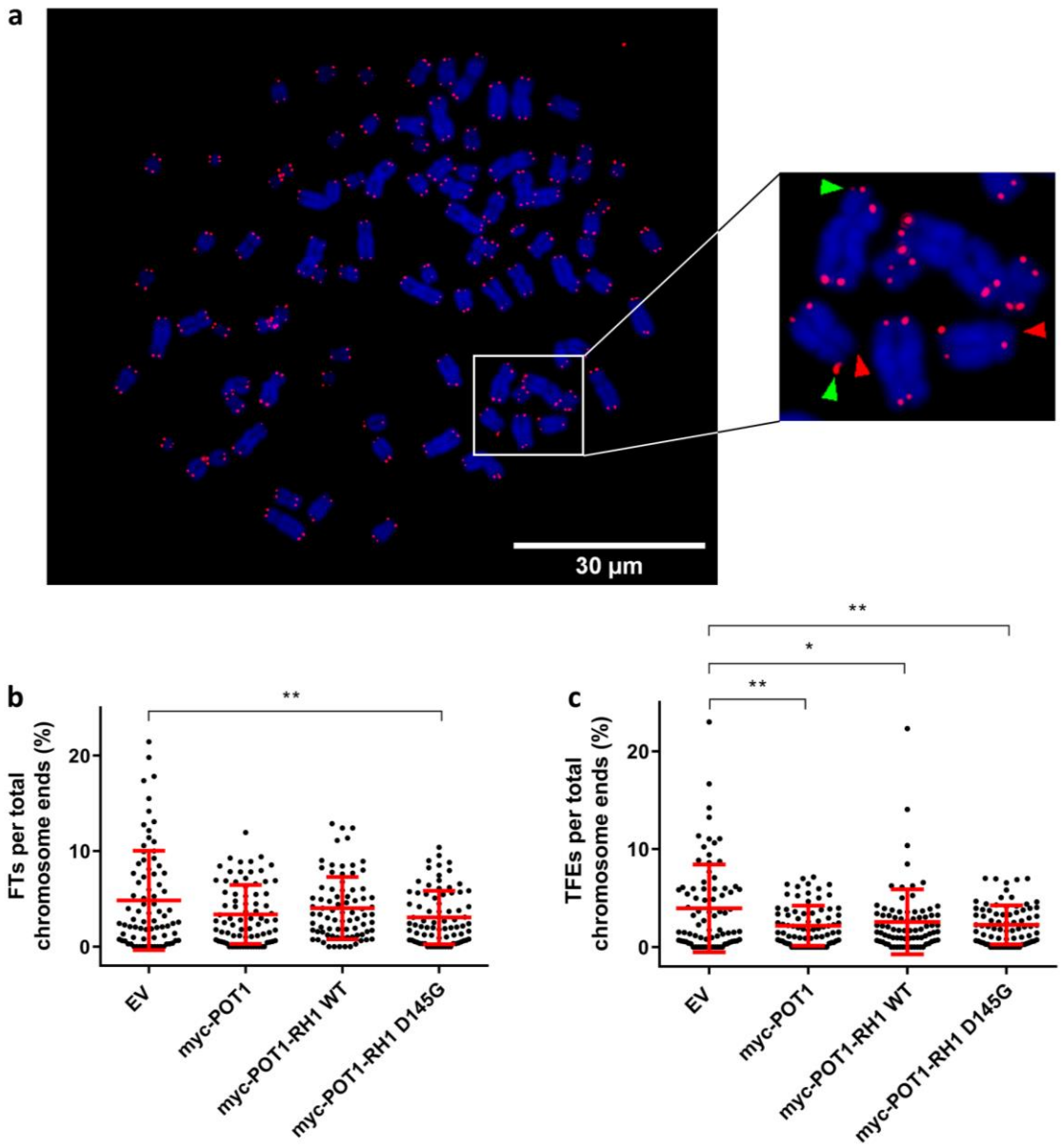

Figure 15 | Transgenic POT1-RH1 WT does not affect telomere stability in HT1080 cells

(a) Example from FISH experiments performed on metaphase spreads of HLF cells stably expressing the target transgenes. Telomeric DNA is shown in red and DAPI-stained total DNA is in blue. Arrowheads from the magnification framing point to FTs (green) and TFEs (red). Scale bar: 30 µm. Quantification of **(b)** FTs and **(c)** TFEs from the FISH experiments indicated in **(a)**. Each dot symbolizes a fraction of FTs and TFEs per chromosome end in one metaphase. Bars and error bars represent means and SDs from three independent experiments with a total of 90 metaphase nuclei for each condition. *P* values were calculated with a one-way ANOVA test. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

4. Concluding remarks

With this study, we have established an efficient telR-loop removal system by directly targeting the catalytic activity of RNase H1 to telomeres via POT1 binding. Furthermore, while we believe that the association of RNase H1 could be enhanced in the presence of its HBD, our preliminary findings suggest that the inherent interaction of this domain with RPA (Nguyen et al., 2017a) most likely hinders our attempts at improving POT1-mediated telomere colocalization by directing the transgene to other R-loop-containing-sites throughout the genome **(Figure 7)**. We also confirmed that the catalytic activity of RNase H1 is the main source by which this endonuclease promotes RNA-DNA hybrid degradation, since its catalytical inactivation not only suppresses the ability to resolve telR-loops but also leads to their accumulation **(Figure 9)**. While these results were not entirely conclusive, given the limited number of tested chromosome ends and their associated non-significant statistical values, they confirm previous data obtained from RNase H1 overexpression assays in ALT-positive and yeast cells (Balk et al., 2013; Arora et al., 2014; Yu et al., 2014). As a consequence of telR-loop degradation, we have reported increased levels of telomere-associated DNA damage in both telomerase-positive (HLF-hTERT and HT1080) and negative (HLF) human cells **(Figure 10,11,12)**, but only a significant rise of deprotected chromosome ends in the latter cell type **(Figure 13)**. Conversely, the levels of TIFs and telomere fragility were mitigated in all myc-POT1-RH1 D145 cell variants **(Figures 10-15)**.

Altogether these observations suggest that, while telR-loops may functionally protect telomeres from DNA damage-associated dysfunction, their role becomes more significantly crucial in telomerase-negative human cells, which rely on these hybrids to maintain telomeres and ensure their stability. Therefore, we hypothesize that TERRA-mediated telR-loops promote telomere extension in the absence of a telomere maintenance mechanism. Furthermore, and in accordance with previous studies performed with ALT-positive cells, telomerase-negative pre-senescent yeast cells, and yeast survivors (Balk et al., 2013; Arora et al., 2014; Fallet et al., 2014; Yu et al., 2014; Graf et al., 2017; Misino et al., 2018), this elongation process is stimulated by HDR in a telomere state-dependent manner, given that it is only essential at critically short lengths, i.e., when HDR is required to counteract the progressive loss of telomeric sequences and stably maintain telomeres, thus delaying the early onset of cellular senescence. This differential regulation may represent a cellular signature of telomere length dynamics that reflects altered levels of TERRA and, consequently, of TERRA-telomere association to form telR-loops, depending on the state of chromosome ends. As such, the roles of

TERRA may vary between short and long telomeres or be altered at telomeres that require recombination for telomere elongation when compared to those that are maintained by telomerase. In agreement with this, TERRA (and subsequently telR-loops) is specifically upregulated at both shortened or damaged and recombining telomeres (Arnoult et al., 2012; Lovejoy et al., 2012; Cusanelli et al., 2013; Arora et al., 2014; Episkopou et al., 2014; Porro et al., 2014; Yu et al., 2014), whereas in telomerase-positive cells it is kept at comparably lower levels (Ng et al., 2009; Arnoult et al., 2012; Yu et al., 2014). This is due to the fact that heavily eroded and deprotected telomeres lose their functional ability to control TERRA expression, possibly due to the dissolution of higher-order structures like T-loops and G4 complexes associated with displaced G-rich ssDNA strands (e.g. from a T-loop or telR-loop) (Tarsounas & Tijsterman, 2013), or the absence of the protective function of shelterin, since TRF2 depletion also results in increased TERRA levels (Porro et al., 2014). Furthermore, the chromatin status of telomeres is a considerably relevant factor, as H3K9me3 density inversely correlates with TERRA expression. Higher levels of TERRA promote heterochromatin formation in a negative feedback loop mechanism by which TERRA represses itself in a H3K9me3-dependent manner to limit the transcription of telomeres of normal length (Arnoult et al., 2012). This model is consistent with the relatively low levels of TERRA expression found in telomerase-positive cells. In sum, the regulation of TERRA transcription functionally interplays with telR-loop formation as a major determinant of both telomere length status and the proliferative potential in the absence of telomerase. Given this, future efforts should be directed at better understanding how TERRA specifically accumulates at recombination-dependent short and damaged telomeres, rather than at normal-length ones. Additionally, it could be interesting to assess if the cell-cycle regulation of TERRA is disrupted in pre-senescent telomerase-negative human cells, given their closer similarity with ALT models.

Finally, we speculate that telR-loops promote the stability of shorter HLF telomeres in an HDR-dependent manner via the BIR pathway, which has already been implicated in telomere maintenance in eukaryotes in the absence of telomerase. Indeed, given that BIR may arise from one-ended DSBs, cells lacking a telomere maintenance system represent a perfect substrate for this type of DNA lesions as a result of continued erosion of the telomeric tracts that exposes the extremities of linear chromosome ends (Anand et al, 2013; Sakofsky & Malkova, 2017). Furthermore, the persistence of replicative stress due to replication fork stalling from telR-loops in the absence of telomerase can promote BIR. While BIR is not activated at one side of a DSB and therefore proceeds as normal for early HDR events (DNA resection, homology search, strand capture and

invasion), the presence of a telR-loop on the other side blocks the capture of the second resected strand, leading to the engagement of this repair mechanism. In a recent study, RNase H1 overexpression has been shown to limit the interference of RNA-DNA hybrids in promoting BIR-mediated unstable repair of DSBs (Amon & Koshland, 2016), which could explain why resolving telR-loops under the same condition led to a significant increase of deprotected chromosome ends in HLF cells **(Figure 13)**. Besides, we hypothesize that telR-loops may delay the uncapping of chromosome ends by further blocking the nucleolytic processing of telomeres. However, this may prove to be an otherwise secondary effect of the recombination promoted by these structures, since BIR has been associated not only to the repair of replication stress but also of nuclease-induced DSBs at telomeres (Dilley et al., 2016; Zhang et al., 2019).

BIR has been long identified in yeast cells and distinguished between two phenotypically different telomere sub-types that resort to different recombination machineries to mediate ALT-related survival - while both rely upon Rad52 and POL32 (Lundblad & Blackburn, 1993; Lydeard et al., 2007), Type I survivors require the canonical Rad51-dependent pathway for DNA strand homology search but Type II do not (Lundblad & Blackburn, 1993; Teng & Zakian, 1999; Teng et al., 2000). In parallel, ALT HDR has also been shown to occur through BIR (Dilley et al., 2016; Roumelioti et al., 2016), and like their Type II yeast survivor counterpart, it does not require Rad51 for ssDNA annealing between homologous sequences (Bhowmick et al., 2016; Sotiriou et al., 2016). Although this process remains functionally unclear, it has been proposed that Rad52 facilitates ssDNA annealing from eroded telomeres to partially single-stranded telomeric C-circles (Tomaska et al., 2009; Dilley & Greenberg, 2015), or that shelterin proteins enable intratelomeric strand invasion (Verma & Greenberg, 2016). Further insights into the recombinatory phenotype of short telomeres in telomerase-negative human cells that we state in this study could improve our understanding on how different BIR pathways are orchestrated, and even what additional recombination-associated factors may be involved depending on telomere length and its cellular context in the presence of TERRA and telR-loops.

Although there has been substantial development on the knowledge regarding TERRA regulation and biogenesis in eukaryotic cells, including yeast and humans (Maicher et al., 2014; Azzalin & Lingner, 2015; Cusanelli & Chartrand, 2015), the functional relevance of telomere transcription remains to be determined. Yet, several studies have demonstrated the physiological relevance of telR-loops at telomeres (Pfeiffer & Lingner, 2012; Balk et al., 2013; Pfeiffer et al., 2013; Arora et al., 2014; Yu et al., 2014; Rippe & Luke, 2015; Graf et al., 2017; Nanavaty et al., 2017; Sagie et al.,

2017). Previously, we had detailed that the catalytic activity of RNase H1 prevents telomere loss due to aberrant telR-loop accumulation specifically in ALT cells. While restricting telR-loop accumulation stimulates semiconservative replication, it restrains the ability of ALT telomeres to recombine, which is essential to regulate the characteristic HDR-mediated telomere maintenance of ALT tumors via the excision of considerable telomeric tracts in the form of C-circles (Arora et al., 2014). Similarly, the results in this study suggest that telR-loops may sustain a decisive role in the regulation of telomere length in human telomerase-negative cells, thus bestowing them the necessary stability to avoid premature senescent stages. In the absence of a telomere-maintenance mechanism, telomeres become progressively shorter with each replication round until reaching a critical stage, where the regulation of TERRA expression, degradation and/or displacement may be lost. The possible inability of repressing TERRA binding to short or deprotected telomeres, together with loss of heterochromatin and higher-order protective DNA structures, leads to the upregulation of TERRA and subsequently of telR-loops. Accumulation of telR-loops aggravates replication fork stalling and possible collapse within the telomeric tract, as well as associated replicative stress and DSB formation (Gan et al., 2011; Aguilera & García-Muse, 2012), which may also cause TERRA build-up in a feedback loop mechanism (Rippe & Luke, 2015). While these events could be detrimental to the integrity of pre-senescent telomerase-negative human cells, we believe that, just like in ALT and telomerase-negative yeast cells, they create the ideal environment for a counteractive DDR based on BIR-mediated HDR. Our current results suggest that, in the absence of telomerase, telR-loops may positively contribute towards telomere stability of human cells in an HDR-dependent manner by promoting replication fork restart and telomere elongation of shorter and damaged chromosome ends through *de novo* synthesis of telomeric DNA, which is most likely supported by the BIR pathway. Therefore, the existence of an alternative telomere maintenance mechanism based on TERRA functionality marks an important, yet fairly undisclosed process by which human chromosome ends may rely on to postpone premature senescence.

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