# Telomeres, elements of transcriptional regulation in senescent cells

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# THÈSE DE DOCTORAT

# Les télomères, des éléments de régulation de la transcription dans les cellules sénescentes


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Les télomères, des éléments de régulation de la transcription dans les cellules sénescentes.

Les télomères sont des structures nucléoprotéiques, localisés à l'extrémité de l'ADN des cellules eucaryotes, qui préservent l'intégrité des chromosomes. Ces structures subissent des changements au cours du développement et du vieillissement, comme un raccourcissement de leurs tailles ou une altération dans leur composition en protéines (notamment le complexe shelterin). Cependant, les télomères n'ont pas qu'une fonction protectrice et peuvent aussi réguler l'expression des gènes présent dans les régions adjacentes, *i.e.* subtélomères, voir plus distantes. Ce processus est connu comme l'Effet de Position Télomérique (TPE), découvert initialement chez la levure *Saccaromyces cerevisiae*, puis chez la drosophile et *Schizosaccharomyces pombe* et plus récemment chez l'humain. Dans ces organismes, les gènes situés en position subtélomérique sont réprimés par un mécanisme épigénétique qui est dépendant de la taille des télomères et des protéines les composant ainsi que des structures tridimensionnelles adoptées par la chromatine télomérique et subtélomérique. Le TPE peut être décrit comme un mécanisme de propagation de l'hétérochromatine, du télomère vers le centromère, accompagné de boucle de chromatine permettant d'étendre la répression de la transcription dans des régions plus interne.

Dans ce contexte, le but de ma thèse est de déterminer l'impact des télomères sur les changements transcriptionnels observés lors de la sénescence cellulaire. A cette fin, nous avons séquencé les ARNm de fibroblastes de poumon (MRC-5) jeunes et sénescents (RNAseq). Nous avons observé un enrichissement subtelomérique des gènes dont l'expression est augmentée en sénescence. Ce résultat suggère une levée de la répression induite par le TPE à la sénescence. Cette dérépression ne concerne que certains gènes et certains subtélomères.

Nous avons aussi testé l'hypothèse que les protéines shelterin puissent prendre part au TPE, notamment TRF2 (Telomeric Repeat Binding Factor 2) dont l'expression est diminuée à la senescence. Nous avons donc augmenté le niveau d'expression de TRF2 dans les cellules sénescentes. Ainsi, nous avons pu observer que TRF2 modulait l'expression de certains gènes subtélomériques dans les cellules sénescentes. Par 3D-FISH, nous avons montré que cet effet de TRF2 s'accompagnait d'un remodelage spatial des subtélomères.

Dans son ensemble, ce travail révèle la contribution des télomères au programme transcriptionnel des cellules sénescentes et jette ainsi les bases de l'importance du TPE dans le processus de la senescence.

**Mots Clefs :**
Télomère, Sénescence, Effet de Position Télomérique, Transcription, Épigénétique, TRF2.

Telomeres, elements of transcriptional regulation in senescent cells.

Telomeres, the nucleoprotein structures located at the end of eukaryotic DNA, protect chromosomal integrity. These structures undergo changes during development and aging, including length shortening and alterations in the levels of the proteins associated to them called sheltering, all this affecting genome stability as the cells age. However, telomeres also behave as transcriptional regulators acting not only on genes present at subtelomeres but also on more distantly located genes presented throughout the genome. This process is referred as Telomere Position Effect (TPE) and was initially discovered in budding yeast, but also seen in drosophila, fission yeast, plasmodium and more recently in humans. In all these organisms, genes located in the subtelomeres are repressed by an epigenetic mechanism that is dependent on telomere DNA length, telomere nucleoprotein composition and higher order chromatin organization adopted by telomeres and subtelomeres. The TPE mechanism can be described as the spreading of a heterochromatin-like structure toward the centromere most likely accompanied by the formation of large chromatin loops to further extend the transcriptional regulation emanating from a telomere to genes internally located.

In this context, the goal of my thesis is to decipher whether telomeres are involved in the transcriptional remodelling occurring in human cellular senescence. For that, we performed RNA-sequencing in young versus replicative senescent lung fibroblast MRC-5 cells. Interestingly, we found an enrichment of upregulated genes in the subtelomeric regions of senescent cells suggesting a TPE alleviation. This alleviation is not homogeneous in the genome, as only some subtelomeres were enriched in upregulated genes at senescence.

Finally, we tested the hypothesis that shelterin proteins may also be part of the TPE regulation. For that, we re-stored the levels of the shelterin protein TRF2 (Telomeric Repeat Binding Factor 2) whose expression is decreased as the cells approach senescence. We found that TRF2 is indeed modulating the expression of subtelomeric genes in senescent cells, and this is in part mediated by a long-range chromatin reorganization of subtelomeres as observed by conformation changes in 3D chromatin conformation by FISH.

Overall, this work reveals the contribution of telomeres in the transcriptional program of senescent cells and set the basis for the relevance of TPE in the senescence/aging process.

**Key Words:**

Telomere, Senescence, Telomere Position Effect, Transcription, Epigenetic, TRF2.

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# TABLE OF CONTENT

« *La révolution scientifique a été non pas une révolution du savoir, mais avant tout une révolution de l'ignorance. La grande découverte qui l'a lancée a été que les hommes ne connaissent pas les réponses à leurs questions les plus importantes* »

Yuval Noah Harari. Sapiens.

# INTRODUCTION

# Chapter 1: Telomeres, a protective structure with signalling properties:

## The protective functions of telomeres:

Telomeres, from the greek “telos”, end, and “meros”, part, were conceptualized as caps, protecting linear eukaryotic chromosome termini from fusion, by Muller and McClinton in the late 1930’s. The first telomeric sequence, a tandemly repeated sequence of $(T_2G_4)n$ was discovered by Blackburn and Gall in *Tetrahymena thermophila* in 1978 (Blackburn and Gall, 1978). In human, the telomeric sequence is $(T_2AG_3)n$ (Moyzis et al., 1988). Interestingly, this G-rich sequence constituting chromosome termini was shown to be conserved in most eukaryotes; in vertebrates and most metazoans with $(T_2AG_3)n$ (Gomes et al., 2011; Silvestre and Londoño-Vallejo, 2012), in *Saccharomyces cerevisiae* with $(TG_{1-3})n$, in *Schizosaccharomyces pombe* with $(TTAC(A)G_{2-5})n$ (Teixeira and Gilson, 2005), and in plants with $(T_3AG_3)$ (Richards and Ausubel, 1988). Of note, some organisms differ greatly from this consensus, such as *Drosophila melanogaster* which possess array of non-long terminal repeat (LTR) retrotransposons (**Figure 1**).

## The end-replication problem:

The repetitive nature of telomeric sequences preserves genome integrity and tackles the end-replication problem. During DNA replication, while the leading strand is fully duplicated, the lagging strand is replicated through discontinuous Okazaki fragments and needs short RNA molecules to prime the replicative machinery. When the most distal primer is removed, a 5’ terminal gap is created, leaving a 3’ G-strand overhang. On the leading strand, a 3’ G-strand overhang is also created by a resection of the 5’ strand by the nucleases Apollo and Exo1 in human (Gilson and Géli, 2007; Olovnikov, 1973; Wu et al., 2012). This “end-replication problem” results in a gradual shortening of telomeric sequences at each cell division, but the gene content of the chromosome is preserved.

Nonetheless it remains important to elongate telomeres and compensate this ineluctable loss. A conserved mechanism relies on Telomerase. Telomerase is composed of a catalytic subunit TERT with a reverse transcriptase activity that synthetizes telomeric DNA repeats and an RNA subunit TERC that serves as a template by binding the 3' overhang (Chan and Blackburn, 2004; Greider and Blackburn, 1985). In organism lacking telomerase, such as *Drosophila*, telomere elongation depends on recombination events (Cacchione et al., 2020; Sasaki and Fujiwara, 2000). In human somatic cells, telomerase repression starts during embryonic life and is maintained repressed during the life of the organism (Wright et al., 1996). Consequently, telomeres shorten; for instance they were shown to lose an average of ~70pb per year, in human lymphocytes (Canela et al., 2007). This mechanism of telomere shortening is associated with aging (López-Otín et al., 2013). Some recombination-based events, known as ALTs (Alternative Lengthening of Telomeres), can elongate telomeres in human cells, but these mechanisms are often found in cancer cells (Cesare and Reddel, 2010; Gilson and Géli, 2007). Telomere elongation is a regulated process mediated by the protein complexes protecting telomeres.

## Telomeres and protection:

Due to their linearity, chromosome termini need to be protected against the repair machinery to avoid fusion and consequently genomic instability. This function is ensured by specialized protein complexes that bind telomeric repeats together with non-coding RNA (Telomeric Repeat-containing RNA, or TERRA). They regulate telomere length homeostasis and "hide" the chromosome end to avoid its recognition as double strand breaks (Giraud-Panis et al., 2013; Vega et al., 2003).

However, when telomerase activity is loss (whether its inhibition is developmentally regulated, or whether a loss of function mutation occurs), telomeres shorten gradually until they reach a limit and become exposed. Consequently, the DNA Damage Response (DDR) is triggered, leading to the activation of p53, and cells enter into replicative senescence or apoptosis (d'Adda di Fagagna et al., 2003). Replicative senescence is a permanent growth arrest accompanied by apoptosis resistance and

an altered gene expression. This cellular state, by preventing division of cells with short telomeres, susceptible to chromosomal rearrangements, preserves genome integrity (Campisi and d'Adda di Fagagna, 2007).

The protein complexes protecting chromosome ends are known as the shelterin complex (or shelterin-like) and CST (or CST-like) in vertebrates and yeasts or the terminin complex in fruit flies ((Giraud-Panis et al., 2013; Lange, 2005; Raffa et al., 2011) (**Figure 1**).

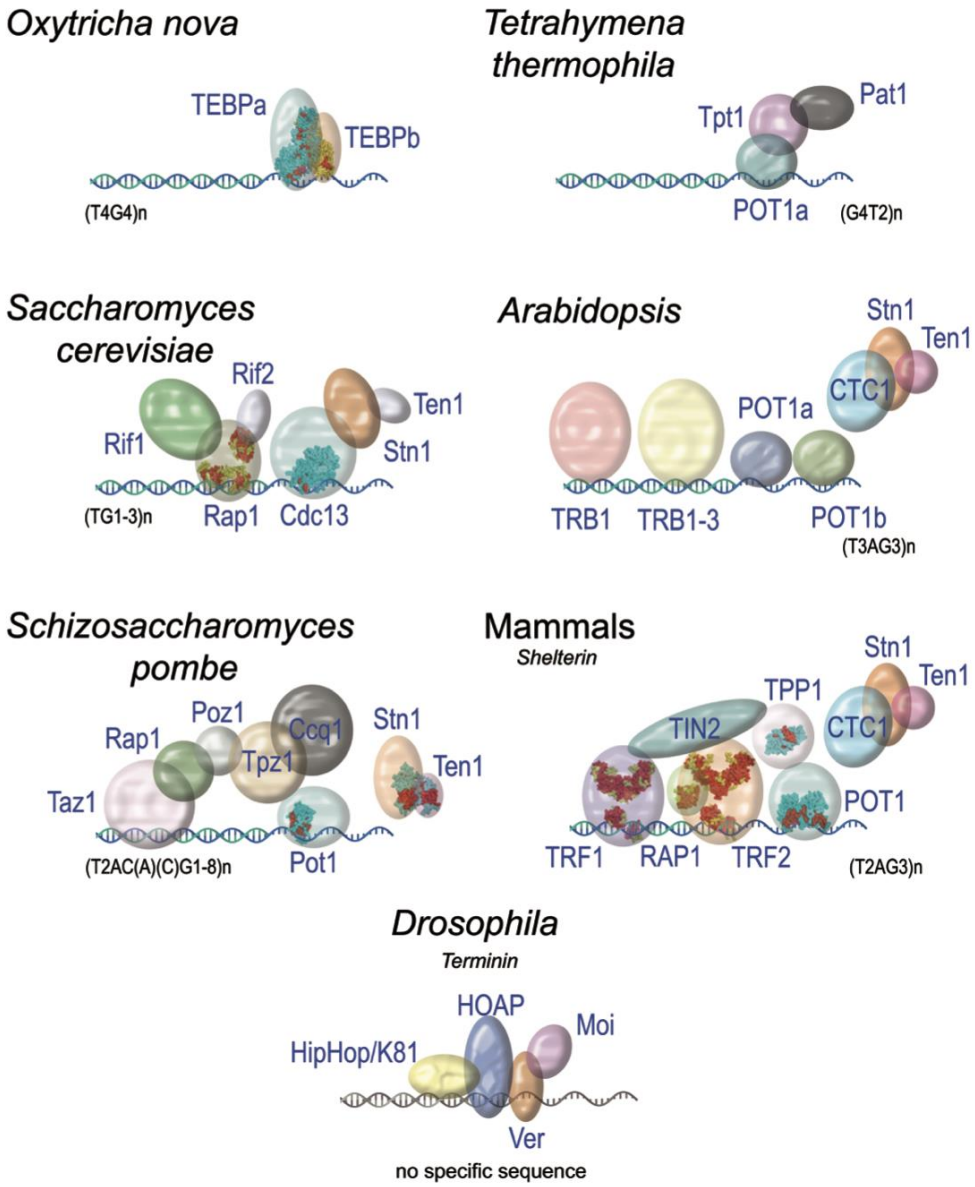


**Figure 1: Telomere sequences and telomere-associated proteins in different species.**
Extracted from Giraud-Panis *et al*., 2013.

The human telomere complex:

Human telomere sequences are between 5-15kb long and are bound by six proteins known as the shelterin complex that functions as a hub by recruiting proteins, regulating enzymatic activities, and controlling DNA conformation (Ghilain et al., 2021). The shelterin complex is composed of TRF1 (Telomeric Repeat binding Factor 1, (Chong et al., 1995)), TRF2 (Bilaud et al., 1996, 1997), TIN2 (TRF1-Interacting Nuclear protein 2,(Kim et al., 1999)), RAP1 (Repressor Activator Protein 1, (Li et al., 2000)), POT1 (Protection Of Telomere 1, (Baumann and Cech, 2001)) and TPP1 (previously named Tint1 – Ptop – Pip1 (Houghtaling et al., 2004; Liu et al., 2004; Ye et al., 2004a)). TRF1 and TRF2 directly bind the dsDNA as homodimers, POT1 binds the ssDNA while TPP1 and TIN2 form a protein bridge linking TFR1, TRF2 and POT1 together, and RAP1 binds TRF2 (**Figure 2**).

Telomeres were shown to adopt a folding back structure in several organisms, including human, named T-loop (Griffith et al., 1999). This structure, resulting from the invasion of the 3' overhang into the double strand telomeric DNA, create a closed conformation, protecting chromosomes from being recognized as DNA breaks and DDR activation. Other structures can be formed at telomere ends to ensure their protection (Giraud-Panis et al., 2013). The formation and maintenance of the T-loop is mediated by TRF2 thanks to its ability to wrap DNA (Benarroch-Popivker et al., 2016; Doksani et al., 2013).

POT1 binds the ssDNA G-rich and interacts with TPP1 (Rice et al., 2017). Together, they protect the telomeric tail from fusion by preventing the activation of DDR pathways via ATR (Ataxia–Telangiectasia and Rad3-related kinase), or alt-NHEJ (alternative Non Homologous End Joining) (Brambati et al., 2020; Kratz and de Lange, 2018). TPP1-POT1 are also involved in telomere length regulation by controlling the resection necessary to form a 3' overhang and by regulating the recruitment and the activity of telomerase with TIN2 (Grill et al., 2018; Pike et al.; Wu et al., 2012). TRF1 is mainly involved in replication and elongation of telomeric chromatin and protection against ATR. TRF2 is the major protector of telomeric ends by promoting the T-loop formation, inhibiting ATM (Ataxia–Telangiectasia-Mutated)-dependent repair, classical

NHEJ or homologous recombination (Karlseder et al., 2004; van Steensel et al., 1998; Wang et al., 2004). Importantly, the loss of TRF2 by proteasomal degradation mediated by SIAH1 (Seven In Absentia Homolog 1) in cells with short telomeres triggers DDR and p53 activation, leading to replicative senescence (Fujita et al., 2010).TRF2 also controls the resection to form the 3' overhang. RAP1 was also shown to have a redundant activity, by protecting short telomeres in the absence of TRF2 against NHEJ (Lototska et al., 2020). TIN2, is essential for the recruitment of TPP1 and POT1 and stabilizes TRF1 and TRF2 on telomeres (Takai et al., 2011; Ye et al., 2004b) (**Figure 2**).

Altogether, these proteins composing the shelterin complex are essential to protect telomeres from DDR and regulate lengthening and replication by serving as a hub to recruit diverse proteins required for their actions (Ghilain et al., 2021). Another protein complex is also at play to maintain telomere homeostasis. The CST complex is composed of three subunits, CTC1 (Conserved Telomere maintenance Component 1), STN1 (Suppressor of CDC Thirteen homolog) and TEN1 (Telomere length regulation protein homolog). It is localized of the ssDNA overhang and limits telomerase action to prevent over-elongation of telomeric DNA (Giraud-Panis et al., 2010; Stewart et al., 2018).

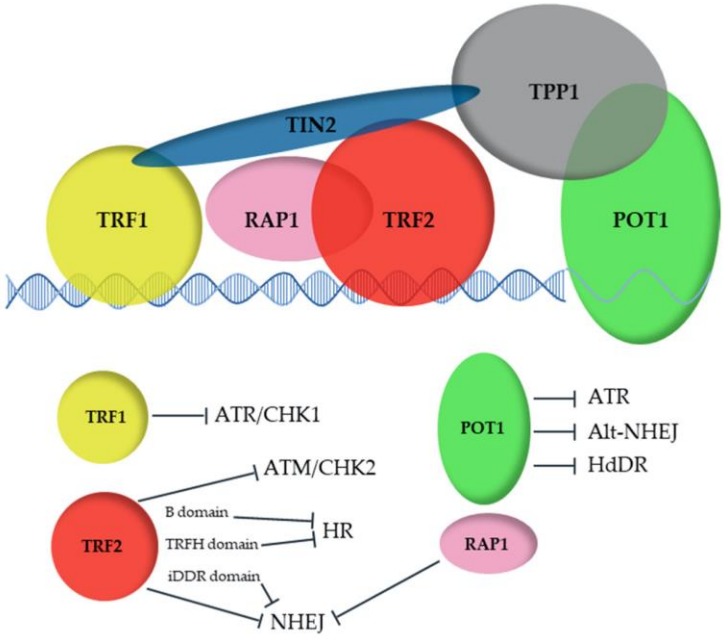


**Figure 2 : Protection against illegitimate activation of DDR pathways and repair pathways by the human shelterin complex.**
Extracted from Ghilain *et al.*, 2021.

Additionally, telomeres are transcribed by RNA polymerase II from the subtelomeric region towards the chromosome ends, using the C-rich strand as template, into long non-coding RNA named TERRA (telomeric-repeat containing RNA, (Azzalin et al., 2007; Schoeftner and Blasco, 2008)). This RNA component has been shown to be an essential component in telomere homeostasis as its deregulation causes telomere dysfunctions (Deng et al., 2009; López de Silanes et al., 2014). By promoting POT1 binding on ssDNA, TERRA was shown to participate in telomere protection (Flynn et al., 2011). TERRA has the ability to form DNA:RNA hybrids with telomeric DNA known as telomeric R-loops, to bind several proteins, and to form G-quadruplexes. With these functions, TERRA was shown to facilitate telomeric replication by recruiting ORC1 and forming R-loops (Beishline et al., 2017; Deng et al., 2009). By a direct pairing with TERC (the RNA component of telomerase), TERRA could inhibit telomerase (Redon et al., 2010) and therefore telomere elongation. Also, during telomere dysfunction upon TRF2 depletion, TERRA transcription is induced and can promote DDR activation (Porro et al., 2014a, 2014b). Upregulation of TERRA can also result in telomere dysfunction via the increased formation of R-loops (Sagie et al., 2017). Consequently, TERRA needs to be properly regulated in cells to favour telomere maintenance.

Altogether, telomere protection is regulated by different mechanisms, tertiary structure of the telomeric DNA, association with different protein complexes and involvement of RNAs. This complex protection mechanism prevents unwanted DDR activation and subsequent repairs, but it can also promote it, when necessary, to conduct cells into senescence or apoptosis for instance. It also promotes telomere maintenance and replication, by regulating telomerase activity or helping the recruitment of the replication machinery.

<u>The budding yeast telomeric complex:</u>

In the budding yeast *Saccharomyces cerevisiae*, the mammalian shelterin complex is poorly conserved.

Chromosome ends, in budding yeast, are composed of repetitive subtelomeric sequences known as the X element, found in all subtelomeres, and the Y' element in 0 to 4 tandem copies found between the X element and the telomeric tract (Louis and Haber, 1990). Telomeric sequences are ~300pb long and consist of simple $TG_{1-3}$ tandem repeats with a 3' G-rich overhang (Larrivée et al., 2004) (**Figure 3A**).Telomerase is constitutively expressed, and yeasts do not experience replicative senescence in a wild-type background. Importantly, yeast telomeric repeats are not embedded into nucleosomes, instead the dsDNA repeats are heavily bound by Rap1 (a homologue of human RAP1 with DNA binding properties) (Conrad et al., 1990; Gilson et al., 1993; Wright et al., 1992). Rap1 is a key telomeric protein and is required to prevent telomere fusions (Conrad et al., 1990; Lustig et al., 1990). Rap1 serves as an interacting partner recruiting Rif1/Rif2 (Rap1-Interacting Factor 1/2), involved in length regulation; and Sir3/4 (Silent information regulator 3/4) involved in silencing of subtelomeric regions (Hardy et al., 1992; Moretti et al., 1994; Wotton and Shore, 1997). The yeast Ku complex (Ku70 and Ku80) is thought to mediate the junction between dsDNA and ssDNA and is also critical for telomere protection (Gravel et al., 1998). The terminal ssDNA is bound by a CST complex composed of Cdc13, Stn1 and Ten1 (Grandin et al., 2001). Altogether, these proteins protect telomeres from undesirable DDR activation and regulate telomere length (**Figure 3B**).

The yeast CST complex prevents telomeres being recognised by an ATR-like dependent repair machinery (mediated by Mec1 kinase,(Hirano and Sugimoto, 2007)) and protects the 3' overhang from degradation (Grandin et al., 2001) during late S-phase and G2/M (Vodenicharov et al., 2010). Additionally, CST, via Cdc13, recruits the telomerase holoenzyme to telomeres (Chan et al., 2008). In non-replicative state (G1/G0), Rap1 is critical to cap telomeres. Together with Rif2, it prevents ATM-like dependent (Tel1 kinase) activation of DDR and limits end resection (Bonetti et al., 2010; Marcand et al., 2008; Vodenicharov et al., 2010). Rif1 is important to maintain viability in cells with compromised CST capping (Anbalagan et al., 2011). The Ku complex also exhibits capping activities by preventing excessive resection of DNA ends (Polotnianka et al., 1998; Vodenicharov et al., 2010). Additionally, these proteins are also implicated in telomerase regulation, Rif1 and Rif2 negatively regulate telomerase while Ku complex and Sir4 can recruit it at telomere ends (Lemon et al., 2019; Wotton and Shore, 1997).

Telomeric transcription can also be seen as a global feature of telomeres (Kwapisz and Morillon, 2020). In *S. cerevisiae*, telomeres are also transcribed into TERRA but it is rapidly degraded by an exonuclease (Luke et al., 2008). Telomere shortening was shown to induce TERRA expression and in return TERRA was proposed to act as a scaffold for the recruitment of telomerase (Cusanelli et al., 2013). Therefore, TERRA is implicated in telomere length homeostasis. In yeast, cells lacking telomerase activity experienced telomere shortening and senescence. Some cells could “evade” this senescence state by telomere elongation via homologous recombination (a phenomenon similar to ALT in human cells) (Lundblad and Blackburn, 1993). Via R-loop formation, TERRA could promote homologous recombination and consequently telomere elongation (Balk et al., 2013; Graf et al., 2017).

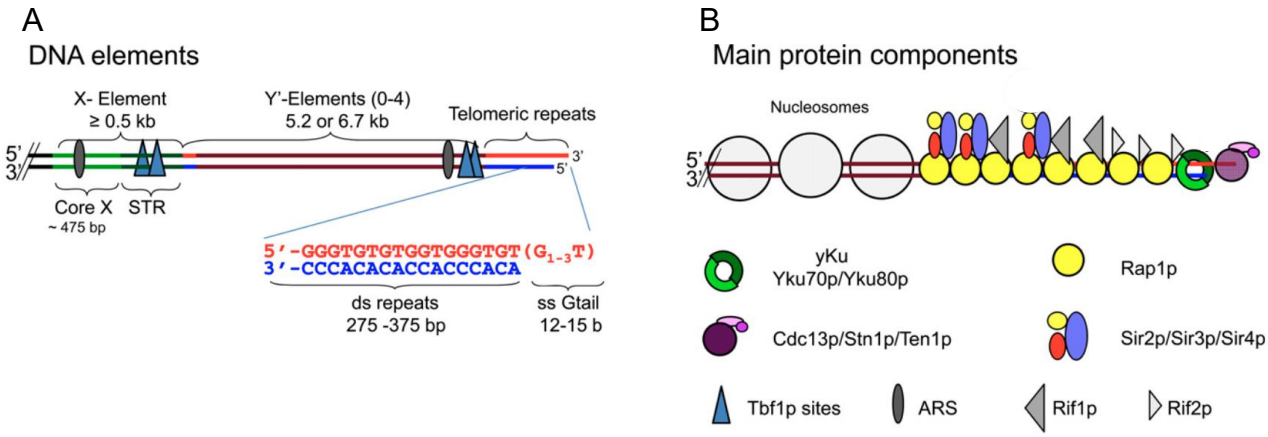


**Figure 3 : The *Saccharomyces cerevisiae* telomeres.**
A: sequences of telomeric and subtelomeric Y’ and X elements.
B: Major protein components of budding yeast telomeres.
Modified from Wellinger and Zakian 2012.

To conclude, telomeres are the nucleoprotein structures protecting the ends of linear eukaryotic chromosomes, preventing loss of genetic material and genome instability. The protein complexes composing telomeres with the addition of telomeric transcripts organize and regulate these different functions, ensuring a proper homeostasis of telomeres. A strong cellular perturbation or stress could lead to a rupture of telomere structure and/or function triggers cellular pathways, notably the DDR that stops cellular proliferation and leads to senescence or apoptosis. However, some mild stress,

sensed by telomeres have been proposed to also triggers cellular responses and ensuring a better adaptation to the environment (Jacome Burbano and Gilson, 2021; Ye et al., 2014).

## The chromatin structure of telomeres:

Originally, the definition of euchromatin and heterochromatin was based on microscopy observations by Heitz in 1928. Heterochromatin was defined as chromatin which remains condensed throughout the cell cycle. These terms are, now, more related to transcription. Euchromatin is associated with an open chromatin state accessible to transcription machinery. Reversely, heterochromatin is associated with transcriptional silencing and a close chromatin compartment with less accessible DNA. A molecular definition is therefore preferred, based on posttranslational modifications borne by histones, referred as the histone code (Jenuwein and Allis, 2001; Strahl and Allis, 2000) and on DNA methylation.

Based on early cytological observations, telomeres were commonly identified as packed in heterochromatin. They share other features of heterochromatin; they are often replicated late in S phase, in yeasts, (Davé et al., 2014; Hayashi et al., 2009) and they can induce an epigenetic repression in adjacent regions, known as TPE (Telomere Position Effect) (Baur et al., 2001; Gottschling et al., 1990; Koering et al., 2002; Nimmo et al., 1994). Moreover, a heterochromatic view of telomeres is in adequation with their repetitive nature and protective functions at chromosome termini. Telomeres are still often referred as constitutive heterochromatic regions, characterized by histone tails hypermethylation and hypoacetylation. However, recent findings described a more complex epigenetic signature of telomeres.

## Telomeric chromatin in yeasts:

In *Saccharomyces cerevisiae*, heterochromatin is characterized by nucleosomes devoid of histone marks and bound by a set of nonhistone proteins called Silent Information Regulators or Sir proteins (reviewed in(Gartenberg and Smith, 2016). As mentioned earlier, telomeric repeats are not wrapped around histones but are bound by Rap1 (Conrad et al., 1990; Wright et al., 1992). Therefore, according to a histone view of heterochromatin, telomeres in budding yeast cannot be defined as such.

Nonetheless, telomeres conserve features of heterochromatin. They replicate late in S-phase, a property regulated by the telomeric protein Rif1 (Davé et al., 2014). They also establish a heterochromatic compartment in subtelomeric regions by recruiting the Sir complex (described in detail in the Chapter 2).

*Schizosaccharomyces pombe* possesses a histone code similar to human with hypermethylated and hypoacetylated heterochromatin. Telomeres in fission yeast are short (~300 bp) and have long been proposed to form a nonnucleosomal structure as in budding yeasts (Chikashige et al., 1989). More recent analysis, based on MNase digestion (a nuclease that preferentially cleaves linker DNA between nucleosomes, revealing nucleosome position) exhibit an atypical, diffused pattern at telomeres. This indicates the presence of alternative nucleosome structures in telomeric position (Greenwood et al., 2018; Wang et al., 2016). It has been proposed that the shelterin proteins of fission yeast occlude the linker region between nucleosomes, impacting the MNase pattern (Greenwood et al., 2018). In addition, histone modifications have been identified at telomeres upon DNA damage (Carneiro et al., 2010). Whether the telomeric nucleosomes bear heterochromatic features is not clear. Greenwood *et al.*, on one side have shown that telomeric chromatin was enriched in H3K9me2, an epigenetic mark found in heterochromatin, however, histone methylation did not contribute to the structure of the telomere (the MNase pattern did not change when the methylation was impaired). On the other side, Wang et al. found that shelterin bound telomeric repeats were refractory to heterochromatin spreading, suggesting a non-heterochromatic state of the telomeric nucleosome.
Even if telomeres of *S. pombe* are late replicating (Hayashi et al., 2009) and can exhibit a position effect (Nimmo et al., 1994), it is still unclear whether they are heterochromatic.

## Telomeric chromatin in human:

The answer is not simpler in higher eukaryotes. In mammals, telomeric DNA is packaged in tightly space nucleosomes, being more compacted than bulk DNA (Lejnine et al., 1995). Despite a proposed model of heterochromatin mammalian

telomeres based on observations in mouse cells (Blasco, 2007), an increasing amount of evidences does not support this view, suggesting that telomeric nucleosome in human are more associated with euchromatin marks (Cubiles et al., 2018; O'Sullivan et al., 2010; Rosenfeld et al., 2009).

In human, somatic cells and telomerase positive cancer cells seem to be preferentially euchromatic. First, telomeres in mammalian cells are devoid of CpG island and cannot undergo DNA methylation. Second, ChIP-seq data from human CD4+ T cells showed that H3K9me3 and H4K36me3 (heterochromatin marks) are the less significant modifications, while the most significant are H2BK36me1 and H3K4me3 (euchromatin marks) (Rosenfeld et al., 2009). In agreement, a low level of heterochromatin marks (H3K9me3, H4K20me3 and H3K27me3) were found in telomeric sequences of fibroblasts by O'Sullivan *et al*. (2010). While Arnoult *et al.* (2012) also found a low density of H3K9me3, they also observed an H3K27me3 enrichment at fibroblast telomeres. Analysis of ENCODE ChIP-seq data from primary somatic cells and cancerous telomerase positive cells revealed that H3K9me3 was not enriched at telomeres, but H4K20me1 and H3K27ac (euchromatic mark) were (Cubiles et al., 2018). In this study, to avoid any "contamination" by non-telomeric $T_2AG_3$ repeats (named Interstitial Telomeric Sequences, ITS), a more stringent method was used in the ChIP-seq analysis (Cubiles et al., 2018; Vaquero-Sedas and Vega-Palas, 2019). Additionally, the epigenetic state of telomeres seems to be highly dynamic with variations during the cell cycle (O'Sullivan et al., 2010), with the recruitment of different histone modifiers at different phases. For instance, SIRT6 an H3K9 and H3K56 deacetylase (associated to heterochromatin) is recruited specifically during S-phase (Michishita et al., 2008, 2009). Altogether, it suggests that even if telomeres in somatic human cells exhibit a compacted structure, it is probably not due to their heterochromatin nature since treatment with inhibitors of histone deacetylases or methylases does not induce telomere decompaction (Bandaria et al., 2016; Vancevska et al., 2017).

On the contrary, telomerase negative cancer cells, which expand their telomeres through an ALT mechanism (depending on recombination), exhibit a different chromatin state. Cubiles *et al.* observed that U2OS cells, contrary to telomerase positive cells, exhibit an enrichment in H3K9me3 heterochromatic mark at

their telomeres. In agreement, Gauchier *et al.* have shown that SETDB1 (SET Domain Bifurcated 1), an H3K9 methyltransferase, was responsible for the deposition of H3K9me3 at ALT-telomeres; and that H3K9me3 was necessary to promote recombination and telomere lengthening. Similarly, the decrease of repressive H3K9me3, H4K20me3 and H3K27me3 marks, leads to telomere shortening in ALT-positive cells (Montero et al., 2018). It has also been observed that telomeres were enriched in H3K36me3, a histone mark associated with polymerase processivity, suggesting an atypical chromatin state promoting both compaction and transcription (Gauchier et al., 2019). In line, TERRA seems to be a central player in ALT. First, TERRA levels have been shown to be elevated in ALT-positive cells compared to telomerase positive cells. Second, through the formation of DNA:RNA hybrids, TERRA is thought to promote telomere recombination and telomere elongation (Arora and Azzalin, 2015). Third, TERRA has been shown to promote heterochromatin formation at telomeres, notably via the recruitment of the polycomb repressive complex PRC2, responsible of H3K27me3 deposition (Arnoult et al., 2012; Montero et al., 2018). Fourth, in cells lacking TERRA, ALT-telomeres were observed to shorten at each division (Arora and Azzalin, 2015; Montero et al., 2018). In ALT-positive cells, a virtuous circle between TERRA, recombination and heterochromatin seems to maintain telomere length homeostasis.

Altogether, these data demonstrate that the relation between heterochromatin and telomeres is not as obvious as initially thought. In human cells, it seems that the heterochromatic feature changes with the type of telomere maintenance used. While telomerase positive cells exhibit telomeres with euchromatic features, ALT-positive cells exhibited heterochromatic features. Additionally, heterochromatin at ALT-telomeres was shown as a prerequisite to maintain their homeostasis. Supporting this idea, in fission yeast, it has been shown that the loss of telomerase can be compensated by the amplification of heterochromatin blocks at termini (Jain et al., 2010).

Differences can also be noted among mammals; indeed, mouse cells seem to require heterochromatin at their telomeres, for them to be protected (Blasco, 2007).

Nonetheless, heterochromatin at telomeric sequences is not a conserved feature in eukaryotes. However, the epigenetic mechanism known as TPE, resulting in a heterochromatic nature of subtelomeric regions seems conserved.

## The signalling properties of Telomeres:

Telomeres are an essential structure in maintaining genome stability by protecting chromosome ends. However, it is not their sole function in cells. Telomeres can regulate gene expression.

Telomeres were shown to respond to a wide variety of stress, *i.e.* factors that lead to a rupture of homeostasis. Indeed, both endogenous and environmental factors were shown to modulate telomere length positively or negatively (reviewed in (Jacome Burbano and Gilson, 2021)). Of note, telomere length is one marker of telomere homeostasis, widely studied, notably because of the relative technical facility by which it can be assessed. Nonetheless disruption of telomere integrity can result in changes in telomere composition (shelterin factors), accumulation of damages at telomeres, change in TERRA expression levels…

One of the primary responses of dysfunctional telomeres is processed through DDR pathways. In somatic human cells, telomeres will shorten as a consequence of the end-replication problem, of replication fork collapse or t-loop excision. When telomeres can no longer protect chromosome ends, DNA-damage signalling accumulates at the dysfunctional telomeres and activates the DDR/p53 senescence pathway (d'Adda di Fagagna et al., 2003). Interestingly, it has been shown that five damaged telomeres are enough to trigger p53 activation and replicative senescence entry (Kaul et al., 2011). Telomeres do not have to be short to be sensed as dysfunctional. Due to their ability to repress DDR and prevent chromosomal fusions, the telomeric tract is irreparable. As a consequence, under genotoxic insults or oncogenic stress (two conditions that increase DNA damages), telomeres accumulate persistent damages and drive a senescence response (Fumagalli et al., 2012; Suram et al., 2012).

However, other mechanisms have been described to explain how stress-sensitive telomeres could translate their dysfunctions in an appropriate transcriptional response by the cell. Telomeres have been shown to control the redistribution of telomeric factors that could directly regulate gene expression at extra-telomeric loci. TERRA has also been proposed to regulate gene expression (Ye et al., 2014).

## The redistribution of telomeric proteins:

In *Saccharomyces cerevisiae*, Rap1 binds telomeric sequences, mediates telomere protection, and recruits silencing factors. Interestingly, Rap1 was initially described as a transcriptional regulator, binding upstream promoter sequences, and interacting with various coactivators (Schawalder et al., 2004; Shore and Nasmyth, 1987). Additionally, telomeres in yeast were shown to cluster at the nuclear periphery and to sequester large amounts of Rap1. This led to the hypothesis that a decrease in telomere length drives a dispersion of Rap1 molecules throughout the genome allowing their binding at extra telomeric sites to regulate gene expression (Gotta et al., 1996; Maillet et al., 1996; Marcand et al., 1996; Ye et al., 2014).

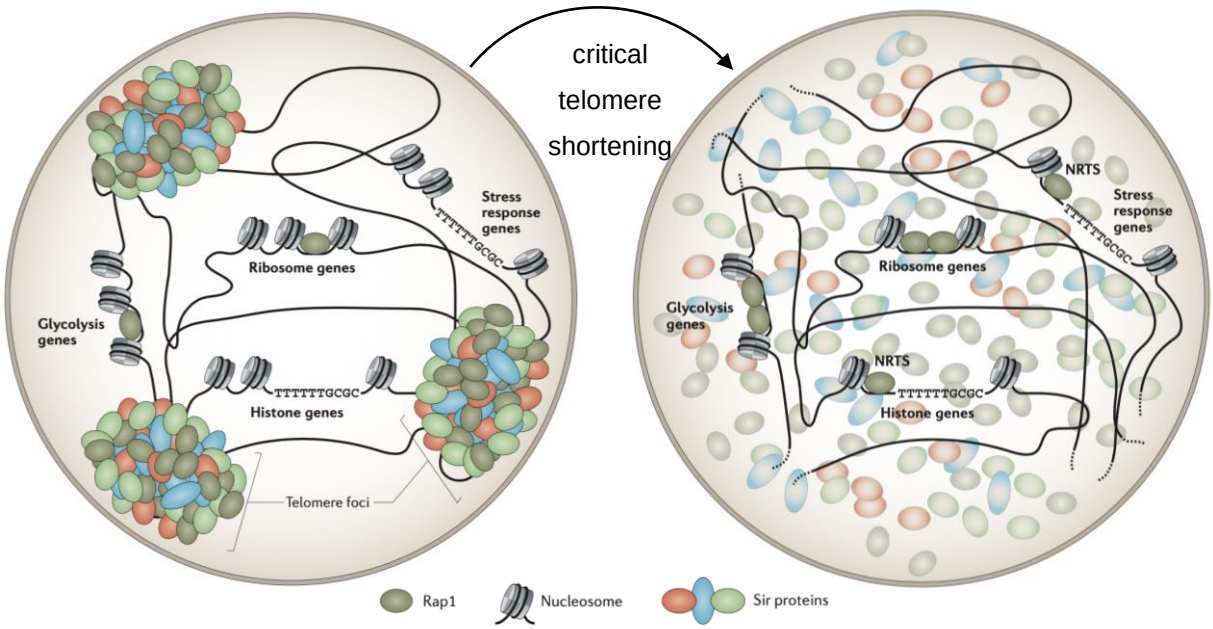


**Figure 4 : Redistribution of Rap1 in the nucleus upon telomere shortening in *Saccharomyces cerevisiae***
In budding yeast, telomeres cluster at nuclear periphery and sequester Rap1. When telomeres became critically short, Rap1 binds new target in the nucleus (NRTS) and regulates their transcription. Redistribution of Rap1 at replicative senescence participate to the remodelling of transcription. Modified from Ye *et al*., 2014

In 2013, Platt *et al.* used a telomerase-deficient yeast, a common model to study telomere shortening-driven senescence, to observe how Rap1 localisation is affected. In senescent cells (with short telomeres), Rap1 enrichment increases at internal loci already targeted by Rap1 in WT cells, Rap1 occupancy decreases in subtelomeric

regions, and interestingly, they identified ~500 new Rap1 targets at senescence (NRTS). These new targets of Rap1 are not strongly regulated but are usually associated with an upregulation in senescent cells; with the notable exception of several histone genes which are repressed by Rap1 (Platt et al., 2013; Song et al., 2020) (**Figure 4**). This downregulation of histone genes by Rap1 was shown to affect the rate of senescence, as downregulation of Rap1 or overexpression of histones delay senescence. However, the upregulated NRTS genes, involved in metabolism and stress responses, were shown not to impact the pace of senescence (Song et al., 2020). It suggests that, even if implicated in transcriptional changes seen at senescence, their regulation is more a consequence than a cause. With this view, it could be interesting to understand which cellular adaptation are drove by the transcriptional regulation of Rap1.

Interestingly, *S. cerevisiae* Rap1 is not the only telomeric protein that could regulate the transcriptome. Taz1 (a homologue of mammalian TRF proteins) in *Schizosaccharomyces pombe* was also shown to regulate heterochromatin at non-telomeric sites. Taz1 binds telomeric repeat-like elements and recruits the other shelterin proteins. Together, they interact with Clr4, the methyltransferase responsible of H3K9me3, resulting in the epigenetic silencing of adjacent genes (Zofall et al., 2016). To my knowledge, it is not known whether telomere dysfunction influences gene regulation mediated by Taz1. However, heterochromatin islands contain genes that are repressed during normal growth conditions but are induced under stress. Whether telomeres could be implicated is intriguing (Chen et al., 2003).

Human shelterin proteins were also shown to impact gene regulation at extra-telomeric sites. Indeed, TRF2, TRF1 and RAP1 can bind extra-telomeric sequences, usually corresponding to Interstitial Telomeric Sequences (ITS) and are preferentially located in genic regions (Simonet et al., 2011; Yang et al., 2011). The interstitial binding of telomeric proteins is associated with transcription regulation of the nearby genes. For instance, TRF2 was shown to bind and transactivate PDGRFβ in endothelial cells (El Maï et al., 2014), as well as HS3ST4 in transformed fibroblasts (Biroccio et al., 2013; Cherfils-Vicini et al., 2019). Recently, telomere elongation was shown to induce a reduction of TRF2 occupancy at gene promoters leading to an alteration of gene transcription in association with epigenetic changes (Mukherjee et al., 2018).

Taken together, telomeric proteins have non-telomeric activities and can notably regulate gene expression. It has been shown, at least for TRF2 in human and Rap1 in budding yeast, that telomere length influences the redistribution of telomeric factors throughout the genome, resulting in telomere-dependent regulation of transcription.

## TERRA and gene transcriptions:

It has been shown that long non-coding RNA could regulate transcription through interaction with chromatin modifiers and transcription machinery (reviewed in (Yao et al., 2019)). Therefore, I shall discuss what could be the potential functions of TERRA in gene transcription regulation.

The regulation and biogenesis of TERRA is still under investigations (reviewed in (Barral and Déjardin, 2020)). However, the state of the telomere seems to influence its transcription. First, TERRA transcription in human cells, seems to be dependent on telomere length. Indeed, telomere elongation was shown to repress TERRA expression depending on H3K9me3 and HP1α deposition (Arnoult et al., 2012; Kim et al., 2016). However, those two repressive marks were not enriched at TERRA promoters, nor did they impair Pol II recruitment, but were found in telomeric repeats, suggesting an impairment of TERRA elongation. Additionally, TERRA length was shown to be proportional to telomere length (Arnoult et al., 2012; Viceconte et al., 2021). TERRA expression was also observed to increase upon telomere deprotection, when TRF2 was depleted (Porro et al., 2014a) or upon heat-shock (Koskas et al., 2017). Therefore, TERRA transcription seems to be driven by different stressors and to respond to the telomeric state.

As other long non-coding RNAs, TERRA was also shown to interact with chromatin modifying enzymes. At telomeres, TERRA has been shown to interact with SETDB1; with EZH2 (Enhancer of Zest Homologue 2), a subunit of the Polycomb Repressive Complex 2 (PRC2) responsible of H3K27me3; with SUV39H1 (Histone-lysine N-methyltransferase SUV39H1), another H3K9me3 methylase; and LSD1 (Lysine-specific demethylase 1) known to remove methyl groups from H4K9 and H3K9 (Gauchier et al., 2019; Montero et al., 2018; Porro et al., 2014a, 2014b). Because in

mouse cells, TERRA has been shown at the inactive X (Zhang et al., 2009), it has been proposed that it could regulate gene expression in *trans*. One possible mechanism could be through the interaction between TERRA and TRF2 (Deng et al., 2009) at extra telomeric sites. However, this interaction remains to be demonstrated at intra-chromosomal loci.

To my knowledge, investigation on TERRA dependent transcriptional regulation has been done only in cancer cells. This study reveals that telomere elongation increases TERRA signal, a result that is in discrepancy with others, and results in an alteration of the transcriptome. They proposed that TERRA regulates gene expression through the formation of G-quadruplex (G4) structures which are recognized by G4 binding proteins which in turn silenced the targeted locus (Hirashima and Seimiya, 2015).

These interesting properties of TERRA need further exploration to understand whether TERRA is a general transcription regulator in normal cells. However, the lack of understanding in its regulation and the difficulty to modulate *in vivo* its expression, complicate the investigations.

# Conclusion:

Taken together, these results highlight the interaction between the protective nature of telomere and its ability to sense stress and induce a transcriptional response. Telomeric factors, proteins, or RNA, were shown to be dispersed in response to, at least, change in telomere length. This dispersion allows extra-telomeric functions of telomeric components. Even if some have transcriptional properties, it is not the only response that could be driven by telomeres in cells. For instance, telomeres and mitochondria seem closely intertwined, as telomere shortening is exacerbated by oxidative stress, and a knock-out of each shelterin shows an impact in mitochondrial metabolism (Kim et al., 2017; von Zglinicki, 2002).

The first discovered mechanism by which telomeres influence gene expression is the Telomere Position Effect (Gottschling et al., 1990). This epigenetic mechanism regulated by telomeres is the main focus of my thesis and will be extensively described in the following chapters. Of note, redistribution of telomeric factors and TERRA functions are found in cooperation with TPE ensuring several layers of regulation driven by telomere.

Because in human somatic cells, telomerase is inactivated, and telomeres shorten gradually, and because somatic cells cannot proliferate indefinitely, as observed by Hayflick (Hayflick, 1965), telomeres have long been proposed to act as a mitotic clock, governing the number of cell division. Because telomeres are proposed to govern transcription, one can ask whether this mitotic clock and the regulation of transcription are linked. Could the theory of programmed aging, originally developed by Weismann in 1890, find part of the answer in this link?

# Chapter 2: Telomere Position Effect, a peculiar Position Effect of Variegation:

Position Effect Variegation (PEV), an epigenetic mechanism, was first defined as such because the change in gene location, *i.e.* epigenetic environment, had an effect on its expression resulting in a variegated phenotype. It was first observed in 1930 by Hermann Muller in *Drosophila melanogaster*. After X-ray irradiation, a mutant, $w^{m4}$ exhibited an inversion of the *white* gene locus, resulting in its switch from euchromatin to pericentromeric heterochromatin. This positional change resulted in cell-to-cell variation in gene expression, leading to a mosaic phenotype of drosophila eye colour with white and red adjacent patches (Muller, 1930). Indeed, *white* functions as an ATP binding cassette transporter, carrying brown/red pigment precursor in drosophila's eyes. Therefore, its inactivation results in white eyes. It was among the first evidence of the role of localisation and epigenetics in the regulation of gene expression. Screening for mutations enhancing (*E(var)*) or suppressing (*Su(var)*) variegation of *white* gene allowed the identification of epigenetic factors and a better understanding of epigenetics in general (Elgin and Reuter, 2013) (**Figure 5**).

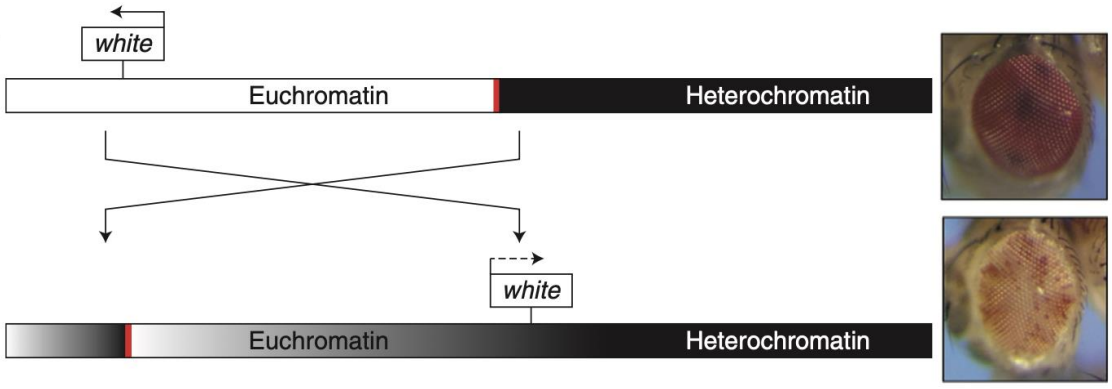


**Figure 5** : **Position Effect of Variegation**
Position Effect of Variegation of *white* in drosophila.
Rearrangement attributable to an X-ray-induced inversion places the white locus, normally located in the distal euchromatin (white bar) of the X chromosome (see top line), ~25 kb from a breakpoint in the pericentric heterochromatin (black bar; bottom rearranged line). Spreading of heterochromatin packaging into the euchromatic domain results in silencing (causing a white eye in this case); loss of silencing in some cells during differentiation results in a variegating phenotype (bottom line, right). Extracted from Elgin and Reuter, 2013.

PEV, in drosophila, was described to take place in constitutive heterochromatic regions such as centromeric and pericentromeric regions, as well as in the Y and fourth small chromosomes. In 1984, it was reported for the first time by Hazelrigg and colleagues that the *white* gene inserted in a subtelomeric region exhibited a variegated expression. Moreover, they observed that when the *white* gene was removed from its subtelomeric location and placed elsewhere in the genome, they reverted the variegated phenotype. It was already described that *Drosophila* telomeres were morphologically heterochromatic. However, it was the first time that an effect of telomere chromatin environment on gene expression was observed (Hazelrigg et al., 1984).

In 1990, Gottschling *et al.* observed in *Saccharomyces cerevisiae* a transcriptional repression of transgenes when inserted near telomeres. They introduced *URA3* (required for uracil biosynthesis) into the *ADH4* locus, located at 15kb from the telomere of chromosome VII-L, of a haploid yeast strain that contained *ura*$^-$ alleles. When *URA3* with an adjacent 81bp stretch of ($TG_{1-3}$) is inserted, eliminating the distal DNA part, and allowing a newly formed telomere at 1.3kb from the promoter, they observed a repression of *URA3* transcription. Repression that did not occur when *URA3* was inserted in the wild-type *ADH4* locus. This repressed state conferred by telomeres was mitotically inherited over generations, but an epigenetic switch could change subtelomeric genes to an active transcriptional state and therefore showed a variegated phenotype. Moreover, when *URA3* is inserted in the *ADH4* locus with an internal tract of ($TG_{1-3}$), they did not notice a decrease in mRNA level. Nevertheless, this internal telomeric sequence can spontaneously become a chromosomal end in a few clones, resulting in a repression of *URA3* expression. These observations demonstrate that ($TG_{1-3}$) sequences *per se* were not sufficient to cause a position effect and that only the telomere itself is responsible of this regulation (Gottschling et al., 1990).

Altogether, they demonstrated that telomeres were able to repress gene expression through an epigenetic mechanism baptised Telomere Position Effect (TPE). It was the first formal proof that, together with its protecting role, telomeres could also influence gene expression.

Ten years later, Telomere Position Effect was identified in human cell lines by two independent teams. They observed that introducing a reporter gene (either luciferase or eGFP) at the vicinity of a newly formed telomere, but not at an internal location, reduced its expression. Moreover, they observed that trichostatin A (TSA) treatment, an inhibitor of histone deacetylases, increased the expression of the telomeric reporter but not the internal one. Thus, that TPE in human cells is also an epigenetic mechanism. These pioneering works demonstrated that human telomeres, by influencing the epigenetic state of its neighbour chromatin, could impact gene expression (Baur et al., 2001; Koering et al., 2002). This discovery linked the mitotic clock to gene regulation, opening a new field of investigation on whether subtelomeric genes could be regulated by “aging”.

## TPE mechanisms, a process of heterochromatinization:

Telomere Position Effect is an epigenetic mechanism that is dependent on telomere length, telomere nucleoprotein composition and higher order chromatin organization adopted by telomeres and subtelomeres. It is described as the spreading of heterochromatin, nucleated at telomeres, toward centromeres. This heterochromatinization process of subtelomeric regions results in silencing of subtelomeric genes in a telomeric-dependant manner.

In this part, I shall describe how telomeres recruit heterochromatin factor, how heterochromatin spreads and how the spreading is regulated in different model organisms.

The heterochromatin formation emanating from telomeres was discovered and extensively studied in the budding yeast *Saccharomyces cerevisiae*. Therefore, I will take the time to describe what is known in this species.

Because heterochromatin formation in *Schizosaccharomyces pombe* is closer to what is found in higher eukaryotes, I will describe the epigenetic state of fission yeast subtelomeres and its regulation.

Finally, TPE has been discovered in human, however little is known on how telomeres regulate the epigenetic state in their surrounding regions. Therefore, I will detail what is known and/or still puzzling in human TPE.

## TPE mechanisms in *S. cerevisiae*:

As described previously, telomeres in budding yeast are nucleosome free regions, constituted of $TG_{1-3}$ repetitions bound by Rap1. The heterochromatic feature of the budding yeast subtelomeres is due to the recruitment of the Sir (Silent information regulator) complex at telomeres (Aparicio et al., 1991; Kyrion et al., 1993) that spread towards the centromere, compacting nucleosomal DNA. Importantly, heterochromatin in budding yeast is devoid of post-translational histone modifications thus nucleosomes are hypomethylated and hypoacetylated. Heterochromatin is found in subtelomeric regions, silenced mating-type loci, and ribosomal DNA arrays (rDNA). The Sir complex at telomeres is composed of 3 proteins: Sir2 a $NAD^+$-dependent histone/protein deacetylase (or sirtuin); Sir3 a nucleosome binding protein; Sir4 a scaffold protein necessary for the complex assembly.

### Telomeric recruitment of silencing factors initiate the spreading of heterochromatin:

Sir complex is recruited to the centromeric part of the telomeric tract by Rap1. It has been shown that Sir4 and Sir3 colocalize with telomeres (Cockell et al., 1995; Gotta et al., 1996) and interact directly with the C-terminus domain of Rap1 in a double hybrid system (Moretti et al., 1994). This interaction is essential for the subtelomeric heterochromatin formation, as expression of C-terminus truncated Rap1 mutant abolished position effect (Kyrion et al., 1993). Additionally, Sir4 persists even in the absence of the other Sir proteins at the centromeric extremity of telomeres (Hoppe et al., 2002; Luo et al., 2002) and the artificial tethering of Sir4 at telomere enhances TPE even in the context of a mutated Rap1 (Marcand et al., 1996). Even if Sir3 also interacts directly with Rap1, it has been shown that disruption of this interaction had little impact on silencing in the subtelomeric region contrary to Sir4 (Moretti and Shore, 2001). It has been proposed that the high abundance of Rap1 protein at telomeres (Gilson et al., 1993) favoured and stabilized Sir4 binding (Luo et al., 2002). Concomitantly with a requirement of numerous Rap1-Sir4 interactions to promote TPE, Sir4 has been shown to be in competition with Rif1 and Rif2 (Rap1-interacting factor) to bind Rap1.

Increasing Rif proteins decrease TPE whereas their deletion increase TPE (Bourns et al., 1998; Kyrion et al., 1993; Moretti et al., 1994). In addition, it has been shown that Sir4 could also binds telomeres through its interaction with the Ku70/80 complex and this interaction is sufficient to nucleate the silencing complex and induce TPE (Boulton and Jackson, 1998; Martin et al., 1999; Tsukamoto et al., 1997). It has been proposed that Ku heterodimer helps the recruitment of Sir proteins at telomeres by impacting the competition between Sir4 and Rif1/2 to bind Rap1. Ku70/80 helps but do not replace Rap1 to induce silencing (Mishra and Shore, 1999). All together, these results support that binding of Sir4 at telomeres through Rap1 and/or Ku complex is the first step allowing the nucleation of the Sir complex before its spreading. Sir4 interaction with telomeres allows the recruitment of Sir2 and Sir3. Indeed, Sir4 binds Sir2 and Sir3, but the last two do not interact directly (Moretti et al., 1994; Strahl-Bolsinger et al., 1997), forming *in fine* the silencing complex (**Figure 6**).

The spreading of the sir complex toward centromeres relies on the histone deacetylase activity of the Sir2 and the Sir3 affinity for unmodified histone H4. Indeed, Sir2 lacking catalytic activity, abrogates the spreading (Ellahi et al., 2015; Hoppe et al., 2002; Imai et al., 2000). Deacetylation of histone H4, specifically of the Lysine 16, allows the docking of Sir3 (Aparicio et al., 1991; Hecht et al., 1995, 1996; Johnson et al., 2009) that in turn recruits Sir4/Sir2 complex, expanding the growing of the silent chromatin on adjacent histones. Loading of the Sir complex on nucleosomes increases the steric hindrance, as demonstrated by a reduced accessibility for restriction enzyme digestion or Dam DNA methyltransferase (Gottschling, 1992; Oppikofer et al., 2011), resulting in a repression of the underlying genes (**Figure 6**).

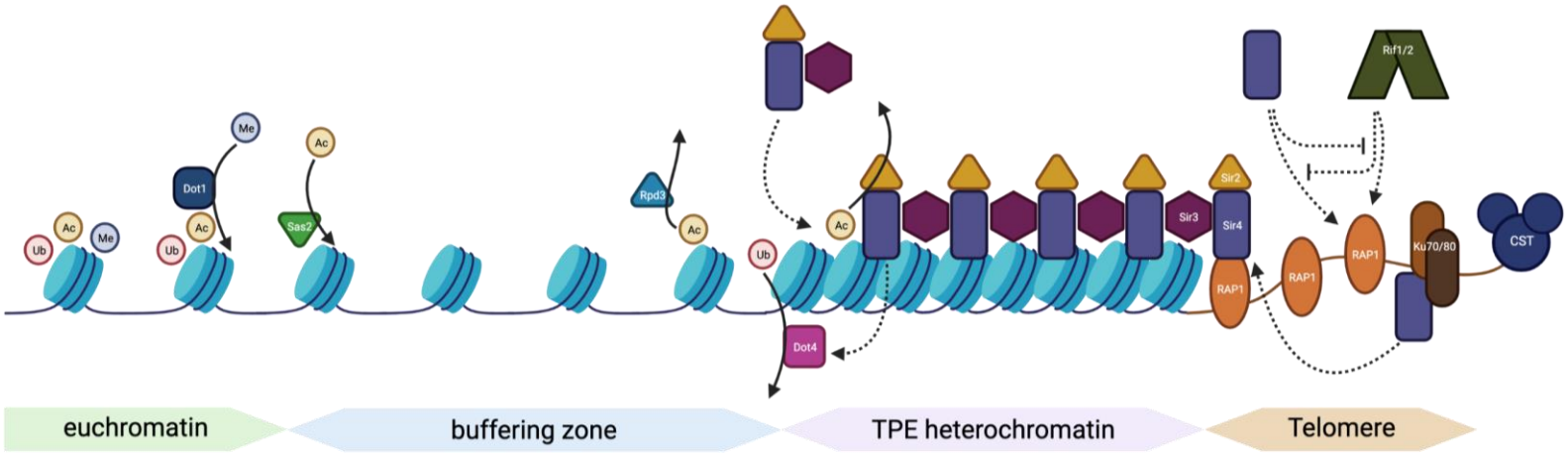


**Figure 6: Epigenetic of TPE in *Saccharomyces cerevisiae***
Epigenetic regulation of telomeric position effect in *Saccharomyces cerevisiae.* TPE is regulated by telomeric-bound proteins. Rap1 recruit the Sir complex that propagate and condense chromatin in centromeric direction to form heterochromatin. Sir spreading is counteracted by a buffering zone established by Rpd3 deacetylase. Sas2 and Dot1 ensure the deposition of euchromatin mark and form a strong boundary to Sir spreading.
Inspired from Ottaviani et al., 2008 and created with BioRender.com

## A discontinuous spreading of heterochromatin:

This sequential-spreading model of the Sir complex along chromatin in subtelomeric regions predicts an uninterrupted silencing in subtelomeric regions. However, analysis of chromatin distribution of Sir proteins at natural budding yeast subtelomeres showed a discontinuous binding of the complex (Ellahi et al., 2015; Radman-Livaja et al., 2011). It has been shown that X and Y' subtelomeric regions contained subtelomeric repeats named STAR (SubTelomeric Antisilencing Region) that could act as an insulator and disrupt Sir complex spreading whereas core X and internal Y' sequences could promote silencing. This effect of subtelomeric elements results in a discontinuous silencing emanating from telomeres (Fourel et al., 1999; Lebrun et al., 2001). Another explanation for this discontinuous silencing is the involvement of 3D chromatin structure, like telomeric loops. Indeed, it has been shown that telomeres could form a back-folding structure to inhibit a reporter gene (de Bruin et al., 2001; Poschke et al., 2012). Moreover, it has been observed, that Rap1 could also be associated punctually with subtelomeric chromatin together with the Sir complex but did not spread and this association was lost upon telomeric transcription, probably due to the disruption of a looping structure (Bruin et al., 2000; Strahl-Bolsinger et al., 1997). Recently, thanks to chromosome conformation capture, Wagner *et al.* demonstrate that telomeres formed a fold-back structure promoted notably by chromatin modifiers, including Sir2 (Wagner et al., 2020). Although the transcriptional effect of this folding has not been tested in a natural subtelomeric context, it is tempting to propose it as part of an explanation to the discontinuous spreading of Sir silencing (**Figure 7A**).

## A tight control of the heterochromatinization process in subtelomeres:

Sir mediated silencing in subtelomeric regions has been shown to be restricted in the first kilobases from a telomere and to be discontinuous. Considering the ability of an autonomous spreading of the Sir complex, endogenous mechanisms exist to counteract the spreading and to avoid silencing of essential genes (**Figure 6**). Due to Sir2 activity and Sir3 affinity for naked nucleosomes, silenced chromatin in budding

yeast is usually devoid of histone marks. Consequently, post translational modifications on histone residues have been shown to exhibit an anti-silencing effect, the most common being H4K16Ac and H3K79me1/2/3. Sas2 (Something about silencing 2), a histone acetyltransferase, accounts for H4K16 acetylation. A barrier to spreading in subtelomeric regions is created due to the competition between Sas2 and Sir2 for the acetylation status of H4K16 (Kimura et al., 2002; Suka et al., 2002). Intriguingly, domains without H4K16Ac extend far beyond Sir complex binding which is counter-intuitive regarding this competition model. It has been proposed that Rpd3L deacetylase complex creates this transition zones devoid of acetylation impairing the binding of the Sir complex. Indeed, active deacetylation by Sir2 has been shown to be necessary for a stabilized binding of Sir complex on nucleosomes (Ehrentraut et al., 2010; Oppikofer et al., 2011; Zhou et al., 2009). In addition, H4 dependent acetylation by Sas2 was shown to be important for the deposition of H2A.Z, a histone variant exhibiting barrier activity (Raisner et al., 2005; Shia et al., 2006). H4K16Ac and H2Bub (H2B ubiquitination) has also been shown to be essential for the deposition of methylation marks on H3K79 by Dot1 (Disruptor of telomeric silencing 1) (Altaf et al., 2007; Gardner et al., 2005). H3K79 methylation impairs binding of Sir complex and blocks silencing, therefore, Sir4 was shown to recruit Dot4 to deubiquitinate H2B and impair Dot1 binding and H3K79me deposition (Gardner et al., 2005). The competition between chromatin marks create a buffering zone. Moreover, Hocher *et al*. 2018, observed that upon Sir3 over-expression, silenced chromatin from telomeres extended in centromeric direction but was blocked by the H3K79 trimethylated mark. Deletion of Dot1 in this context allowed the silencing domain to encompass essential genes and caused cell lethality (Hocher et al., 2018). Altogether, the robustness and redundancy of mechanisms regulating the Sir complex spreading illustrates the importance of a proper regulation of the TPE in *Saccharomyces cerevisae*.

## The spatial organization is essential for a proper TPE:

Last but not least, the spatial organization of telomeres in the nucleus and TPE are closely intertwined in budding yeast. Indeed, the 32 telomeres are clustered to the nuclear membrane within 3 to 7 foci (**Figure 7**). This clustering is dependent on Sir

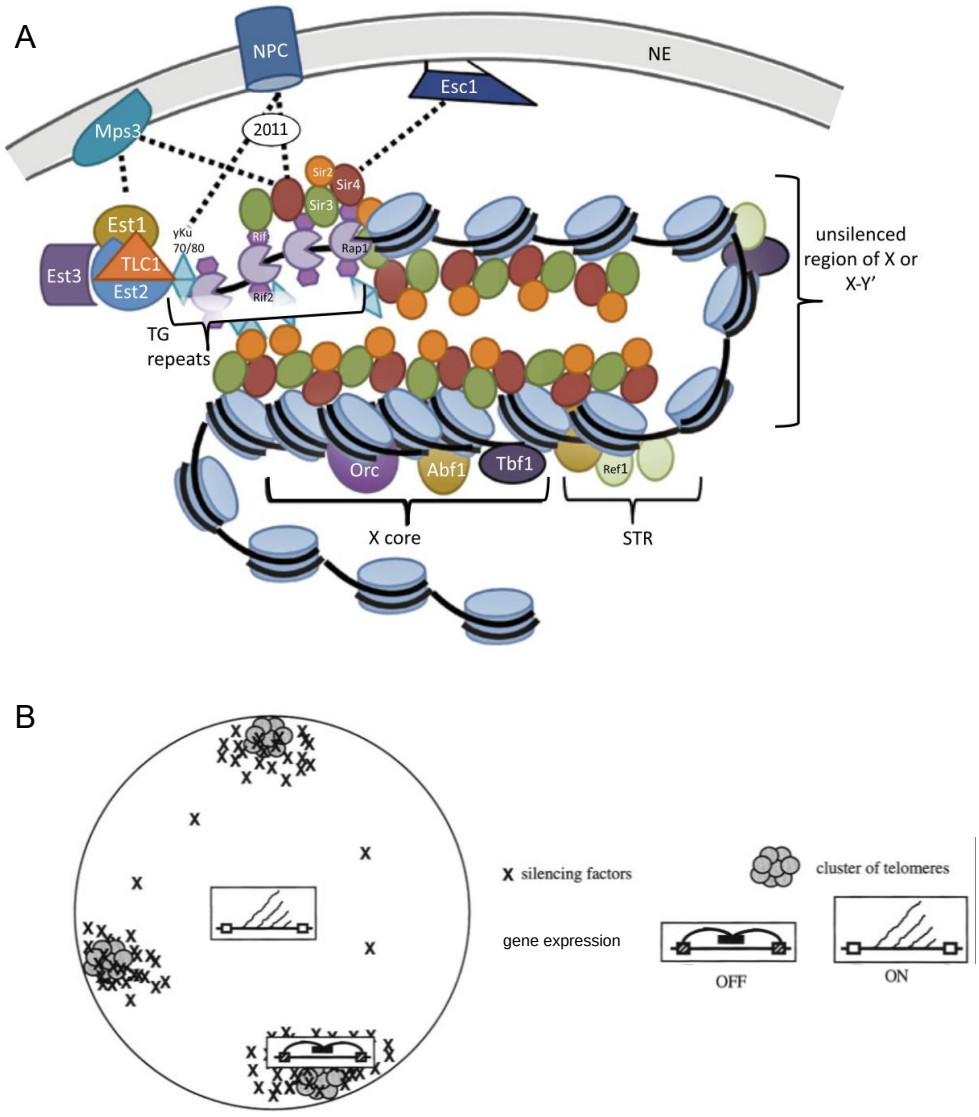


**Figure 7 : 3D chromatin organisation of *S. cerevisiae* telomeres**
A: Model for telomere and subtelomere nuclear architecture and links with molecular components of the nuclear periphery (dashed lines) in *Saccharomyces cerevisiae*. From Fabre and Spichal, 2014.
B: Reservoir model of silencing factor sequestrated at telomeres near the nuclear periphery. Adapted from Maillet *et al.*,1996.

protein binding to Rap1 as deletion of Sir3 and Sir4 disrupted this phenotype and decrease contact between telomeres (Gotta et al., 1996; Maillet et al., 1996; Palladino et al., 1993; Ruault et al., 2021). This peripheral localization is dependent on redundant pathways involving Ku complex and Sir4 (Laroche et al., 1998; Taddei et al., 2004). Anchoring properties of Sir4 involved several docking partners such as Esc1 (a protein associated to the nuclear pore complexes and the inner nuclear membrane), Nup170

(a nucleoporin) and Mps3 (a nuclear envelope protein) (Bupp et al., 2007; Deshpande et al., 2019; Taddei et al., 2004; Van de Vosse et al., 2013). Since Sir4 and Ku complex interact directly, the two anchoring pathways might crosstalk. Additionally, without anchoring properties, Sir3 has been shown as an important player of the clustering, increasing contact between telomeres and subtelomeres both in *trans* and in *cis* (Ruault et al., 2021). Conversely, localization of telomeres at the nuclear membrane is important for a proper repression of subtelomeric regions. When the telomere anchoring pathways were disrupted, a loss of TPE (an increase in subtelomeric gene expression) was shown, whereas internal genes were misregulated dependent on SIR proteins (Taddei et al., 2009). Telomeric clustering acts as a reservoir compartment sequestering and concentrating silencing factors at its vicinity (Maillet et al., 1996) (**Figure 7B**). Indeed, the supply of Sir proteins is limited, and it appears that it is necessary to achieve a critical local concentration for silencing (Gotta et al., 1996; Maillet et al., 1996; Marcand et al., 1996).

## Conclusion:

The ongoing elucidation of TPE in *Saccharomyces cerevisiae* points out the interconnection between spatial organization of the nucleus, regulation of histone post-translational modifications, protein-protein interactions, and telomeres. A perturbation occurring in one of these regulation processes has an impact on the silencing properties emanating from telomeres. Subsequently, telomere length is an important factor regulating TPE. Telomere elongation or telomere shortening provoke changes in the protein content resulting in an enhancement or loss (respectively) of subtelomeric heterochromatin (Kyrion et al., 1993; Nautiyal et al., 2002; Renauld et al., 1993). Later in this manuscript I shall address the relevance of this repressive mechanism in terms of stress response and aging in several organisms, including *S. cerevisiae*.

## Atypical telomeres in drosophila exhibit TPE:

*Drosophila melanogaster* is at the basis of genetic studies. As mentioned at the beginning of this chapter, this model organism shed light and stets the fundament of what we know today on epigenetics. Importantly, in *Drosophila*, telomerase is absent (Sasaki and Fujiwara, 2000) and their telomeres are not composed of short GC-rich repeats. Instead, telomeres consist of head-to-tail arrays of three non-LTR retrotransposons, *Het-A*, *TART* and *TAHRE*, collectively named HTT (Cacchione et al., 2020; Levis et al., 1993). Due to this particularity, telomeres are capped by a particular complex named terminin (in reference to Rome's train station) that shares almost no sequence homology with the shelterin or CST complex (Raffa et al., 2011). However, it shares the structural organization and functions of a shelterin complex by protecting telomere from end-to-end fusion. Briefly, terminin complex is composed of HOAP (HP1/Orc-associated Protein, encoded by *caravaggio*,(Cenci et al., 2003)) and HipHop (HP1-HOAP-interacting Protein, (Gao et al., 2010)), which bind double strand repeats in a non-sequence specific manner, and with Moi (Modigliani,(Raffa et al., 2009)), Ver (Verrocchio, (Cicconi et al., 2017)) and Tea (Telomere ends associated, (Zhang et al., 2016b)), which interact with ssDNA. Additionally, several non-telomeric proteins are required for proper telomeric homeostasis (Raffa et al., 2011) (**Figure 9A**). Adjacent to the HTT array, subtelomeres XL (left), 2L, 2R (right), 3L and 3R carry a cluster of repetitive DNA elements known as TAS (Telomere Associated Satellites/Sequences, Cryderman 1999). Whereas the subtelomeres YL, YS, XR and 4L contained *bona fide* constitutive heterochromatin features similarly to what is found in pericentromeric regions. 4R subtelomeric region is more particular and share both euchromatic and heterochromatic features (Marzullo and Gatti, 2015) (**Figure 9B**).

Despite their differences with yeast and human telomeres, *D. melanogaster* also presents a subtelomeric silencing. Insertion of a *white* transgene in TAS results in a variegated phenotype (Hazelrigg et al., 1984; Karpen and Spradling, 1992; Wallrath and Elgin, 1995). Importantly, silencing in TAS known as canonical Telomeric Position Effect is different from silencing in other constitutive heterochromatic regions, such as pericentromeric regions or subtelomeric regions of the Y or 4$^{th}$ chromosome, known as Position Effect Variegation (PEV). When *white* is subjected to PEV, the fly's eyes are

a mosaic of white and red facets meaning that *white* is either completely repressed or normally expressed. When *white* is subjected to TPE, eyes present a yellow/orange coloration suggesting a strong but not complete repression of the transgene (Cryderman et al., 1999). Concomitantly, subtelomeric regions are not as compact and as late replicating as centromeric regions (Andreyeva et al., 2005) (**Figure 9B**). The chromatin factors involved in PEV differ from the ones involved in TPE. For instance, HP1a (Heterochromatin protein 1a) known to be an important actor in PEV, is not involved in TPE despite is implication in telomere protection (Cryderman et al., 1999; Doheny et al., 2008; Fanti et al., 1998; Wallrath and Elgin, 1995). Some others, such as the histone methyltranferase Su(var)3-9, responsible of the deposition of the heterochromatin mark H3K9me3 are implicated in both TPE and PEV (Doheny et al., 2008; Donaldson et al., 2002; Ner et al., 2002). By doing a genetic screen of the known Su(var) (suppressor of PEV), Doheny *et al.* identified three factors that were also implicated in TPE. Interestingly, all three, Hdac1, Su(var)3-9 and Su(var)2-10, are enzymes involved in H3K9 methylation and are respectively, a histone deacetylase, a H3K9 methyltransferase, a SUMO ligase important for H3K9me3 deposition (Czermin et al., 2001; Ner et al., 2002; Ninova et al., 2020; Rubertis et al., 1996). It demonstrates that nucleosome modification, and more particularly H3K9me3, is an early step in the establishment of heterochromatin. The majority of the other mutated loci identified in this screen, named Su(TPE) (suppressor of TPE) did not modify PEV (Doheny et al., 2008).

Altogether, these studies highlight an important difference between subtelomeric heterochromatin and constitutive heterochromatin found elsewhere in the genome (usually at pericentromeric regions). Of note, even if I shall not go into detail here, in the other organisms described here (*Schizosaccharomyces pombe* and human cells) this difference between subtelomeric heterochromatin and centromeric heterochromatin is present both in term of modifications and establishments.

To conclude, despite its atypical chromosome end, drosophila also exhibit subtelomeric silencing. The silencing mechanism found in TAS is different from the one found in constitutive heterochromatic regions and seems more permissive. There are still a lot of unknow concerning the establishment of TPE in *Drosophila*. For instance, the link between telomeric proteins and heterochromatin is still undetermined. It seems that, as in yeast, yKu70/80 paralogs (*Irbp* and *mus309*) are

involved in the control of telomere length and the regulation of TPE (Doheny et al., 2008; Melnikova et al., 2005). However, whether terminin complex is implicated in TPE is not known. Similarly, whether telomere length influences TPE has not been investigated to my knowledge.

Nonetheless these remaining question marks, subtelomeric silencing exists in drosophila. It suggests that adjacent heterochromatin is a conserved feature of telomeres.

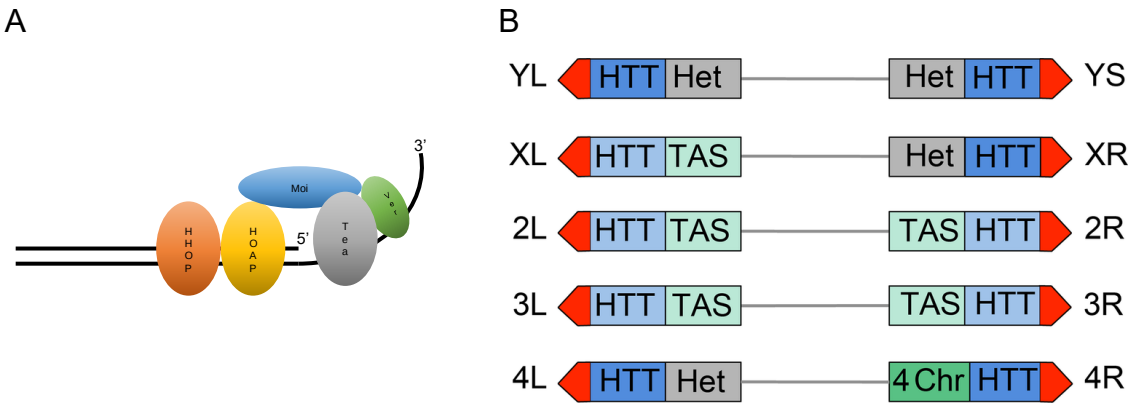


**Figure 8** : **Structure of drosophila telomeres and subtelomeres**
A: Schematic representation of the Terminin complex. Adapted from Raffa *et al*. 2013.
B: Schematic representation of *Drosophila* chromosomes. Terminin-associated region are represented in red; HTT array are represented in blue and can be either repress (dark blue) or not repress (light blue); TAS, subjected to TPE are represented in light green; and subtelomeric region with constitutive heterochromatin similar to peri-centromere are represented in grey. From Cenci *et al*., 2015.

## TPE mechanisms in *S. pombe*:

Soon after the discovery of TPE in budding yeast and fruit fly, it was also identified in *Schizosaccharomyces pombe*, a fission yeast. Indeed, introduction of a reporter gene near a newly formed telomere in a minichromosome resulted in its epigenetic silencing (Nimmo et al., 1994). An interesting point to study the establishment of heterochromatin in this model organism is its resemblance to the one found in humans/mammals. Heterochromatin formation required an active RNAi mechanism and/or deposition of heterochromatin marks on histones, two phenomena absent in budding yeast. In fission yeast, constitutive heterochromatin is found in pericentromeric regions, in silent mating-type region and subtelomeric regions. It is characterized by hypoacetylated histones and di- and trimethylation of histone H3 at lysine 9 (H3K9me2/3). However, fission yeast lacks DNA methylation, a commonly used system to silence gene expression and establish heterochromatin in other eukaryotes. Additionally, *S. pombe*, on the contrary to *S. cerevisiae* has nucleosomal DNA at its telomeres. In the following section, I shall detail how fission yeast silences its subtelomeric regions.

## Both telomeric recruitment of silencing factors and RNAi machinery regulate TPE:

As in *Saccharomyces cerevisiae*, components of the shelterin complex bound to telomeres are important for subtelomeric silencing. Deletion of Taz1, the telomeric double strand binding protein related to TRF proteins in mammals, was the first protein to be shown to impair TPE (Cooper et al., 1997; Nimmo et al., 1998). However, all shelterin proteins, Rap1, Poz1, Tpz1, Pot1 (similar to RAP1, TIN2, TPP1 and POT1 in mammals, respectively) and Ccq1 (involved in telomerase recruitment and activation), impact subtelomeric silencing (Fujita et al., 2012; Kallgren et al., 2014; Kanoh and Ishikawa, 2001; Sugiyama et al., 2007; Tadeo et al., 2013). Briefly, Taz1 binds the telomeric dsDNA, Tpz1-Pot1 subcomplex binds the ssDNA, Rap1 with Poz1 bridge Taz1 with Tpz1-Pot1 and lastly, CCq1 associates with Tpz1 (**Figure 1**). It has been shown that Ccq1 interacts directly with the CLRC complex (Wang et al., 2016). This

complex contains the only histone H3K9 methyltransferase identified in fission yeast, Clr4 (Cryptic loci regulator 4) and is responsible of the deposition of the H3K9me2/3 heterochromatin marks (Nakayama et al., 2001). Binding of CLRC on Ccq1, thus on the single strand portion of telomeres, is not sufficient to nucleate heterochromatin formation in subtelomeric region. Instead, a proper connexion between shelterin components is necessary to bypass telomeric repeats, and to promote spreading of CLRC in subtelomeric regions (Kanoh et al., 2005; Liu et al., 2021; Wang et al., 2016). In addition to the recruitment of CLRC, shelterins also recruit SHREC (Snf2/Hdac-containing Repressor Complex), a histone deacetylase complex composed of Clr1, Clr2, Mit1 and Clr3 (responsible of the deacetylase activity of the complex). SHREC is recruited at telomeres through a direct interaction between Clr3 and Ccq1 and participates to subtelomeric silencing (Armstrong et al., 2018; Sugiyama et al., 2007). After this nucleation step of the chromatin remodelling factors at telomeres, as in budding yeast, it has been proposed that heterochromatin spreads toward centromeres (**Figure 9**).

The spreading of heterochromatin in subtelomeric regions requires Swi6, a chromodomain protein, orthologue of HP1 mammalian proteins (Ekwall et al., 1995; Kanoh et al., 2005). Moreover, cooperation between histone methyltransferase activity of CLRC and histone deacetylase activity of SHREC is necessary. SHREC deacetylates H3K14 thank to Clr3 while CLRC methylates H3K9. This allows the recruitment and the binding of Swi6 on methylated histones. In turn Swi6 promotes the recruitment of additional CLRC and SHREC complexes to further extend heterochromatization (Hall et al., 2002; Sugiyama et al., 2007; Yamada et al., 2005). In addition, CLRC is also able to bind H3K9me, thanks to its chromodomain, and modify adjacent histones to spread (Zhang et al., 2008). It seems that histone deacetylase activity of Sir2 is also required for subtelomeric silencing but, how it is recruited to subtelomeres and participates to silencing remain elusive (Shankaranarayana et al., 2003). It has been proposed that Sir2 deacetylation of H4K16 increases histone compaction, thus facilitating heterochromatin spreading at least in centromeric regions (Wang et al., 2013). Nevertheless, this spreading model is supported by the continuous location of Swi6 and H3K9me2/3 over the silenced regions in subtelomeres (Cam et al., 2005; Kanoh et al., 2005).

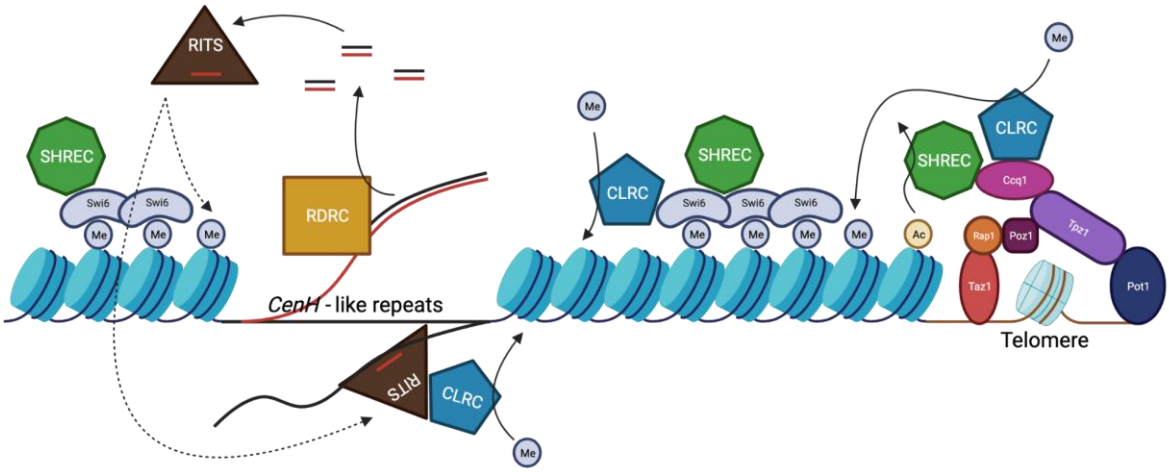


**Figure 9** : **Both telomeric recruitment of silencing factors and RNAi machinery regulate subtelomeric heterochromatin in *Schizosaccharomyces pombe*.**
The connection between shelterin proteins allows CLRC and SHREC, initially recruited by Ccq1, to skip telomeric DNA and to reach nucleosomes in subtelomeric regions to initiate methylation on H3. H3K9me2/3 is bound by Swi6 which in turn recruits SHREC and CLRC to extend heterochromatin. In parallel, CenH-like repeats, located in subtelomeric regions are transcribed by PolII, processed into siRNA by RDRC and loaded into the RITS. RITS is targeted to the repetitive regions and anchored on methylated H3K9, in turn it recruits CLRC and establishes and/or propagates heterochromatin. Created with BioRender.com

However, recruitment of CLRC directly by the selterin complex is not the only way to promote heterochromatin in subtelomeres and a redundant pathway involving the siRNA machinery is also at play. It has been shown that deletion of shelterin impacted subtelomeric silencing to a lesser extent than deletion of the methyltransferase Clr4 or HP1 protein Swi6. And similarly, when components of the RNAi machinery were deleted (Cam et al., 2005; Hansen et al., 2006; Kanoh et al., 2005). Indeed, Cam *et al.* (2005) identified in the left subtelomere of chromosome I (tel I-L) a *cenH*-like repeat sequence in a Telomere-linked helicase (*thl*) gene, later found in the other subtelomeric regions in tel I-R, tel II-L and tel II-R (Hansen et al., 2006; Oizumi et al., 2021). Similar repeats are not found in chromosome III as the telomeric repeats are immediately adjacent to rDNA sequences. These repeats were shown to be transcribed allowing the recruitment of the RNAi machinery. Briefly, after transcription, double strand RNA is synthetized by the RNA-dependent RNA polymerase complex (RDRC) and processed by Dcr1 (Dicer) to produce small interfering RNAs. These siRNAs are loaded in the Ago1 protein that is part of the RNA-induced transcriptional gene silencing (RITS) complex together with Chp1 and Tas3. Through base pairing between siRNAs and nascent transcripts, RITS is targeted to the

repetitive regions and anchored to methylated H3K9 nucleosomes by Chp1. It recruits in turn CLRC, histone deacetylases and HP1 proteins to established and/or propagate heterochromatin (reviewed in (Allshire and Ekwall, 2015; Bhattacharjee et al., 2019).

Altogether, these data demonstrate that two redundant pathways regulate subtelomeric silencing in *Schizosaccharomyces pombe*, one relying directly on the recruitment of silencing factors by telomeric proteins and the other taking advantage of the repetitive sequences found in subtelomeric regions (**Figure 9**).

## Two distinct chromatin domains in subtelomeric regions:

As in *Saccharomyces cerevisiae*, spreading of heterochromatin needs to be constrained to specific regions to avoid anarchical silencing of essential genes. In fission yeast, subtelomeres of chromosomes 1 and 2 can be divided in two distinct domains (for chromosome 3, rDNA repeats directly adjacent to telomeres make them behave differently) (**Figure 10**).

The region adjacent to telomeres, named subtelomeric homologous (SH) sequences, span the first 50 kb, are highly polymorphic and contain the *cenH*-like repeat sequences (Oizumi et al., 2021). This region is enriched in heterochromatin marks H3K9me2 and Swi6 binding (Cam et al., 2005; Tashiro et al., 2017) due to the spreading emanating from telomeres. These SH sequences were shown to function as a buffer region against spreading of heterochromatin. Indeed, their deletion allows the spreading of heterochromatin in the neighbouring gene-rich regions (Tashiro et al., 2017). It is not known how they achieve this function, but involvement of Epe1 (a H3K9 demethylase) in cooperation with Mst2 (a H3K14 acetyltransferase) seems to be required to limit the propagation of heterochromatin (Flury et al., 2017; Wang et al., 2015).

In the vicinity of the SH sequences, a telomere-distal region composed of unique sequences spans an additional 50 kb. This peculiar gene rich region, showing a conformation and histone mark content different from euchromatin and constitutive heterochromatin, is named “knob” or ST-chromatin (for subtelomeric chromatin). This knob is seen as a highly condensed chromatin body located near telomeres microscopically (Matsuda et al., 2015). Concomitantly, it presents a high H3 density.

However, the level of histone modification is particularly low for both heterochromatin marks (H3K9me2/3) and euchromatin marks (H3K4me2, H4K5Ac, H4K12Ac, H4K16Ac and H4K14Ac and H2A.Z variant) (Buchanan et al., 2009). Probably due to its condensed state, ST-chromatin inhibits transcription of its endogenous genes (Buchanan et al., 2009; Matsuda et al., 2015). The knob condensation is independent of silencing factors, as deletion of Swi6, Clr4, Clr3 or Dcr1 do not affect its formation. However, despite a low level of methylation on H3K36, epigenetic marks on H3K36 play a crucial role, as deletion of Set2 (H3K36 methyltransferase) or Clr6 (H3K36 deacethylase) eliminate knob formation and gene repression (Matsuda et al., 2015). Additionally, FACT (Facilitates Chromatin Transcription), an essential histone chaperon containing the mono-ubiquitinated H2B is required for knob formation. It has been proposed that FACT and Set2/H3K36me work together to maintain nucleosome density (Murawska et al., 2020). Additionally, it has been shown that Sgo2 (Sugoshin 2), a centromeric protein switched localization after mitosis and localized, during interphase, in subtelomeric regions independently of telomeric sequences. Sgo2 association to subtelomeres is dependent on the phosphorylation of serine 121 on histone H2A (H2AS121p) by Bub1. Interestingly, deletion of Bub1 or mutation in H2A preventing its phosphorylation completely abrogate Sgo2 subtelomeric localization and knob formation. Therefore, Sgo2 plays a crucial role in the establishment of this unique chromatin region (Tashiro et al., 2016).

The telomere-distal knob is flanked by a nucleosome-free region that prevents chromatin spreading. Indeed, when SH sequences are deleted, heterochromatin spread in the knob region but is stopped at this nucleosome free region (Tashiro et al., 2017). Conversely, when this boundary is affected, euchromatin spreads toward telomeres inducing a derepression of the genes located in the knob region (Steglich et al., 2015; Strålfors et al., 2011). In all four subtelomeres (of chromosome 1 and 2), the nucleosome free region is in LTR (long terminal repeat) sequences. It has been demonstrated that LTRs in this region are bound indirectly by Fft3 (Fission yeast fun thirty), a chromatin-remodeling factor. Fft3 was shown to be required to exclude nucleosome in this region to establish boundaries (Steglich et al., 2015).

Altogether, it appears that the subtelomeric repressive environment can be divided in two distinct formations. The telomere proximal one, that bears heterochromatin marks, is established dependently of telomeres. The telomere distal

one, a highly condensed chromatin environment devoid of chromatin marks, is formed and maintained independently of telomeres. The knob regions could act as a buffering zone preventing heterochromatin spreading in the centromeric direction as well as euchromatin spreading in the telomeric direction. This redundancy in security mechanisms suggest the important role of the epigenetic state of subtelomeres.

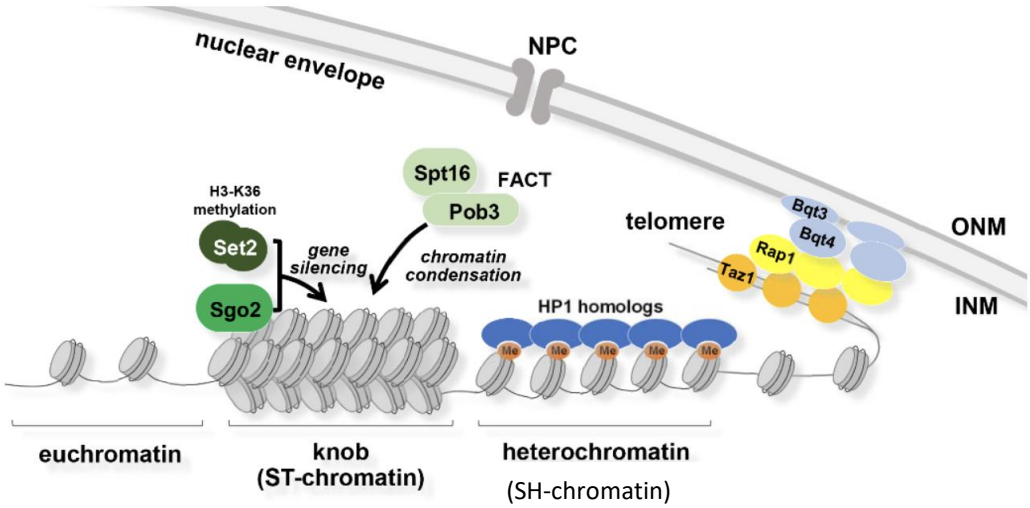


**Figure 10 : Two chromatin domains in subtelomeric region of *S. pombe*:** Subtelomeric Homologous sequences (SH-chromatin), enriched in heterochromatin marks, H3K9me3 and Swi6 (HP1 homolog) depending of a telomeric and RNAi recruitments. ST-chromatin or knob is a highly condensed chromatin formed by a cooperative action of Sgo2, Set2 and FACT-dependent nucleosome assembly pathway.
Extracted from Hirano *et al*., 2020

## The spatial organization is determinant for a proper TPE:

As in *Saccharomyces cerevisiae*, telomeres are not randomly distributed inside the nucleus of *Schizosaccharomyces pombe*. In interphase nuclei they are clustered in two foci at the nuclear periphery while centromeres are anchored at the spindle pole body, a conformation known as Rabl orientation (Funabiki et al., 1993). Several protein-protein interactions allow the telomeric subnuclear localization. It has been found that Bqt4 (bouquet formation protein 4), embedded in the inner nuclear membrane and stabilized by Bqt3, was associated with Rap1 to anchor telomeres (Chikashige et al., 2009). However, in cells lacking Bqt4, telomeres were not

completely disrupted from peripheral localization and subtelomeric silencing was not affected (Chikashige et al., 2009; Steglich et al., 2015). Indeed, another member of the inner nuclear membrane proteins, Lem2, is also important for telomere anchoring at the nuclear envelope. Lem2 was not found in interaction with telomeric/subtelomeric chromatin except with the tel III-L by ChIP. However, all telomeres detached from the nuclear envelope in the absence of Lem2 (Banday et al., 2016; Barrales et al., 2016; Gonzalez et al., 2012). Moreover, subtelomeric regions exhibited a silencing defect in the absence of Lem2. It was suggested that Lem2 regulated silencing by balancing SHREC and Epe1 binding at heterochromatin loci (Banday et al., 2016; Barrales et al., 2016). Lastly, it was shown that Fft3, found at the centromeric boundary of the knob, anchored subtelomeres to the nuclear envelope, indirectly (Steglich et al., 2015). Whether the anchoring of the knob regulated its chromatin state was not assessed. Nevertheless, it seems that telomeric clustering and tethering at the nuclear envelope is important for heterochromatin assembly (**Figure 10**).

Interestingly, it has been shown that heterochromatin defects at centromere could be rescued if telomeric or mating-type silencing were impaired (Barrales et al., 2016; Tadeo et al., 2013). Therefore, it has been proposed that, as in budding yeast, silencing factors, notably Swi6 and Clr4, were limiting for silencing (DiPiazza et al., 2021; Tadeo et al., 2013). Redundancy of pathways to form heterochromatin in subtelomeres, pericentromere and silenced mating-type loci, highlight the necessity to restrain Swi6 binding to specific loci to prevent ectopic heterochromatin assembly. Additionally, it has been shown that a proper density of H3K9me2/3 was necessary to promote propagation and inheritance of heterochromatin (DiPiazza et al., 2021). These results argue that clustering of heterochromatin loci could facilitate the sequestration of heterochromatin factors to avoid ectopic heterochromatin formation while maintaining them at a proper concentration to favour epigenetic inheritance.

Altogether, these results show that subtelomeric silencing is finely regulated not only to properly silence subtelomeric genes but also to maintain a proper homeostasis of silencing factors inside the nucleus.

## TPE mechanisms in *H. sapiens*:

As presented earlier, TPE also occurs in human (Baur et al., 2001; Koering et al., 2002), however, contrary to yeasts, the epigenetic mechanism by which telomeres regulates gene expression is still puzzling.

## Identification of TPE in human cells:

TPE in human was initially studied using plasmids containing a reporter gene adjacent to telomeric repeats. Introduction of such constructs in cells can result in breakage of the chromosome at the insertion site, followed by extension of the telomeric sequence by telomerase, recruitment of shelterin proteins and establishment of a functional telomere (Hanish et al., 1994). Therefore, this telomere seeding technic allows the monitoring of the expression of a reporter gene at telomere vicinity.

It was shown that insertion of a luciferase reporter gene adjacent to a newly formed telomere repressed the expression of luciferase whereas its insertion at an internal position did not. Moreover, it has been demonstrated that hTERT overexpression increased the repression of the transgene indicating a length dependent action of the telomere on TPE (Baur et al., 2001). Similarly, stable transfection of an eGFP reporter adjacent to a seeded telomere showed a stable repression over time. However, transient transfection of the same plasmid with 1.6kb of telomeric repeat did not exhibit silencing properties. Thus, silencing is not due to the binding of repressive factors on the $T_2AG_3$ tract itself but requires a fully functional telomere (Koering et al., 2002). Moreover, as observed in drosophila and yeasts, human cells with a transgene insertion adjacent to a telomere exhibited a variegated phenotype confirming that an epigenetic mechanism is at play (Baur et al., 2004). All together, these data demonstrate that human cell lines exhibit a *bona fide* TPE on subtelomeric reporter genes. Importantly, the first endogenous gene demonstrated to be regulated by TPE was *ISG15* (interferon stimulated gene 15), a ubiquitin-like protein (Lou et al., 2009). To distinguish a telomere length effect from confounding effects such as time in culture, DNA damage signals arising from short telomeres, *ISG15* expression was monitored in different cell lines. Young primary cells (with long

telomeres) had a low *ISG15* expression. On the contrary, old cells (with short telomeres) exhibited enhanced transcription. Finally, overexpression of hTERT in old cells (with re-elongated telomeres) repressed *ISG15*, demonstrating the telomere length dependent regulation of ISG15 (Lou et al., 2009).

## Epigenetic signature of human TPE:

How telomeres influence the epigenetic state of the sutelomeric regions is still unclear. Trimethylation on H3K9 and H4K20, two histone marks of constitutive heterochromatin, has been shown to be enriched in repressed transgenes inserted near a telomere (Majocchi et al., 2014; Rincón-Arano et al., 2007; Tennen et al., 2011). It was also confirmed that subtelomeric genes naturally subjected to TPE were enriched in H3K9me3. Upon telomere shortening, this repressive mark was observed to decrease (Robin et al., 2014). H4K20me3 enrichment was not assessed in promoters of genes regulated by TPE. To date, it is not known which of the five mammalian SET-domain containing methyltransferases regulates H3K9me3 deposition in subtelomeric regions and how it is recruited. As in fission yeast, H3K9me3 allows the binding of HP1 proteins (three isoforms exist in mammals: HP1α, HP1β, HP1γ), resulting in a self-propagation of heterochromatin. However, it is not clear whether HP1 isoforms are involved in TPE. Indeed, a correlation was observed between TPE alleviation and the reduction of HP1α and HP1β punctiform pattern (Koering et al., 2002). However, enrichment of HP1α by ChIP at TPE regulated genes' promoters was not a general feature (Robin et al., 2014). More experiments should be performed to determine the implication of HP1 in telomere silencing in mammals.

On the other hand, SIRT6, an $NAD^+$-dependent histone/protein deacetylase (or sirtuins), a paralogue of Sir2 in yeast, was shown to have an H3K9 and H3K56 deacetylase activity, notably at telomeres during the S phase of the cell cycle (Michishita et al., 2008, 2009). Interestingly, it has been shown that the downregulation of SIRT6 derepressed the expression of a reporter gene adjacent to a newly formed telomere as well as *ISG15*. Furthermore, SIRT6 inhibition led to an increase of H3K9 acetylation marks and a decrease of H3K9 trimethylation at the reporter gene locus while hTERT overexpression increased H3 density and H3K9me3 and decreased

H3K9Ac. Thus, SIRT6 has been proposed to be a key enzyme in the establishment and maintenance of TPE in human cells (Tennen et al., 2011). Concomitantly, Trichostatin A (TSA), first described as a histone deacetylase class I and II inhibitor, was shown to inhibit also SIRT6 (Wood et al., 2018), and TSA treatment on cells derepressed TPE on reporter genes (Baur et al., 2001; Esnault et al., 2009; Koering et al., 2002). Therefore, even if we cannot rule out the effect of other histone deacetylases, it reinforces the involvement of SIRT6-dependent deacetylation of histones in the formation of subtelomeric heterochromatin in human. However, how SIRT6 is recruited by telomeres and propagated in centromeric direction is still an open question. Interestingly, it has been shown recently that SIRT6 could interact with TRF2. Upon DNA damage, this interaction was enhanced, resulting in a deacetylation of TRF2 by SIRT6 and a proteasome-dependent degradation of TRF2. Nevertheless, in non-treated cells the interaction between TRF2 and SIRT6 still exists (Rizzo et al., 2017). It is tempting to suggest a role of TRF2 in the recruitment of SIRT6 to induced TPE; as the shelterin complex recruits SHREC, a deacetylase complex, in fission yeast.

Lastly, heterochromatin in mammals is also associated with DNA methylation on cytosine. It has been observed that high levels of DNA methylation in subtelomeric sequences was associated with long telomere in leukocytes of 44-45 year-old men (Buxton et al., 2014). Also, an association between subtelomeric hypomethylation and age-related telomere shortening in the normal Japanese population was shown (Maeda et al., 2009). In contradiction with these results, a recent study using a large European population cohort, observed that subtelomeric DNA methylation increased with age (Bacalini et al., 2021). However, they focused only on two subtelomeric regions (chr 5p and chr 21q) contrary to Maeda *et al.* who evaluated the global subtelomeric methylation. It is therefore possible that some subtelomeres exhibit different behaviours regarding DNA methylation. Even with the use of a reporter gene adjacent to a newly formed telomere, the DNA methylation status in TPE is not clear. Indeed, several studies reported that the use of 5-Azacytidine (5-azaC), an inhibitor of DNA methyltransferases, does not reactivate the expression of the subtelomeric transgene in human cells (Esnault et al., 2009; Koering et al., 2002). Whereas one study done in chicken erythroblasts showed a derepression of the transgene upon 5-azaC treatment (Rincón-Arano et al., 2007). All together more investigations are

needed to know if telomeres regulate subtelomeric DNA methylation and whether methylated DNA contributes to TPE.

## Chromatin loops regulate TPE:

To decipher to which extend heterochromatin could spread from a telomere toward the centromere, Kulkarni *et al.* inserted a plasmid with reporter genes located at 100kb from a newly formed telomere. They observed that silencing of transgenes was greatly reduced when placed at 100kb instead of 3kb from a telomere. Therefore, influence of a telomere on gene expression seemed limited in range (Kulkarni et al., 2010). However, as in yeast, spreading of heterochromatin toward the centromere in human could be discontinuous. Indeed, *ISG15* is located at more than 1Mb from the chromosome 1p extremity, and yet is still regulated by TPE. Moreover at least five genes between *ISG15* and the telomere were not regulated by telomere length, arguing in favour of a discontinuous spreading of TPE at natural telomeres (Lou et al., 2009).

As in yeast, some DNA elements could act as an insulator in subtelomeric regions, preventing aberrant heterochromatinization. It has been initially demonstrated in chicken erythroblasts that heterochromatin spreading from telomere could be counteracted by an insulator such as cSH4, a chicken β-globin insulator (Rincón-Arano et al., 2007). Indeed, an insulated transgene inserted near a telomere was enriched in histone marks corresponding to an open conformation such as H3K79me2 and H3K4me2. On the contrary, an uninsulated transgene was enriched in H3K9me3 repressive marks. Additionally, other DNA elements were found to counteract TPE. Scaffold/Matrix attachment regions (S/MARs) are AT rich regions that tether chromatin to the nuclear matrix, and that stabilize and enhance transgene expression trough boundary activity (Girod et al., 2007). Ubiquitous chromatin opening element (UCOEs) are CpG-rich regulatory elements found in housekeeping gene promoters, that maintain chromatin in a permissive state. Both DNA elements (S/MARs and UCOEs) were found to exert boundary activity when placed between a telomere and a transgene (Majocchi et al., 2014). These findings reinforce the epigenetic nature of

TPE. However, it is not clear whether these DNA elements are naturally present in subtelomeric regions and whether they participate to the discontinuous nature of human TPE. Recently, a genome-wide distribution of S/MARs has been published. It could therefore be of interest to see where those elements are present in subtelomeric regions (Narwade et al., 2019).

Additionally to a potential role of insulators, it has been demonstrated that 3D chromatin conformation and telomeric loops were primordial for the spreading of heterochromatin in subtelomeric regions (**Figure 11**). Robin *et al.* observed that in primary fibroblasts and myoblasts, telomeres and subtelomeres formed fold-back structures up to 10Mb away from the telomeric sequence to repress gene expression, named TPE-OLD (Telomere Position Effect Over Long Distances). These loops are telomere length dependent and are lost upon telomere shortening (but regain upon telomere elongation). In respect with the folding, they identified 144 genes in myoblast, whose expression are telomere length dependent. Interestingly, not all subtelomeres are enriched in TPE regulated genes, suggesting a heterogeneity in subtelomeric nature, *i.e.,* epigenetic marks, gene content, DNA repeat content… This could explain some discrepancies observed in different studies deciphering the epigenetic nature of subtelomeric regions. Nevertheless, repressed subtelomeric genes were enriched in H3K9me3 chromatin marks and TRF2 shelterin protein. These enrichments were lost upon looping disruption and absent in genes located within the loops (Robin et al., 2014). Mechanisms allowing these long-range interactions remain elusive. It has been shown that human telomeres could interact with interstitial telomeric sequences (ITS), an internal repetition of the TTAGGG motif. This interaction is mediated by TRF2 and stabilized by its direct interaction with lamin A/C (Wood et al., 2014). Indeed, TRF2, was shown to bind ITS (Simonet et al., 2011; Yang et al., 2011). Concomitantly, at the *TERT* promoter in chromosome 5p, TRF2 was observed to mediate loops and to bring in close proximity ITS and telomeres resulting in a transcriptional repression of *TERT* (Kim et al., 2016). CTCF (CCCTC-binding factor) and LDB1 (LIM domain-binding protein 1) two factors commonly involved in chromatin looping structures (Deng et al., 2012; Zhang et al., 2011) were also shown to be required for the loop formation (Kim et al., 2016). TRF2 was also demonstrated to mediate a long subtelomeric loop to regulate *SIRT3* expression (in the chromosome 11p) in myotubes (Robin et al., 2020).

Therefore, it appears that TRF2 could be a central player in TPE establishment, linking telomeres, heterochromatin formation and 3D chromatin conformation.

Altogether, these data demonstrate that, as in yeast, chromatin organization plays an important role in subtelomeric chromatin and TPE.

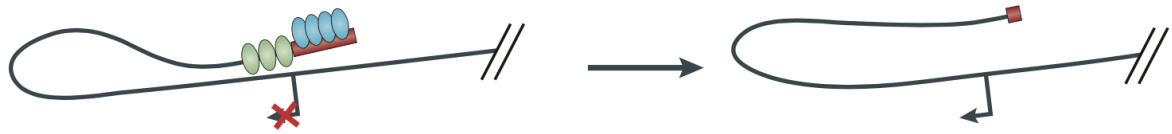

**Figure 11 : chromatin loops regulate TPE in human cells:**
Long telomeres are engaged into chromatin loops and silenced physically close loci. Telomere shortening disrupted 3D chromatin conformation and derepressed gene expression.
From Ye *et al.*, 2014.

## Conclusion:

Overall, Telomere Position Effect is a common feature of chromosome ends shared by multiple eukaryotes. Yeasts, trypanosome, drosophila, human cells and others exhibit a repressive chromatin state in their subtelomeres, driven by telomeres.

However, as described in chapter one, telomeres are not generally heterochromatic. The deposition of heterochromatin marks and the silencing of reporter genes in subtelomeric regions is not due to a heterochromatic state of telomeres that propagates in subtelomeres. In other words, the vision of a telomere as a reservoir of heterochromatin that passively diffuse in adjacent loci needs to be challenged. At least in *S. cerevisiae* and *S. pombe*, telomeres actively recruit chromatin silencer. Thanks to 3D chromatin conformation, and cis-acting elements, telomeres silence specific subtelomeric regions. In that aspect, TPE seems really different from the PEV described in pericentromeric regions. This vision of an active recruitment of silencing factor by telomeres also suggest a more regulated process. Telomeres in this aspect, could be view as an integrative part of the transcriptional regulation machineries via their chromatin remodeling properties.

Nonetheless, this more proactive definition of TPE can be seen as purely semantic and rely on how telomeres can be seen in the biology field; a protecting cap, or an important actor in regulation of cellular homeostasis. In any case, the consequences are similar and subtelomeric regions are silenced.

Consequently, another aspect of this discussion is why subtelomeres are silenced? With the initial vision of a passive propagation of telomeric-heterochromatin factors, the silencing of subtelomeric regions was a consequence of the telomeric chromatin state. With a more “pro-active” definition of TPE, it rather suggests that subtelomeric regions need, or initially needed to be silenced. To which end?

If we postulate that subtelomeric regions need to be silenced, is it because of their genes content? In other words, is the transcriptional (silencing) function of TPE, a cause of subtelomere heterochromatin state? It rather seems that the heterochromatin properties given by the telomeres to the subtelomeres has been “exploited” by cells. The heterochromatin state of subtelomeric regions is thought to allows chromatin rearrangement, duplication, break and repair without damaging the cells. Consequently, subtelomeres are seen as an evolutionary reservoir with silenced non-essential genes (Trask 1998, Linardopoulou 2005). I shall not detail this evolutionary discussion furthermore.

In the following chapter, I shall address the relevance of subtelomeric silencing in term of gene regulation and I shall focus on the transcriptional regulation properties of telomeres in respect with Telomere Position Effect.

Given the importance of telomeres in the senescence and aging process, could TPE be a process by which cells modulate the transcriptome in response to aging?

# Chapter 3: Physiological and pathological functions of Telomere Position Effect:

We have seen in the previous chapter that TPE is a common feature of telomeres. From yeast to human, and even in drosophila with atypical telomeres, chromosome ends influence the epigenetic state of their surrounding sequences. Therefore, in the following section I shall describe what is the biological relevance of this epigenetic mechanism mediated by telomeres. I shall principally focus my attention on the transcriptional regulation by TPE.

## Telomere Position Effect and regulation of pathogenicity:

Interestingly, several evolutionarily distant organisms take advantage of subtelomeric particularities to regulate their pathogenicity. I shall decipher what is the implication of TPE in such regulations.

### Antigenic variation in protozoan and Telomere Position Effect:

Antigenic variation is one survival strategy developed by many evolutionarily distant pathogenic organisms to evade the immune responses of their hosts. By altering their surface antigens *i.e.*, changing the expression of surface proteins, pathogens are able to escape the acquired immune response of hosts (reviewed in (Deitsch et al., 2009)). Antigenic variation requires a large reservoir of antigen genes and precise regulation mechanisms to ensure the presence at the surface of a very small number or one antigen at a time.

Two well-known parasites *Trypanosoma brucei* and *Plasmodium falciparum* escape the human immune system thanks to antigenic variation. *Trypanosoma brucei* is transmitted to human by infected tsetse flies and is responsible for African Trypanosomiasis, also named sleeping sickness. In 2019, only 992 new cases were reported but the population at risk is estimated at 65 million people (WHO).

*Plasmodium falciparum* is transmitted by mosquitos and causes malaria, a disease affecting an estimate of 219 million people worldwide in 2017 (WHO). Regarding the incidence of those two diseases in the world, concerted efforts have been made to understand the mechanisms regulating the immune escape of these two protozoans.

Telomeres of *Trypanosoma brucei* regulate antigenic variation:

In mammals, *Trypanosoma brucei* proliferate as slender Bloodstream Forms (BF) and avoid antibody clearance by changing periodically its Variant Surface Glycoprotein (VSG) coat. A large repertoire of 2500 VSG genes and pseudogenes are present throughout the genome (Cross et al., 2014). However, only one VSG is expressed at a time from one of the ~15 subtelomeric expression sites (ES) (De Lange and Borst, 1982). Each ES is a polycistronic transcription unit and contains a single functional VSG flanked by the telomeric sequence and an upstream 70bp repeat. The antigenic variation *i.e*., switch in VSG expression, can be the result of an *in-situ* switch. It occurs at the transcriptional level, when an active ES is silenced, and another ES becomes fully transcribed. However, antigenic variation is mainly mediated by DNA homologous recombination. A previously silenced VSG is recombined into the active ES to replace the previously active VSG ((Myler et al., 1984), reviewed in (Cestari and Stuart, 2018; Horn, 2014)). Although a double strand break (DSB) is not absolutely required to initiate homologous recombination, it remains a good inducer. Therefore, it has been hypothesized that DSB targeted in ES are an important trigger of VSG switch (Boothroyd et al., 2009). For VSGs both chromosomal location and position within the nucleus appear critical for their regulation of expression. The active bloodstream ES is transcribed by Pol-I in an extranucleolar structure named ES body (Navarro and Gull, 2001) whereas the other VSG loci are located in closed chromatin compartment, resulting in their silencing (Müller et al., 2018) (**Figure 12**).

Because VSGs are mainly in subtelomeric position, it was rapidly asked whether TPE was involved in their silencing. Telomeres in *T. brucei* are composed of $T_2AG_3$ repeats (Blackburn and Challoner, 1984) bound by tbTRF (functionally related to mammalian TRF1/2, (Li et al., 2005)) in association with tbTIF2 (homolog of mammalian TIN2, (Jehi et al., 2014a)), tbRAP1 (homologue of telomeric RAP1

proteins, (Yang et al., 2009)), tbTRAF (TTAGGG Reapeat-Associated Factor, a telomeric protein, (Glover et al., 2016)) and tbTelAP1 (Telomere-Associated Protein 1, (Reis et al., 2018)). Interestingly, telomeres were not randomly distributed in the nucleus but formed several clusters in the bloodstream form of *T. brucei* (Pérez-Morga et al., 2001).

Reporter genes inserted in a subtelomeric position were observed to be epigenetically repressed in *T. brucei* (Horn and Cross, 1995). Additionally, insertion of a newly formed telomere at several loci resulted in a repression of the reporter gene, depending on telomere length (Glover and Horn, 2006). However, even if deletion of one telomere resulted in a derepression of a subtelomeric reporter gene depending on tbSIR2IP (a member of sirtuin family analogue of Sir2); VSG silencing at ES was preserved (Glover et al., 2007). This is due to the redundancy of mechanisms to silence subtelomeric ES. Concomitantly, several telomeric associated proteins known to regulate TPE in yeasts and human were found to have little impact on VSG silencing, including tbKU70/80 (Conway et al., 2002), tbTRF (Jehi et al., 2014b; Li et al., 2005) and tbTIF2 (Jehi et al., 2014a). In contrast, tbRAP1 was shown to be essential for a proper repression of subtelomeric VSG in ES (Yang et al., 2009). tbRAP1 downregulation induced a partial derepression of ES loci, disrupted the monoallelic expression of VSG and resulted in the presentation of several VSG at the surface of the cells (Afrin et al., 2020; Yang et al., 2009). It was shown that tbRAP1 bound directly to telomeric DNA, independently of its interaction with tbTRF. This binding activity was required for VSG silencing and can be regulated by phosphorylation of its DNA binding site (Afrin et al., 2020). Herein tbRAP1 seems to have a conserved role in regulating TPE in trypanosome, and posttranslational modifications could regulate its activity. Although not much is known about tbTRAF, it has been shown that knock-down of this telomeric protein was associated with VSG derepression (Glover et al., 2016), supporting the importance of telomere integrity in VSG silencing.

Additionally, both tbTRF, tbTIF2 and tbRAP1 depletion as well as telomere shortening increased the switch in VSG expression (Dreesen and Cross, 2006, 2008; Jehi et al., 2016; Nanavaty et al., 2017) due to an increase in telomere/subtelomere instability (Jehi et al., 2014b, 2014a, 2016; Nanavaty et al., 2017). The long non-coding telomeric RNA (TERRA) has been shown to be transcribed in *T. brucei* from the telomere downstream of the active ES (Nanavaty et al., 2017; Rudenko and Van der Ploeg, 1989). TERRA has the ability to form DNA:RNA loops (R-loops) with telomeric

DNA and an increased amount of R-loops was observed to increase DSBs in the adjacent subtelomere and therefore to increase VSG switch. Therefore, by regulating TERRA levels, telomeric proteins (at least tbTRF and tbRAP1) control the formation of R-loops, the induction of DSBs and the VSG switching rate (Nanavaty et al., 2017; Saha et al., 2021) .

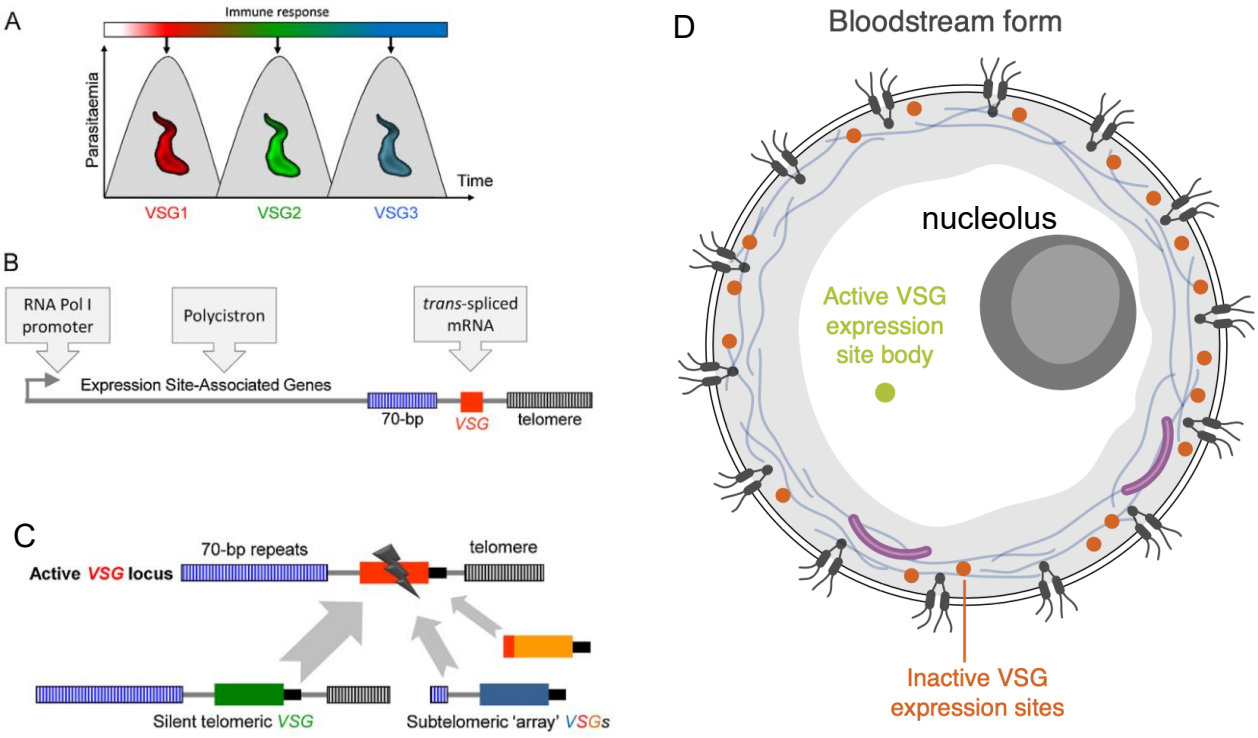


**Figure 12 : VSG expression and switching:**
A: VSG switch brings about antigenic variation and allows escaping from host immune response.
B: Schema of the Expression Site
C: Switch in VSG expression can result from a switch at the transcriptional level from one ES to another, or by homologous recombination between the active VSG and another VSG. (A, B, C, extracted from Horn 2014.)
D: Nuclear organisation of the VSGs loci in the slender bloodstream fom of *Trypanosoma*. Inactive VSG and ES are located in the silenced periphery and the active VSG is located in the expression site body. (Extracted from Glover *et al.*, 2013).

Altogether, these data support that telomeres are involved in VSG silencing redundantly with other silencing processes (reviewed in (Cestari and Stuart, 2018)). Thereby, perturbation in telomere homeostasis results in an increase in the antigenic variation or a partial loss of VSG silencing. Interestingly, it was observed that recently laboratory-adapted strains, coming from a stressful environment, had shorter

telomeres and consequently a higher switching rate of VSG than the commonly used laboratory strain, grown in controlled conditions for decades (Dreesen and Cross, 2008). This suggest that a proper telomere length regulation could serve the adaptability of Trypanosome to its environment. Deciphering what links environment and telomeres has yet to be determined. Because repressive chromatin is well compartmented in *T. brucei*, and telomeres cluster together, telomeres probably participate to the sequestration of heterochromatin factors as it was observed for *Plasmodium falciparum*.

Telomeres of *Plasmodium falciparum* and establishment of a closed chromatin compartment:

*Plasmodium falciparum*, infects and proliferates in human blood circulation system for an extended period suggesting the involvement of an immune-escape mechanism. Indeed, this parasite undergoes antigenic variation when it infects red blood cells (Hommel et al., 1983) by expressing a unique immunodominant and clonally variant of pfEMP1 (Erythrocyte Membrane Protein 1) at the cell surface (Leech et al., 1984). pfEMP1 is encoded by the *var* genes present in ~60 copies. Interestingly, the majority of the *var* members are in subtelomeric regions (Gardner et al., 2002). Moreover, silenced *var* genes are not randomly located in the nucleus but clustered at the nuclear periphery (Freitas-Junior et al., 2000; Ralph et al., 2005).

In *P. falciparum* few transcription factors were identified, suggesting that additional levels of gene regulation are involved to govern the complex gene expression program (Coulson et al., 2004). Epigenetic regulation and 3D chromatin conformation were indeed shown to play a central role in the regulation of the *var* gene family and invasion genes (reviewed in (Abel and Le Roch, 2019)). Heterochromatin regions are clustered together at the nuclear periphery creating a silencing compartment (Ay et al., 2014; Freitas-Junior et al., 2000). Telomeres are thought to participate to this sequestration.

Telomeres in Plasmodium falciparum are composed of tandem $G_3T_2$(T/C)A repeats and can regulate gene expression through a TPE mechanism (Figueiredo et al., 2002). Telomeres were shown to recruit chromatin factors such as pfALBA3 (Acetylation

lowers binding affinity, (Goyal et al., 2012)) and pfSIR2A (an orthologue of S. cerevisiae Sir2, (Freitas-Junior et al., 2005)) to promote H3K9me3 propagation in subtelomeric regions. H3K9me3, as in other species, is bound by pfHP1 to establish a silenced compartment (Ay et al., 2014; Freitas-Junior et al., 2000). To further extend heterochromatin, telomeres were proposed to form a fold-back structure to bring pfSIR2 in *var* gene vicinity (Mancio-Silva et al., 2008). Additionally to the recruitment of heterochromatin factors, telomeres *per se* were shown to be essential for a proper anchoring at the nuclear periphery (Figueiredo et al., 2002). This anchoring has been proposed to facilitate the occurrence of ectopic recombination events between *var* genes, promoting the diversity of pfEMP1 (Hernández-Rivas et al., 2013) (**Figure 13**).

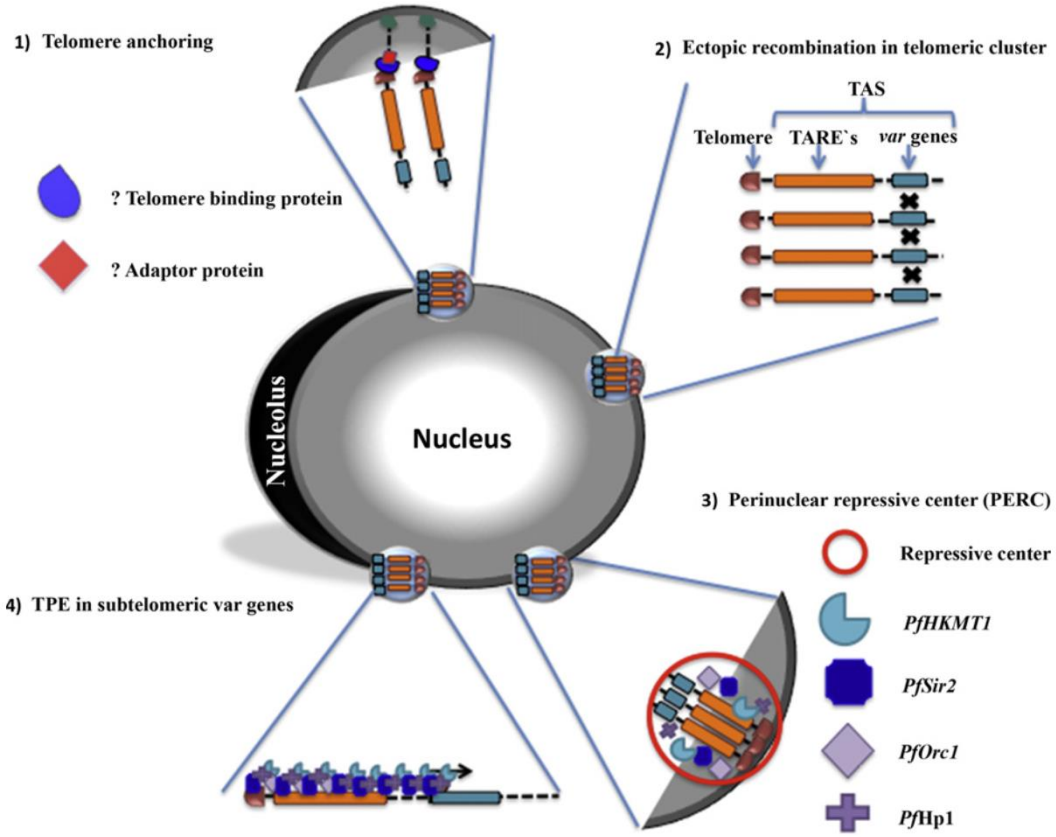


**Figure 13 : Role of *P. falciparum* telomeres in silencing and antigenic variation:** Telomeres were shown to be important for the anchoring of chromosome ends at the nuclear membrane (1), and the establishment of a repressive compartment (3). Telomere were also shown to regulate gene expression through a TPE mechanism (4). Telomere clustering is also thought to be important for recombination of *var*, and consequently of the diversity and the plasticity of the antigen (2).
Extracted from Hernandez-Rivas *et al*., 2013,

Taken together, these data demonstrate that telomeres are important in *Plasmodium falciparum* silencing and therefore regulation of antigenic variation. Telomeres clustering at the nuclear periphery and telomere-dependent recruitment of silencing factors participate in the establishment of a closed chromatin compartment; creating a gradient of gene expression across the nucleus. Repressed *var* genes and other invasion genes are clustered in this region of the nucleus and through 3D chromatin changes can be moved to a more open compartment to be expressed (Ay et al., 2014; Bunnik et al., 2018). Therefore, it strengthens the reservoir model developed in *S. cerevisiae* (Maillet et al., 1996). To understand deeper the functional role of telomeres in antigenic variation, further characterization of the yet to be defined protein complex protecting telomeres has to be done. It could be of interest to observe if natural telomere variations occur relative to parasite life-stages or stressed parasites and whether it play a role in its adaption.

*Plasmodium* and *Trypanosoma* are two evolutionary distant human parasites. Nevertheless, they adopted a similar strategy to escape adaptative immunity taking advantage of the peculiar chromatin state of their subtelomeric regions. It reveals the important role of telomeres and subsequently TPE in the regulation of gene expression.
In the following section I shall continue to explore how TPE is involved in the regulation of pathogenicity in the *Candida* fungi family and how aging could play a role.

## *Candida*, a yeast genus with an enhanced pathogenicity at old age?

*Candida* is a budding yeast genus belonging to the family of *Saccharomycetaceae* together with *Saccharomyces* genus. *Candida* spp. are human commensals or endosymbionts but can cause opportunistic infections when mucosal barriers are disrupted, or the immune system is compromised. Two species, *Candida albicans* and *Candida glabrata* were responsible of 65.3% and 11.3% of the case of candidiasis, respectively, in 2007 (Pfaller et al., 2010). Consequently, most research efforts have been focused on those two species to understand by which mechanisms they improved their pathogenicity. Subtelomeric regions have gained interest in the

study of pathogenic fungi due to their content in genes known to be involved in virulence.

*TLO*, a subtelomeric gene family that regulates virulence in *C. albicans*:

In *C. albicans*, 14 out 15 members of the *TLO* (telomere-associated) gene family are located within 12kb from a telomere (Anderson et al., 2012; van het Hoog et al., 2007) whereas less pathogenic *Candida* spp. possessed one to two *TLOs* (Butler et al., 2009). *TLO* genes encode subunits of the mediator complex, a large protein complex used in the initiation of transcription at targeted promoters (Haran et al., 2014; Zhang et al., 2012). It has been shown that loss of *TLO* in *Candida dubliliensis*, a close relative to *C. albicans*, impaired virulence and stress response. Notably, cells lost their ability to form hyphae, a peculiar state that facilitates adherence, penetration of tissues and formation of a biofilm (Haran et al., 2014; Nobile and Johnson, 2015). This is due to the defect in activation of transcriptional responses at genes regulating virulence traits. Reversely, adding *C. albicans TLO* in the *C. dubliliensis* genome improved its virulence. Consequently, it has been proposed that expansion of the *TLO* gene family contributes to the enhanced adaptability and virulence of *C. albicans* (Flanagan et al., 2018).

It has been shown that insertion of a reporter gene in subtelomeric regions caused its silencing and that caSir2 (homologue of *S. cerevisiae* Sir2) is required to maintain this transcriptionally repressive state. Deletion of caSir2 resulted in a mild transcriptional upregulation of subtelomeric genes within the first 10/15kb from a telomere, including some *TLO* genes (Freire-Benéitez et al., 2016a). Importantly, subtelomeric heterochromatin was found to be responsive to environmental changes. Indeed, while yeast kept at 30°C (a temperature corresponding to the host's skin) exhibited a mild subtelomeric gene repression, yeast kept at 39°C (mimicking a moderate fever in a host) presented an enhanced subtelomeric silencing (Freire-Benéitez et al., 2016b). This effect was specific of subtelomeric chromatin and was not observed in other constitutive heterochromatin regions. It suggests that the subtelomeric heterochromatin component evolved to sense environmental variations.

Subsequently, modulation of the transcription of subtelomeric genes, like *TLO*, participated to the rapid adaptation of *C. albicans* to its environment.

However, whether this effect is regulated by telomeres is still puzzling. *C. albicans* telomeres are bound and protected by caRap1 (Yu et al., 2010). Contrary to its homologue in *S. cerevisiae*, caRap1 lacks the C-terminal domain which is known to mediate interaction with the Sir complex and regulate TPE (Biswas et al., 2003). This species lacks as well other components of the Sir complex (Sir3 and Sir4 notably,(Freire-Benéitez et al., 2016b)) suggesting a different way to mediate subtelomeric silencing. Intriguingly, *rap1* deletion mutants exhibit the formation of pseudohyphae under condition that promote growth in yeast form (Biswas et al., 2003). Knowing that derepressed *TLO* participates to the formation of hyphae, a virulent form of *C. albicans*, it is tempting to hypothesis that caRap1 conserves its role in subtelomeric chromatin regulation and participates in the regulation of *TLO*.

Even if more studies are required to decipher how telomeres regulates TPE in *Candida albicans*, it has been shown that the subtelomeric position of virulence-related genes is important for a proper adaptation of this fungus species to environmental changes. In addition, the peculiar subtelomeric environment has been proposed to favour the generation of genotypic diversity in the *TLO* gene repertoire enhancing the adaptability of *C. albicans* (Anderson et al., 2015).

<u>The adhesion properties of *C. glabrata* regulated by TPE:</u>

*Candida glabrata* is an important nosocomial pathogen associated with a high mortality rate. One factor that contributes to its virulence and pathogenicity, is its ability to strongly adhere to surfaces and host cells, to form a biofilm. *In vitro*, this adherence is mainly mediated by Epa1, an adhesin (Cormack et al., 1999). Epa1 is encoded by the *EPA1* (Epithelial Adhesin 1) gene, the first member of a large family of ~23 genes encoding cell wall proteins (EPA genes), several of which encode functional adhesins (Castaño et al., 2005; Cormack et al., 1999; de Groot et al., 2008). Most *EPA* genes were found to cluster in several subtelomeric regions (Castaño et al., 2005; De Las Peñas et al., 2003). Usually silenced, *EPA* genes can be expressed in response to various stresses, such as oxidative stress, glucose starvation, osmotic stress or

limitation in NAD+ precursor, leading to biofilm formation and an enhanced survival rate of *C. glabrata* (Domergue et al., 2005; Juárez-Cepeda et al., 2015; Roetzer et al., 2008).

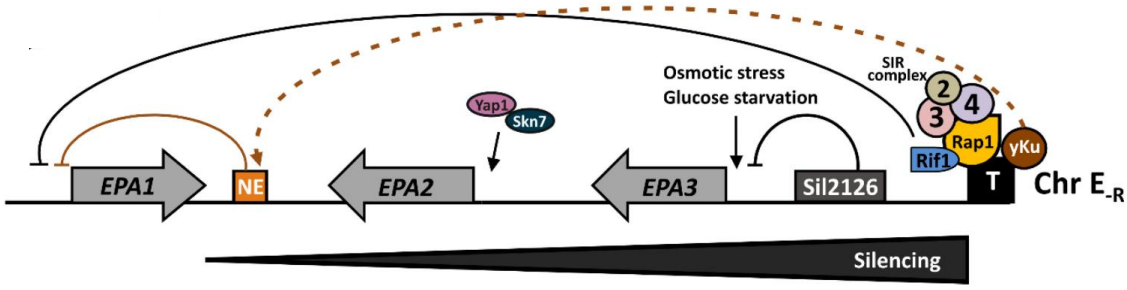


**Figure 14 : Map and regulation of the *EPA* cluster in the right end of chromosome E.**
The silencing complex (Sir2, 3, 4), recruited by Rap1 at telomere, propagates in centromeric direction and silences the subtelomeric *EPA*s (indicated by the horizontal black triangle). Additionally, *EPA1* is repressed by a cis-acting negative element (NE) with the genetic requirement of Ku complex (brown dash arrow). *EPA2* can bypass TPE silencing with the requirement of stress-response transcription factors (Yap1, Skn7). *EPA3* and *EPA1* are also silenced thanks to the proto-silencer Sil2126.
Modified from Lopez-Fuentes *et al*., 2018.

Telomere Position Effect was found to be essential for the repression of subtelomeric *EPA* clusters. Phylogenetically, *C. glabrata* is closer to *S. cerevisiae* than *C. albicans* (Dujon et al., 2004). Telomere protein complex and Sir silencing machinery are conserved between the two species. Telomere-bound cgRap1 recruits cgSir4 which interacts with cgSir2 and cgSir3 to extend heterochromatin in subtelomeric regions (De Las Peñas et al., 2003; Haw et al., 2001; Rosas-Hernández et al., 2008). Overall, all identified subtelomeric EPA genes were shown to be repressed depending on TPE and telomere length. Additionally, it has been shown that cis-acting elements could participate in the regulation. *EPA1* repression and tight regulation, for instance, is also dependent of a negative element located downstream of the gene as well as a protosilencer called Sil2126 located in the same subtelomere (Gallegos-García et al., 2012; Juárez-Reyes et al., 2012; López-Fuentes et al., 2018a). On the other hand, *EPA2* can be expressed and bypass TPE in oxidative stress conditions thanks to the binding of several transcription factors to its promoter (Juárez-Cepeda et al., 2015). Although TPE in *C. glabrata* is a basal silencing mechanism for the *EPA* gene family, silencing induced by telomeres is not homogeneous at the different subtelomeres.

Additional levels of regulations provided by cis-acting elements, transcriptional repressors and activators, allow plasticity in the regulation of *EPA* expression (**Figure 14**). These increase the resistance of *C. glabrata* to a variety of stresses and a better adaptive response (reviewed in (López-Fuentes et al., 2018b)).

Interestingly, it has been shown that replicative aging in *Candida glabrata* enhances the resilience of the cells (Bouklas et al., 2017). In budding yeasts, the replicative lifespan (RLS) is defined as the number of asymmetric mitotic divisions a mother cell can do before it stops dividing and dies (He et al., 2018). Bouklas *et al.* observed that in human patients with candiduria (urinary tract infections), the *C. glabrata* population includes a large proportion of cells with multiple budscars, a hallmark of advanced replicative age. *In vitro*, old cells exhibit an increased cell wall thickness together with an increase resistance to oxidative stress, which was proposed to enhance *in vivo* their resistance to neutrophile. Interestingly, *EPA1* and *EPA6* expression, known to be repressed by TPE, were shown to increase in old cells compared to young ones (Bouklas et al., 2017). cgSir2, an NAD+ dependent deacetylase, is essential for TPE. NAD+ limitation (an environment found during urinary tract infection) was shown to impair *EPAs* silencing in a Sir2 dependent manner (Domergue et al., 2005). Additionally, in *S. cerevisiae* and *C. albicans*, Sir2 level controls replicative lifespan and decreases in old cells (Fu et al., 2008; Kaeberlein et al., 1999). Because Sir2 is a central component of TPE, and because TPE represses genes involved in stress resistances, we could ask if age-related TPE disruption impacts yeast fitness and enhances its pathogenicity.

Overall, it appears that in several *Candida* spp. (and also in other species), stress response genes are located in subtelomeres. The peculiar chromatin environment procured by telomeres allows several levels of gene regulation and permits a tight regulation in response to stress. It also permits a greater evolvability of the stress responsive genes and therefore a greater plasticity to face rapid environmental changes. In this context, Telomere Position Effect appears to be a stress response mechanism.

## Telomere position effect, a stress response mechanism in Saccharomyces cerevisiae:

To understand which biological functions Telomere Position Effect could exert, it is important to know which type of genes are present in subtelomeric regions in *Saccharomyces cerevisiae*. Subtelomeres are devoid of essential genes, but they are enriched in specific gene families important for the interaction of the organism with the environment (Brown et al., 2010; Fabre et al., 2005).

The subtelomeric *MAL* (maltose fermentation) loci, for instance, are composed of three genes encoding for maltose transporters (to import maltose in cell), maltases (maltose hydrolases to transform maltose into glucose) and transcription factors that induce the expression of the MAL loci genes in presence of maltose (Brown et al., 2010; Charron et al., 1989). The diversity of *MAL* paralogues allows the cells to use different carbon sources to grow (Brown et al., 2010). Other polymorphic gene families such as *SUC* (sucrase), *MEL* (alpha-galactosidase)*, IMA* (isomaltase) are also found in subtelomeric positions and are required for alternative carbon source utilization, sucrose, melibiose or isomaltose, respectively (Carlson et al., 1985; Teste et al., 2010; Turakainen et al., 1993). In the usual glucose media, these genes are usually repressed. Additionally to carbon intake-related proteins, *PAU* (seripauperin) genes are the largest gene family in *S. cerevisiae* with 24 members of which 19 are in subtelomeric positions (Luo and van Vuuren, 2009). Their exact functions are still unknown but seems related to cell-wall composition; nevertheless, they are expressed following a wide range of environmental stress (Luo and van Vuuren, 2009; Rachidi et al., 2000). The *FLO* (flocculin) gene family encodes cell-wall glycoproteins that regulate cell-cell and cell-surface adhesion (Teunissen and Steensma, 1995; Willaert, 2018). *FLO* family is related to the *C. glabrata*'s *EPA* adhesin family (Cormack et al., 1999). *FLO* can be divided in two groups. The first group is composed of *FLO1*, *FLO5*, *FLO9* and *FLO10* and promote cell-cell adhesion and the formation of multicellular clumps (flocculation) in response to environmental changes. All are adjacent to telomeres and silenced in normal laboratory condition ((Guo et al., 2000), reviewed in (Goossens and Willaert, 2010)). The second group includes Flo11, Fig2 and Aga1 and are important for diploid pseudohyphal and biofilm formation and mating. They are in intrachromosomal positions (Goossens and Willaert, 2010).

## Cooperation between silencing mechanisms, a key for adaptation:

Taken together, subtelomeres are genomic regions enriched in stress response genes. Therefore, these genes should be properly silenced when cells grow in standard conditions but rapidly and precisely activated in response to a stress. A known regulator of subtelomeric silencing is Hda1, a histone deacetylase, that establishes the repressive HDA1-associated subtelomeric domain (HAST). This enzyme was shown to repress about 40% of subtelomeric genes located between 10 to 25kb from a telomere under rich conditions. This repression is selectively relieved at some location under stress conditions (Martin et al., 2004; Robyr et al., 2002). Importantly, many transcription factors involved in the regulation of stress response genes were found to bind subtelomeric regions with some of them cooperating with Hda1 (Mak et al., 2009). Telomere Position Effect, dependent of the Sir complex, was also shown to repress subtelomeric genes, notably encoding metabolic enzymes (Ellahi et al., 2015). TPE is also implicated in a dynamic silencing of subtelomeric genes prompt to respond to environmental changes.

Subtelomeric *PAU* genes were shown to be bound and repressed by the Sir complex (Ai et al., 2002; Radman-Livaja et al., 2011). Upon stress (rapamycin or chlorpromazine treatment), Sir3 was hyperphosphorylated by the map kinase Mpk1/Slt2, reducing its association with subtelomeric sequences, and consequently *PAU* genes were expressed (Ai et al., 2002). *PAU* expression, probably resulted in a remodelling of the cell-wall, conferring an increased resistance to the drug treatment. Sir3 was shown to be hyperphosphorylated under different conditions, including heat shock, nutrient starvation, and pheromone treatment (Stone and Pillus, 1996). Interestingly, upon pheromone treatment, Sir3 was hyperphosphorylated by another map kinase pathway resulting this time, in an enhanced repression in subtelomeres (Stone and Pillus, 1996). It appears that a variety of environmental stresses triggers Sir3 phosphorylation leading to different responses, allowing a plasticity in the remodelling of subtelomeric heterochromatin. Additionally, the Sir4 N-terminal domain was also shown to be phosphorylated *in vivo*, affecting the stability of the Sir complex binding to nucleosomes and resulting in a decrease in subtelomeric silencing (Kueng

et al., 2012). Mutation of the phosphorylation sites of Sir4 also decreased the ability of cells to grow with rapamycin treatment. It has been proposed that similarly to Sir3, Sir4 phosphorylation could be dependent on environmental signalling pathways to regulate TPE.

In industrial yeast strains, the flocculation process is tightly regulated by a complex network of signalling cascades driven by environmental change. The timing of flocculation is exploited by the brewery industry to separate the yeast from the beer at the end of the primary fermentation. In laboratory yeast strains of *S. cerevisiae*, the subtelomeric *FLO*s are constitutively repressed by chromatin silencing. Interestingly, in condition that reduce the repression of the *FLO10* locus, *FLO10* expression is variegated. Both promoter-specific (binding of Sfl1, Suppressor gene for FLocculation), and telomere position-dependent signalling (Sir3 and Ku70/80) cooperate to regulate its silencing (Halme et al., 2004).

Cooperation between transcription factors responding to environmental signalling and heterochromatin factors are essential for a proper repression of the stress-response genes present in subtelomeric regions, in rich conditions (Halme et al., 2004; Mak et al., 2009; Smith et al., 2011). Interestingly, Smith *et al.* observed that several environment-responsive transcription factors could bind conditionally X and Y'-elements. This binding was shown to participate in the regulation of the proto-silencing activity of X-elements, enhancing or diminishing the Sir2-dependent repression of adjacent genes (Smith et al., 2011). Together with the posttranslational modification on Sir3, this process highlights how the environment can influence the subtelomeric heterochromatin to trigger an appropriate response.

Altogether, Telomere Position Effect, in *Saccharomyces cerevisiae*, can be seen as a component of stress the response mechanism.

## Cellular resilience increases with age:

In yeast, there are two ways to study aging. Replicative lifespan (RLS), as said earlier, is the number of asymmetric divisions a cell can do before it dies (He et al., 2018). Chronological lifespan is the time a cell can survive in a non-dividing state after nutrient exhaustion (Fabrizio and Longo, 2003). Replicative aging in *S. cerevisiae* has

been extensively studied to understand aging in higher order eukaryotes due to the conservation of numerous longevity pathways (McCormick et al., 2015). An interesting feature of replicative aging is the decrease of Sir2 protein levels (Dang et al., 2009). Indeed, Sir2 was shown to be a critical longevity factor for RLS (reviewed in (He et al., 2018; Wierman and Smith, 2014)). Deletion of Sir2 reduced RLS whereas increasing Sir2 dosage improved RLS (Kaeberlein et al., 1999).

Due to the central role of Sir2 in TPE, it can be interesting to ask whether TPE is alleviated in aged cells and what could be its biological relevance. It has been shown that due to Sir2 decrease, H3K16 acetylation level increased in subtelomeric regions of old cells (Dang et al., 2009). Concomitantly, transcriptome analysis in old cells compared to young ones revealed that expression of subtelomeric genes was increased with age (Hendrickson et al., 2018). To my knowledge, TPE disruption in old cells has not been properly assessed, by insertion of subtelomeric reporter genes for instance. Nevertheless, with the genomic data reported here we could hypothesize a TPE alleviation upon yeast aging.

Aged cells are exposed to a wide number of stresses (reviewed in (Dawes and Perrone, 2020)) and a feature of the ageing transcriptome is the activation of the ESR (environmental stress response) (Hendrickson et al., 2018; Janssens et al., 2015; Lesur and Campbell, 2004). ESR was described by Gash *et al.* as a global expression program triggers in response to a variety of environmental stress (heat shock, amino acid starvation, nitrogen depletion…). ESR is a general adaptative response to a suboptimal environment (Gasch et al., 2000). Subtelomeric regions, with genes related to stress, cell surface properties and carbohydrate metabolism, were found to be upregulated both with stress and replicative aging (Hendrickson et al., 2018). These data suggest that TPE alleviation with age participates in a global stress response.

Recently, it has been observed that aged cells, reproducing poorly on glucose compared to young cells, obtained a net advantage over young cells when grown on other carbon sources (Frenk et al., 2017). It was postulated that aging in yeast is associated with a loss of specialization (grow on glucose) over a generalization (grow on other carbon sources). This hypothesis is coherent with the fact that ESR is activated upon aging. This global stress response is probably involved in the resilience of old cells to environmental changes. This is also supported by a mathematical modelling showing that an age-correlated resistance is more beneficial than a random resistance at the scale of the colony (Hellweger et al., 2014). Together with what was

observed with *Candida glabrata* (Bouklas et al., 2017), it seems that aging in yeast could give an advantage to the population allowing a better adaptation to environmental changes. It could be of interest to decipher the contribution of TPE to these phenotypes if they are confirmed.

## Conclusion: subtelomeric regions and stress responses

Telomere Position Effect has been characterized in all species described here and therefore seems to be a global feature of telomeres. Because TPE confer a repressive state to subtelomeric regions, it is legitimate to ask its biological relevance in terms of gene expression.

First, the nature of subtelomeric genes is peculiar. It seems that in most organisms, subtelomeres are enriched in genes related to stress responses. In two protozoans, they confer antigenic variations allowing the escape from the host immune system. In budding yeast (*Candida* spp. and *S. cerevisiae*), they are related to stress responses either by remodelling the cell-wall composition or modulating carbon source intake or regulating transcription machinery. In addition, genes in subtelomeric regions are often found in large families and are prone to rapid evolution. One reason evoked is that subtelomeric regions, following DNA breaks, are tolerant to aberrant repair events due to their little impact on organism fitness (Mason and McEachern, 2018). Nevertheless, this high evolvability confers to subtelomeric genes a high polymorphism associated with diverse functions. Altogether, subtelomeres seem to be a reservoir of evolution to adapt the organism to its environment.

Second, subtelomeric heterochromatin is not fixed. Indeed, subtelomeric heterochromatin seems to respond to environmental variation. Posttranslational modifications affecting key heterochromatin components, change in 3D chromatin conformation, cooperation with transcription factors or silencers, altogether confer to subtelomeric chromatin a plasticity. This cooperation allows a remodelling of subtelomeric silencing to properly regulate subtelomeric genes.

To conclude, TPE has been shown in several organisms to participate in transcriptional remodelling in response to environmental cues. Therefore, TPE has been proposed to be one tool involved in stress response mechanisms. Aging, in

organisms, can be seen as a stressful condition. TPE, if not involved *per se* in regulation of aging, seems to be implicated in the transcriptional changes appearing with age. The relevance of such transcriptional regulation on aging phenotype needs further research.

## Function of Telomere Position Effect in human:

Studies concerning TPE in human are still at the premises. However, even if the mechanism by which telomeres regulate subtelomeric silencing has to be further explored, implications of TPE *in vivo* have been pointed out. As often in science, it is the dysfunction of a biological process that is first identified. This was the case for TPE. The first biological process identified in which TPE was thought to be involved in human was a disease, Facioscapulohumeral Muscular Dystrophy (FSHD). In the following sections, I shall describe several pathologies in which TPE is suspected to be stakeholder, before focusing on genes regulated by TPE in human and discussing the implications of such a mechanism in aging.

## Telomere Position Effect in human pathologies:

### Facioscapulohumeral Muscular Dystrophy, a progressive disease linked to TPE:

Facioscapulohumeral Muscular Dystrophy (FSHD) is the third most common hereditary myopathy with a prevalence of 1 out of 10,000 individuals. Clinical symptoms usually appear during the second decade and strengthen with age. They are characterized by a progressive facial, scapular and humeral muscle weakness but present a high variability among patients and can also affect non-muscle tissues (Richards et al., 2012). Two genetic subtypes of FSHD (FSHD1 and FSHD2) have been identified, with indistinguishable symptoms. FSHD1, the most prevalent form, is linked to a contraction of a microsatellite (D4Z4) array, found in the subtelomeric region of chromosome 4 at the 4q35 region (Wijmenga et al., 1992). Typically, affected patients have between 1-10 D4Z4 repeats whereas healthy individuals have between 11 and 100 repeats. Shortening of the D4Z4 array is associated with an hypomethylated chromatin (Gaillard et al., 2014) implicating epigenetic changes in the disease. FSHD2 patients also exhibit a hypomethylation of the D4Z4 array. They present more than 10 D4Z4 repeats but most of them carry a mutation in the *SMCHD1* gene (Structural Maintenance of Chromosomes Flexible Hinge Domain-Containing Protein 1), an epigenetic regulator involved in D4Z4 silencing (Gurzau et al., 2020;

Lemmers et al., 2012). The open chromatin conformation of this locus allows the expression of *DUX4* (double homeobox protein 4), a gene located in each D4Z4 repeat. However, functional *DUX4* transcripts are only produced by the most telomere proximal D4Z4 in individuals possessing a haplotype A containing a polyadenylation signal (Lemmers et al., 2010) (**Figure 15**). DUX4 protein is thought to be the leading candidate for the pathogenicity of FSHD as its misexpression is toxic to muscle cells (Kowaljow et al., 2007; Rickard et al., 2015).

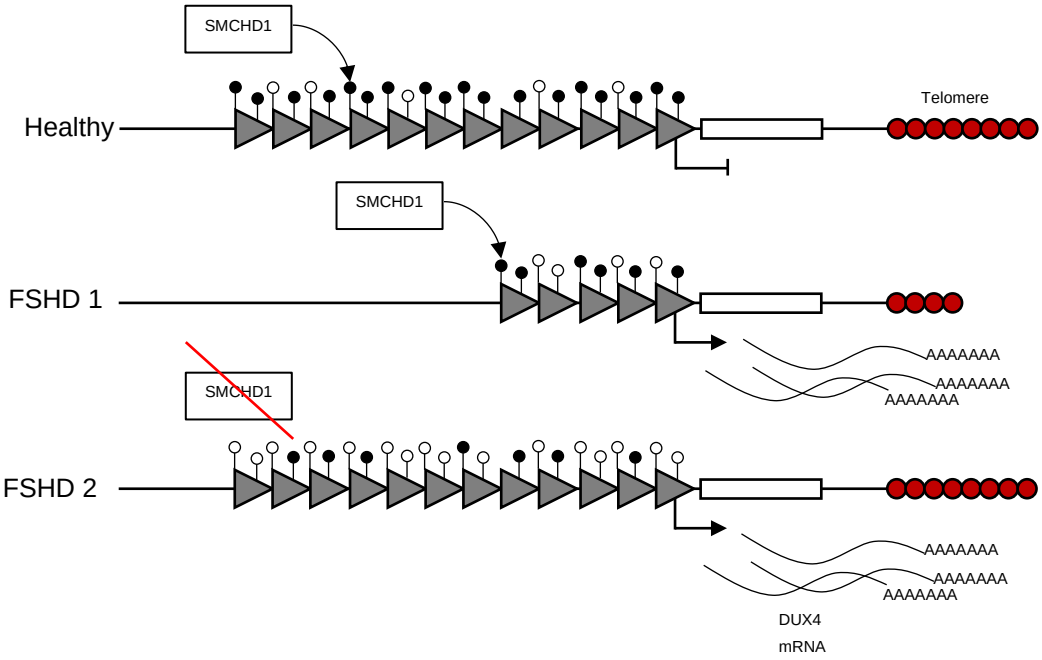


**Figure 15 : Schematic representation of the 4q35 locus in healthy and FSHD patients:**
In healthy patients, the 4q35 locus is composed of >11 D4Z4 repeats (grey triangle) located at ~20kb from the telomere (red dots). The D4Z4 array is methylated and the expression of *DUX4* from the last D4Z4 repeat is repressed.
In FSHD1 patients, the 4q35 locus is composed of less than 11 repeats with the terminal D4Z4 repeat adjacent to a haplotype A bearing a polyadenylation signal and allowing the stable expression of *DUX4*.
In FSHD2 patients, the D4Z4 repeat array is similar to healthy patients. However, a mutation in *SMCHD1* is associated to an hypomethylation of the array and a permissive state allowing the expression of a stable *DUX4* in the presence of the polyadenylation signal.
Inspired from Francastel *et al.*, 2019.

Although FSHD genetic alterations are present since conception, symptoms appear during adolescence, meaning that additional factors or developmentally regulated epigenetic changes, , are involved (Deenen et al., 2014). Among others, telomere length was proposed to affect regulation of *DUX4* via TPE. In differentiated

myoblasts derived from FSHD1 patients (with a short array of D4Z4), telomere shortening was shown to increase *DUX4* expression in association with a decrease in DNA methylation. Thus, telomere length via its control on epigenetic changes regulates *DUX4* expression. Interestingly, in healthy muscle cells (with more than 10 D4Z4), telomere length had no effect on genes located in this locus suggesting an impact of the number of D4Z4 repeats on TPE (Robin et al., 2014; Stadler et al., 2013). Indeed, D4Z4 repeats were shown to be a key factor in the 3D organization of this locus, impacting its nuclear localization (Arnoult et al., 2010), interaction with the lamina (Ottaviani et al., 2009a, 2009b), and long-range interactions (Gaillard et al., 2019; Petrov et al., 2006).

FSHD is the first human disease in which TPE was shown to affect the molecular pathways involved in the pathogenicity. Due to the complexity of the locus, it is still unclear how D4Z4 repeats are involved in the regulation of TPE. Nonetheless, impact on chromatin organization seems central in the D4Z4 regulation by TPE. Since D4Z4 repeats can be found elsewhere in the genome (Lyle et al., 1995), it could be of interest to see their influence on chromatin organization and TPE at other loci.

ICF and telomeric transcription:

Immunodeficiency, Centromeric region instability, and Facial anomalies syndrome (ICF) is a rare autosomal recessive disease. Patients, suffering from immunodeficiency, have a high mortality rate within the first two decades of life. ICF patients display a genome wide DNA hypomethylation, especially striking in pericentromeric regions (Ehrlich et al., 2006). ICF is caused by mutations in four different genes, *DNMT3B* (ICF1, (Xu et al., 1999)), *ZBTB24* (ICF2, (de Greef et al., 2011)), *CDCA7* (ICF3) and *HELLS* (ICF4, (Thijssen et al., 2015)); all are involved in establishment and maintenance of pericentromeric methylation. In addition to pericentromeric hypomethylation, ICF1 patients also exhibit a severe lack of DNA methylation in subtelomeric regions, associated with an enhanced transcription of TERRA (telomeric-repeat containing RNA) (Yehezkel et al., 2008). High TERRA levels in ICF1 cells cause the formation of DNA:RNA hybrids between TERRA and telomeres, leading to telomere dysfunction and shortening and ultimately early senescence (Sagie et al., 2017; Yehezkel et al., 2013). In contrast, ICF2-4 patients with mutations in

*ZBTB24*, *CDCA7* and *HELLS* respectively, display normal subtelomeric methylation levels, telomere length and TERRA expression (Toubiana et al., 2018). Therefore, regulation of methylation in human subtelomeres is dependent of DNMT3B and differs from the pericentromeres.

TERRA transcription starts in subtelomeric regions from promoters located between 10 to 2kb from the telomeric repeats, with some containing CpG islands (Feretzaki et al., 2019; Nergadze et al., 2009). Methylation of CpG islands was shown to regulate TERRA transcription (Le Berre et al., 2019; Nergadze et al., 2009). Due to its implication in ICF1, DNMT3B was shown to be the *de novo* methylase regulating TERRA expression. In addition, telomere length was also demonstrated to regulate telomeric transcription (Arnoult et al., 2012). Whether telomere length influences the recruitment of DNMTB3 in subtelomeric position and therefore whether DNMTB3 is a component of human TPE still needs to be assessed.

Chromosome ring diseases and intellectual disabilities involve subtelomeric abnormalities:

Ring chromosomes are unusual circular chromosomes and generally arise sporadically due to breaks at both chromosome arms with a subsequent fusion of the broken ends resulting in ring formation. Usually, ring formation is associated with loss of genetic information resulting in a large diversity of phenotypes depending on the genes deleted (Guilherme et al., 2011).

However, in a few cases, the rings are formed by telomere-telomere fusions with no losses in terms of gene content and have an intact subtelomeric sequences. The formation of such complete chromosomal rings was reported for several autosomes such as chromosome 14 (Guilherme et al., 2016), chromosome 17 (Surace et al., 2014) chromosome 20 (Peron et al., 2020) or chromosome 22 (Guilherme et al., 2011). Several mechanisms have been proposed to explain the observed clinical phenotypes: (i) somatic mosaicism due to ring instability, (ii) TPE, (iii) other PEV. Concerning TPE, in ring 17 syndrome, a patient with a complete loss of telomeric repeats without other chromosomal alteration has been observed. This patient exhibits *café au lait* skin spots, intellectual disability, epilepsy seizures and dysmorphic traits.

The loss of telomeric sequences causes alterations in the expression of subtelomeric genes which are linked to the phenotype observed. Another patient with ring 17 syndrome and similar symptoms displays a short array of telomeric repeats. Her mother, who also possesses a chromosome 17 ring, presents only a *café au lait* skin spots phenotype, and possesses a long array of telomeric repeats (Surace et al., 2014). It strongly suggests that the severity of the disease is linked to telomere length and differential defect in TPE.

Strikingly, chromosome ring syndromes with defect in subtelomeric regions and/or telomeres are often associated with mental retardation, epileptic seizures… This suggests the importance of a proper regulation of subtelomeric gene content in brain development. Concomitantly, intellectual disabilities are often associated with subtelomeric abnormalities (Michelson et al., 2011; Shao et al., 2008; Soares et al., 2019). About 3 to 16% of the known chromosomal abnormalities are in subtelomeric regions. Usually submicroscopic (smaller than 5Mb), they are not detected with classical karyotyping and need higher resolutive genetic techniques. An interesting study implicating patients with deletions within 0.3 to 2Mb from the telomere of chromosome 16p did not observe a significant alteration in some subtelomeric gene expression. Nevertheless, they observed that long-range interactions between genes adjacent to chromosomal breaks were severely disrupted, highlighting the importance of 3D chromatin conformation in subtelomeric regions (Babbs et al., 2020).

Subtelomeric regions are of particular importance for proper development. Abnormalities are linked to several genetic diseases. Telomeres, through their ability to modulate the epigenetic state of subtelomeric regions as well as chromatin conformations are thought to affect the complex pathogenic traits associated with these diseases. Inversely, studying diseases with subtelomeric deletions could provide insight into the mechanisms by which telomeres influence gene regulation.

## Telomere length and cancers:

In human, somatic cells gradually shorten their telomeres due to the silencing of *TERT*. When telomeres become critically short, DNA damage response is triggered,

leading to cell cycle arrest and replicative senescence (Hayflick, 1965). This biological clock limiting the number of divisions in which cells can acquire oncogenic mutations, is seen as a robust tumour suppressor mechanism (Deng et al., 2008). Paradoxically, critically short telomeres are one of the primary events leading to carcinogenesis (Chin et al., 2004; Hastie et al., 1990). Cells can bypass senescence and resume proliferation if they acquire mutations in genes involved in cell cycle check-point mechanisms, for instance in p53 (Chin et al., 1999). This extension of their cell cycle leads to strong telomere dysfunction, resulting in a high genomic instability during which most cells die. However, some cells overcome this crisis by activating a telomere length maintenance mechanism, allowing them to proliferate indefinitely. Consequently, ~90% of human cancers show telomerase reactivation (Shay and Bacchetti, 1997) whereas the ~10% left activate alternative lengthening of telomeres (ALT) pathways (Heaphy et al., 2011).

In this respect, longer telomeres would be more advantageous to the cancer cells to maintain their immortality. However, even after telomerase reactivation, cancer cells maintain short telomeres compared to surrounding tissues (Meeker et al., 2002; Sommerfeld et al., 1996). Recently, Barthel *et al.* conducted a survey of telomere length in 18,430 samples across 31 types of cancer cohorts. They observed that in 70% of tumors analyzed, telomere length was shorter compared to matched normal samples (Barthel et al., 2017). This result highlights the possibility of a selective pressure to maintain short, instead of long telomeres in cancer.

To explain the discrepancy between the expected long telomere and the observed short telomeres in cancer cells, several ideas has been proposed. One reason is that short telomeres induce a moderate genomic instability advantageous to cancer evolution. A second interpretation is that length doesn't matter if the telomeres stay protected, and the short telomeres observed are due to a particular balance in telomere length regulation specific to cancer cells. For instance, the overexpressed shelterin complex exerting negative feedback on telomerase access to telomeres, could result in short telomeres (Hu et al., 2010; Shore and Bianchi, 2009). Lastly, via their effect on gene expression and TPE, short telomeres could be beneficial.

Hirashima *et al.* addressed the role of telomere length in cancer cells by elongating telomeres in three cancerous cell lines (prostate PC-3, stomach MKN74,

breast HBC-4) that usually maintain short telomeres (Hirashima et al., 2013). They did not observe any effect *in vitro*. However, the xenografts derived from cancer cells with long telomeres were more differentiated and consequently less aggressive. Their gene expression analysis by microarray revealed 151 genes dysregulated in xenografts with long compared to short telomeres. Notably, they explained this difference in differentiation by the overexpression of type I interferon signaling-related genes in cells with short telomeres. Importantly, they did not observe an enrichment of dysregulated genes in subtelomeric regions, but they identified *ISG15*, the first identified TPE-regulated genes, as overexpressed in cells with short telomeres (Hirashima et al., 2013; Lou et al., 2009). Some subtelomeric genes, regulated by telomere length could be potential pro-oncogenes and by maintaining short telomere length, cancer cells allow their expression and tumor progression. In accordance with a potential role of TPE in carcinogenesis, expression of genes near telomeres showed higher expression in tumours than normal tissue (Barthel et al., 2017). And in agreement with what was described by Robin *et al*, the enrichment in upregulated genes in cancer cells was not detectable beyond 10Mb from a telomere. Additionally, *TERT* was also shown to be regulated by TPE, therefore, additionally to the numerous mutations identified in cancer cells to re-express TERT, maintaining short telomeres could add another layer to ensure TERT expression (Kim et al., 2016; Rachakonda et al.).

There is not so many evidence on the role of TPE in cancer cells. However, given the prevalence of short telomeres in tumors, and the associated derepression of subtelomeric regions, TPE could be at play. Did the TPE regulated genes participate to a selective pressure forcing short telomeres in cancer cells? What could be the functions of those genes? To answer these questions, we need to understand the function of TPE, if any, in non-cancerous cells.

## Telomere Position Effect in human aging:

Aging can be broadly defined as a progressive decline of the function of an organism with time. In human cells, telomere shortening, and concomitant replicative senescence has been described as a hallmark of aging (López-Otín et al., 2013). Since

Hayflick's research on primary fibroblast, it is known that human somatic cells have a finite number of divisions before entering senescence. Telomere length, observed to decrease with time in primary cells, (Harley et al., 1990) has been proposed to be part of a mitotic clock regulating the number of cell division (Vaziri et al., 1994). Telomere shortening has been described in several tissues, and even if the telomere length dynamic is tissue specific, the age-related shortening appears to be conserved (Daniali et al., 2013; Demanelis et al., 2020). Moreover, telomere length in three tissues (whole blood, lung, and oesophagus-mucosa), was shown to mediate the expression of age-related genes (Demanelis et al., 2020). Although gene expression changes can result from telomere dysfunction *i.e.* DDR and extracellular stress response pathways, other telomere signalling pathways can be at play (Ye et al., 2014). Because TPE is regulated by telomere length, and telomere length decreases with age, aging is believed to disrupt TPE. In this respect, I shall detail what are the genes known to be regulated by TPE in human cells in relation to aging.

SIRT6 and TRF2 proteins level decrease with age and senescence:

In human cells, TPE mechanism is still puzzling. Nonetheless, SIRT6, an NAD+ dependent deacetylase, and TRF2, a core component of the shelterin complex, were shown to be involved in TPE (Kim et al., 2016; Robin et al., 2014; Tennen et al., 2011). In order to understand how aging could impact TPE, in addition to the decrease in telomere length, it is also important to understand how those two proteins are modulated in response to aging.

SIRT6, is a protein involved in multiple molecular pathways including DNA repair, telomere maintenance, glycolysis, lipid metabolism, inflammation (reviewed in (Chang et al., 2020). Importantly, it has been the first sirtuin (among the seven homologues, SIRT1 to 7, of *S. cerevisiae* Sir2 in human) shown to regulate lifespan in mammals. Male mice overexpressing *Sirt6* exhibited an extended lifespan (Kanfi et al., 2012). In human primary fibroblast cell lines, SIRT6 protein levels were shown to decrease as population doubling increased. Importantly, this decrease was initiated before cells entered senescence (Mao et al., 2012). Overexpressing *SIRT6* was shown to delay senescence (Mao et al., 2012; Zhang et al., 2016a). Using foreskin fibroblasts

from 19 donors of different ages, Xu *et al.* found that SIRT6 expression levels correlate strongly and negatively with age (Xu et al., 2015). Very recently, SIRT6 serum levels and activity were studied in a cohort of 155 participants. It has been found that upon aging, the level of SIRT6 in sera decreases, principally in women, together with its activity, both in man and woman. This age-dependent decrease was confirmed in vascular tissues. In accordance, an increase of H3K9 and H3K56 acetylation was also observed in aged vascular tissues (Zhao et al., 2021). To conclude, it seems that, at least in human fibroblasts, SIRT6 protein levels decrease as cells approach senescence, and decreases with age in serum and in vascular tissues together with its deacetylase activity.

TRF2 protein levels were also shown to vary with aging. During replicative senescence, in fibroblasts, the amount of TRF2 was shown to decrease. The loss of functional inhibition by TRF2 at uncapped telomeres triggers DDR and activates p53. In turn p53 was shown to activate SIAH1, a ubiquitin ligase, that ubiquitinylates TRF2 and induces its proteasomal degradation, resulting in its loss. This TRF2 loss, reinforces DDR signalling and triggers replicative senescence (Fujita et al., 2010). In addition, TRF2 was also repressed in senescent cells by a microRNA, mir-23a (Luo et al., 2015). TRF2 amounts, concomitantly with telomere shortening, were also shown to decrease in human skeletal muscle biopsies from patients with different ages (Robin et al., 2020). TRF2 expression was also observed to decrease in several organs with age in zebrafish (Wagner et al., 2017). Importantly, TRF2 seems to be the only shelterin component that decrease with age and senescence in human (Fujita et al., 2010; Robin et al., 2020). With the exception of skeletal muscles, whether TRF2 decreases globally with age in human tissues is still under investigation.

Altogether, both SIRT6 and TRF2, two proteins involved in the mechanism of TPE, seem to be influenced by age and senescence. Since telomere length, TRF2 and SIRT6 decrease with age, it strongly reinforces the hypothesis that TPE is alleviated upon aging.

<u>TERT, telomere length and aging:</u>

Kim *et al.* observed that *TERT* expression was regulated by TPE. Both in primary fibroblasts, which expressed *TERT* at a very low level, and in HeLa cells, a cancerous cell line with a high *TERT* expression, cells with long telomeres expressed less *TERT* than cells with short telomeres. Long telomeres were shown to fold back on the TERT locus and silenced it, thanks to a TRF2-mediated loop. In aged fibroblasts, with short telomeres, the looping structure was lost and associated with an increase of active epigenetic marks (H3K4me3, H3K9ac) across the *TERT* promoter, suggesting a more permissive state (Kim 2016). Additionally, TRF2 was also found to bind directly the TERT promoter to repress its expression, suggesting a double control of telomere on TERT expression (Sharma et al., 2021). Interestingly, the subtelomeric location of the *TERT* locus was shown to be conserved among higher primates, which also silence *TERT* in their somatic cells. In these species, telomerase is activated at the beginning of embryonic life to ensure a proper telomere elongation and a "reset" of the mitotic clock, but it is silenced afterward. It has been proposed that the negative feedback loop between telomere length and *TERT* expression could explain the silencing of *TERT* during embryonic development when telomere elongation is achieved (Kim et al., 2016; Wright et al., 1996). On the counterpart, as discussed earlier, telomere shortening during aging could favour the permissiveness of *TERT* locus, and therefore facilitate its reactivation in cancer cells.

In addition to its main canonical role, *i.e.* elongation of telomeres, telomerase and notably the catalytic protein TERT could exert non-canonical functions. Among them, TERT was shown to be translocated in mitochondria to reduce oxidative stress (Gordon and Santos, 2010), to have neuroprotective activities (Eitan et al., 2016; González-Giraldo et al., 2016), to participate to the Wnt/β-catenin or NFkB signaling pathways and therefore regulating a wide range of genes to maintain pluripotency (Teichroeb et al., 2016). Consequently, it has been suggested that TERT re-expression could be beneficial in some aspects (Romaniuk et al., 2019).

Nevertheless, TERT expression has not been shown to increase with age. On the contrary, TERT mRNA levels have been observed to decrease with age in serum and vascular tissues of individuals (Zhao et al., 2021). Moreover, across different human tissues from donors with different ages, TERT expression was weakly but positively correlated with telomere length (Demanelis et al., 2020). Altogether, further investigations are needed to know if the permissive state of *TERT* loci induced by telomere shortening allows the re-expression of *TERT* with age. And whether TERT

could be beneficial in normal cells via its stress response-related non-canonical activities.

ISG15, a stress response gene regulated by TPE:

Interferon stimulated gene 15 (ISG15) belongs to the family of ubiquitin-like modifiers. As ubiquitin, ISG15 can be conjugated to other proteins, a process known as ISGylation, and can participate to the posttranslational modification of proteins (Haas et al., 1987). Additionally, ISG15 can be found in an unconjugated intracellular or extracellular from (Knight and Cordova, 1991; Zhang et al., 2015). *ISG15* is constitutively expressed at low levels but its transcription can be enhanced in response to interferon-α and -β and act as an antiviral molecule (reviewed in (Villarroya-Beltri et al., 2017). *ISG15* expression has been shown to be activated following a wide range of stimuli, mostly related to stress conditions such as genotoxic stress (Jeon et al., 2012). Notably, *ISG15* was shown to be regulated by TPE. In primary young fibroblasts with long telomeres, ISG15 is expressed at low levels. These levels are gradually increased upon cell passages and telomere shortening. Conversely, when TERT is induced and telomere length increased in old fibroblasts, ISG15 expression is decreased (Lou et al., 2009; Zhang et al., 2021).

Interestingly in regard to aging, ISG15 was shown to modulate p53. In healthy cells, ISG15 is preferentially conjugated to misfolded p53 to ensure its degradation and therefore promote p53 wild-type function (Han et al., 2018; Huang et al., 2014). Therefore, we could assume that the telomere-length dependent upregulation of ISG15 with age could have a protective effect on aged cells by ensuring a proper function of p53. On the other hand, with its function in immunity, ISG15 could favour a pro-inflammatory environment (reviewed in (Villarroya-Beltri et al., 2017)). It is well known that a large number of age-related diseases are associated with inflammation. In this regard ISG15 could be detrimental to healthy aging. Additional investigations will be needed to know if ISG15 is upregulated with aging and whether telomere length is involved in its regulation. Nonetheless, it is interesting to note that a stress response gene, as in budding yeast, is located in a subtelomeric region and is regulated by TPE.

Overall, what can we say about TPE and aging in human?

As discussed earlier, three components of TPE mechanism *i.e.* telomere length, SIRT6 and TRF2, seem to decrease with age in human and were shown to decrease at replicative senescence. Therefore, it is expected that Telomere Position Effect is disrupted upon aging and replicative senescence and participates to the transcriptional remodelling observed.

In human, TPE was studied essentially in cell lines. Initially, mostly cancerous cell lines with a subtelomeric reporter were used to decipher the presence of TPE in human and its mechanism (Baur et al., 2001, 2004; Koering et al., 2002; Kulkarni et al., 2010; Tennen et al., 2011). Difference in telomere lengths were obtained by overexpressing TERT. To identify which genes are regulated by TPE, different strategies were used. Lou *et al.* identified *ISG15* comparing proliferative young fibroblasts (long telomeres) with proliferative old fibroblasts (short telomeres) with proliferative old fibroblasts overexpressing TERT (long telomeres). Importantly, a custom microarray containing only genes within the first megabase was used for the gene expression analysis (Lou et al., 2009). In order to compare cell populations with a homogeneous telomere length, Robin *et al.* used old proliferative fibroblasts and myoblasts infected with an overexpressing TERT cassette. Excision of the cassette at different time points gave rise to cell populations with different telomere lengths. An early excision resulted in a population with short telomeres while a late excision generated a population with long telomeres (Kim et al., 2016; Robin et al., 2014, 2015). Importantly, in all these situations cells were never in a replicative senescence state. To date, only Ning et al. analysed the expression of subtelomeric genes comparing proliferative fibroblasts (with long telomeres) with senescent fibroblasts (with short telomeres). They measured the expression of 34 subtelomeric genes by qPCR. However, they did not identify a correlation between telomere length and the mRNA level of those genes (Ning et al., 2003). Altogether, even if strongly suspected, it is not known whether subtelomeric transcription is modified in senescent cells and whether TPE is implicated.

At the level of the organismal and tissues aging, some genome-wide studies indicate that TPE could be involved in the transcriptional changes observed. The Genotype-Tissue Expression (GTEx) project aggregates samples from 54 non-diseased tissue sites, collected on nearly 1000 individuals with different ages. Using these data, Demanelis *et al.* observed a decrease in telomere length in correlation with age. Moreover, exploiting the RNAseq data provided, they identified the age-associated genes. Using a causal mediation analysis, to identify which factor could cause the dysregulation of gene expression during aging, they identified that telomere length mediated the effect of age on expression for ~16% of the genes in the three analysed tissues (whole blood, lung, and oesophagus-mucosa). Altogether, they reveal that telomere length is a relevant biological factor in the regulation of age-related gene expression (Demanelis et al., 2020). Also taking advantage of GTEx datasets, another team extract the age-related differentially expressed genes. They observed that genes upregulated with aging were enriched in the first 2Mb from a telomere (Dong et al., 2021). This suggested that TPE was alleviated in aged tissues and therefore subtelomeric genes could be expressed.

# Conclusion:

In human, TPE is not very well understood both at the molecular and at the functional level. Few genes have been identified as being subjected to an epigenetic regulation emanating from telomeres. To date, the influence of telomere regulated transcription is still speculative in replicative senescence and aged tissues in human. To obtain a better understanding on the numerous transcriptional changes associated with a senescent state, it is of interest to understand how telomeres influence gene regulation and if Telomere Position Effect is at play.

From a yeast point of view, TPE can be seen as a stress response mechanism. With this idea in mind, it could be interesting to see if TPE is part of a stress response in human cells. Indeed, telomeres are sensitive to a wide range of stresses that induce telomere shortening but also changes in telomere composition. For instance, telomeres are sensitive to oxidative stress (von Zglinicki et al., 1995), to inflammation (Maekawa et al., 2018), to genotoxic stress (Fumagalli et al., 2012), to psychological stress (Epel et al., 2004) and so on. A modest telomere shortening could alleviate TPE in response to a stress and allow the transcription of stress response genes. Indeed, it has been shown that TPE could be disrupted long before senescence and trigger expression in a telomere length dependent manner, at least for ISG15 (Lou et al., 2009; Zhang et al., 2021). Also, in line with the idea that several stresses could alleviate TPE, two compounds (acacetin and chrysin), were shown to target telomeres, disrupt TPE and allow expression of subtelomeric genes. These drugs did not induce telomere shortening but telomere deprotection. However, telomere deprotection by shelterin downregulation did not strongly induce the expression of a subtelomeric reporter gene, suggesting that deprotection alone is not the leading cause of TPE alleviation in this context (Boussouar et al., 2013). Nevertheless, it suggests that perturbation in telomere homeostasis and not only telomere length could impact subtelomeric silencing. Recently, based on observations in several organisms, a telo-hormesis hypothesis has been proposed. In brief, a limited exposure to a stress affecting telomere integrity will ensure the adaptation of the organism and be beneficial *in fine*, for instance by modulating gene expression (Jacome Burbano and Gilson, 2021). On the contrary, a chronical or high stress will lead to a point of no return, dramatically

damage telomeres and be detrimental to the organism, leading to senescence or cell death. Similarly, a low level of senescence could be beneficial by preventing tumorigenesis whereas senescent cells accumulation could trigger aging and even cancer.

Nevertheless, even if this hypothesis is attractive, further data need to be generated and/or explored to understand the biological function and the mechanism of TPE in human.

# RESULTS

Telomeres are more than just caps protecting chromosome ends. They participate to the regulation of numerous processes and notably, they can regulate gene expression in a DDR-independent manner. It has been shown, in several evolutionarily-distant organisms, that telomeres influence the epigenetic state of adjacent (sequentially and/or spatially) regions. This epigenetic mechanism, known as Telomere Position Effect, and described extensively in the introduction of this manuscript, was shown to regulate gene expression. However, despite its discovery 20 years ago in human cells, we still don't understand how telomeres recruit or influence the recruitment of heterochromatin factors in subtelomeric position; nor we understand the biological function of TPE. More broadly, the influence of telomere on transcription regulation is still puzzling.

In replicative senescence, telomere shortening triggers the DNA damage response and the entry into senescence. Accumulation of senescent cells is seen as a motor of aging resulting in numerous degenerative processes. We know that senescence is accompanied by a profound remodelling of transcription, of chromatin organization and of nuclear structure. The age-related telomere shortening has been proposed to participate in the transcriptional changes associated to aging. However, we still don't know if the DDR-independent signalling properties of telomeres impact the transcriptional outcome of human senescence cells.

In this context, we wanted to investigate how telomeres could influence gene regulation in senescent cells. To this end, we performed RNA sequencing in young fibroblasts, with long telomeres, and in replicative senescent fibroblasts, with short telomeres. We observed that up-regulated genes in senescent cells were enriched in the first 2 megabases from a telomere. We found that those subtelomeric genes upregulated in senescent cells were sensitive to the level of TRF2 and LaminB1, a member of the nuclear lamina. To investigate the importance of chromatin conformation in this up-regulation, we developed a three-dimensional DNA fluorescent in situ hybridization (3D-FISH) using locus-specific subtelomeric DNA probes and PNA telomeric probes.

To get more insight on the potential factors that could influence this derepression of subtelomeric genes at replicative senescence, we took advantage of public datasets. Notably, we observed that the enrichment of upregulated genes in subtelomeric regions was not restricted to replicative senescent cells. Moreover, the

up-regulated genes were found clustered in only a subset of similar subtelomeres and correlate with specific epigenetic marks.

## Specific subtelomeric genes are derepressed during cellular senescence

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### Abstract

**Aging is a continuous process leading to physiological deterioration with age. One of the factors contributing to aging is telomere shortening leading to alterations in the nucleoprotein protective complex named shelterin and consequently to replicative senescence. Here, we report the transcriptional changes occurring in human replicative senescent cells by performing RNA sequencing in young versus senescent lung fibroblast MRC-5 cells. We found that senescence triggered the upregulation of genes enriched in specific subtelomeric regions, restricted to 2 megabases from telomeres. Moreover, we unveiled that the expression of these genes was influenced by the dosage of the shelterin protein TRF2 and the nuclear lamina factor, Lamin B1, both of them being downregulated upon telomere shortening. Overall, these results provide**

**new insights on how telomere shortening contributes to the transcriptional changes associated to the aging process.**

## Introduction

The biology of aging studies the mechanisms leading to a functional decline as a function of age, whether at the molecular, cellular, tissue or systemic level (Gorgoulis et al., 2019; López-Otín et al., 2013). Over the past two decades, our understanding of aging biology has made remarkable progress allowing the identification of unifying features (López-Otín et al., 2013). Among them, the accumulation of senescent cells appears as a major contributor to physiological and pathological aging (Baker et al., 2011, 2016). However, the integration and time-dependent relationship between these aging pathways in different organs and major physiological functions as well as their roles in age-related diseases remain largely unrecognized (Roy et al., 2020). Various aging clocks have been recently described (transcription (Meyer and Schumacher, 2021), methylation (Horvath, 2013) and inflammation (Kozlov, 2021)) that provide interesting tools to assay for biological age but we are still facing a “cause or consequence” conundrum. Thus, there is need to decipher the time-dependent and causality relationship existing between the different aging pathways. This raises the still debated question of whether aging results from developmentally regulated clocks or simply from a cumulative effect of damages over time.

Deprotecting natural chromosome ends is one of the aging effector mechanisms (Blackburn et al., 2015; López-Otín et al., 2013). This deprotection results from alterations of the telomeres, the nucleoprotein structures capping the end of

chromosome to protect them from unwanted DNA damage response (DDR) checkpoint activation and recombinational repair that would otherwise lead to cell cycle arrest and chromosome instability. This protection is ensured in mammals by the shelterin protein complex that specifically binds to telomeric DNA and is composed of six proteins: TRF1 and TRF2, binding the double-stranded DNA part of telomeres; POT1, binding the 3′-overhang, RAP1, binding to TRF2; and TIN2/TPP1, forming a protein bridge between TRF1/TRF2 and POT1 (Ghilain et al., 2021). In addition, telomeric chromatin is associated to the non-coding RNA TERRA transcribed from subtelomeric regions and can adopt peculiar conformations involving telomeric DNA looping (T- loop) (Giraud-Panis et al., 2013). The formation of T-loops, which is facilitated by the shelterin subunit TRF2, contributes to prevent DDR checkpoint and repair (Benarroch-Popivker et al., 2016; Doksani et al., 2013; Sarek et al., 2019). The DNA replication of telomeric DNA requires specific mechanisms to compensate for the inherent inability of the replication machinery to fully duplicate the extremities of the parental DNA molecule (Gilson and Géli, 2007). In many organisms, this involves a specialized reverse transcriptase, the telomerase (Blackburn et al., 2006).

Among the mechanisms leading to aging, telomere shortening is the only one clearly established as being programmed during normal development. Indeed, in human and other vertebrate species, the telomerase expression is downregulated during development in somatic tissues leading to telomere shortening at each cell division, eventually resulting in replicative senescence when a subset of telomere become too short to ensure their anti-checkpoint functions (Hayflick limit) (Abdallah et al., 2009; Bodnar et al., 1998). Over the course of life, in agreement with its link to cell division, the rate of telomere shortening is paced in a tissue specific manner as a function of their regeneration properties (Demanelis et al., 2020). The rate of telomere shortening

can also respond to external stressors, generally by accelerating it as a result of increased oxidative stress which predominantly attacks telomere DNA (Jacome Burbano and Gilson, 2021). Whether telomere shortening can also occur at a low rate in non-dividing cells is suggested by some reports in post-mitotic cell systems and tissues (Daniali et al., 2013; Robin et al., 2020). Telomere shortening can be a driver of human aging since germinal mutations of the telomerase and shelterin complexes, leading to critical telomere shortening and deprotection, causes rare progeroid syndromes such as Dyskeratosis congenita (Armanios and Blackburn, 2012). Moreover, short telomeres are associated in the general population with a broad spectrum of age-related pathologies, including cardiovascular and neurodegenerative diseases, type II diabetes, pulmonary fibrosis, and arthrosis (Blackburn et al., 2015; Martínez and Blasco, 2017).

Understanding how telomere shortening contribute to normal and pathological aging implies the identification of the pro-aging signals emanating from critically short telomeres. The current view is that these signals result from the activation of the DDR checkpoint leading to replicative senescence and therefore aging (d'Adda di Fagagna et al., 2003). Since another function of telomeres is to influence gene expression (Gottschling et al., 1990), it has been proposed that the age-related telomere shortening also contributes to the transcriptional changes associated to aging (Maillet et al., 1996; Wright and Shay, 1992; Ye et al., 2014). In agreement with this hypothesis for human aging, there are genome-wide indications that telomere length is a relevant biological factor in the regulation of age-related gene expression (Demanelis et al., 2020) and that genes upregulated with aging are enriched in subtelomeric regions (Dong et al., 2021). The telomere length-dependent mechanism regulating the expression of subtelomeric genes can be DDR-independent (Lou et al., 2009),

indicating that the transcriptional signaling of telomere dysfunction can be uncoupled from DDR activation and cellular senescence (Ye et al., 2014).

The two established mechanisms by which telomeres influence transcriptional regulation have been discovered and thoroughly studied in the budding yeast *Saccharomyces cerevisiae*. This was first the case for the process leading to the repression of subtelomeric genes, named telomere position effect (TPE) (Gottschling et al., 1990). In brief, the major shelterin subunit in budding yeast, Rap1, serves to recruit a heterochromatin complex at telomeres, including the sirtuin Sir2, followed by its spreading toward the centromere (Kueng et al., 2013). The subtelomeric genes succumbing to this TPE silencing is determined in a chromosome-end-specific manner by specific combinations of insulator and proto-silencer sequences leading to subtelomeric chromatin loops that regulate the heterochromatin spreading in a discontinuous manner (Fourel et al., 1999; Lebrun et al., 2003; Miele et al., 2009). The second form of telomere-mediated transcriptional regulation concerns the sequestration at telomeres of transcription factors (Maillet et al., 1996; Marcand et al., 1996; Ye et al., 2014). In case of telomere shortening, their release throughout the nucleoplasm leads to genome-wide transcriptional regulation of targeted genes (Platt et al., 2013; Ye et al., 2014). Interestingly, the yeast genes whose expression is regulated by telomeres are related to stress responses and could thus contribute to an adaptive response in case of telomere dysfunction (Ai et al., 2002; Platt et al., 2013).

In human cells, telomere proximity also represses gene expression (Baur et al., 2001; Koering et al., 2002). This human TPE involves TERRA (Arnoult et al., 2012), a shelterin subunit (TRF2) (Deng et al., 2009; Kim et al., 2016; Robin et al., 2014), a sirtuin enzyme (SIRT6) (Tennen et al., 2011) and long-range chromatin loops within the subtelomeric region (Robin et al., 2020). The mechanisms allowing long-range

chromatin interactions at subtelomeres could rely on an interaction between telomere and interstitial telomeric sequences, or ITS, in a Lamin A/C and TRF2 dependent manner (Robin et al., 2020; Wood et al., 2014). However, the heterochromatin nature of human telomeres remains controversial since, in many cell types, telomeres do not exhibit classical heterochromatin marks (Cubiles et al., 2018; Gauchier et al., 2019). Thus, despite striking similarities with yeast, no comprehensive model is established yet for human TPE rendering elusive our understanding of the transcriptional role of telomere during aging.

In this work, we analyzed, by RNA-sequencing, the transcriptional changes occurring at replicative senescence in a well-established human cell model of telomere shortening. We found a significant enrichment of up-regulated genes at senescence in regions confined to 2 megabases from telomeres. This allowed us to identify some of the common features of these genes, including their sensitivity to TRF2 and Lamin B1 dosage as well as their clustering in a subset of subtelomeres. Using RNA-seq public databases of oncogene-induced senescence, we found that the same subset of subtelomeres contains genes specifically derepressed during senescence. We discuss these results in term of the broad roles played by telomeres and subtelomeres in the transcriptional changes associated to aging.

## Results

### Subtelomeric genes are upregulated at replicative senescence

To study whether telomere length impacts the transcriptional regulation of cells, we grew human lung primary fibroblasts (MRC-5 cells) at 5 % oxygen until they senesce (population doubling, PD, 71). We defined a senescence culture when cells stop

proliferating, are positive for senescence-associated $\beta$-galactosidase (SA-$\beta$-Gal) staining and EdU is incorporated in less than 1 % of the cells. We harvested cells from young MRC-5 cells (PD 30) and senescence (PD 71) in triplicates, extracted RNA, carried out RNA sequencing (RNA-seq) and performed differential expression analysis. This allowed us to identify 1051 genes whose expression is altered between senescent and young MRC-5 cells. Then, we conducted an enrichment analysis on the differentially expressed genes (DEG) found in senescent versus young MRC-5 cells by establishing intervals of one mega base (Mb) starting from the chromosomal ends and we calculated the fold enrichment of the upregulated and downregulated genes in each segment (Figure 1A). Strikingly, we noticed that upregulated genes in senescence were positively enriched within the first 2 Mb from a telomere, *i.e.* at the subtelomeric regions (fold enrichment = 3.6 and 3.8 with $p = 7.86e^{-15}$ and $7.13e^{-14}$ for the first and second Mb respectively, Figure 1A). On the contrary, no specific enrichment was observed at subtelomeric regions for the downregulated genes.

Next, we studied the expression levels of the DEG in young and senescent cells by analyzing the RNA-seq counts. We used the DESeq2 normalized counts of genes located in the first 2 Mb from telomeres, and we separated them in upregulated and downregulated genes (Figure 1B). This analysis revealed a significant increase of read counts in senescent versus young MRC-5 of the upregulated DEG group ($p < 0.0001$, Kruskal-Wallis test, Figure 1B). This increase is also present in the no-DEG group but in a lesser degree ($p < 0.001$, Kruskal-Wallis test) showing that the expression of subtelomeric genes at senescence is globally increased. Interestingly, there were significantly less reads in the young DEG group compared to the no-DEG group ($p < 0.0001$, Kruskal-Wallis test), suggesting that some genes in the subtelomeric regions are repressed and this effect is alleviated in senescent cells resulting in the

upregulation of gene expression. Regarding downregulated genes (Figure 1B right panel), we confirmed that read counts in the senescent DEG set were significantly lower compared to the young DEG group ($p$ = 0.0151, Kruskal-Wallis test). This suggests that downregulated DEG in senescent cells, at least in subtelomeric regions, is the result of a repression of transcription.

Overall, we found that subtelomeric genes located at the terminal 2 Mb of chromosomes in senescent cells are preferentially upregulated. This phenomenon is caused by an increase in transcription of subtelomeres in senescent cells.

**Increase of subtelomeric transcription is restricted to a subset of subtelomeres**

Next, we studied whether the increase of subtelomere transcription at senescence is a global feature of subtelomeres. We looked for positive fold enrichment of upregulated genes chromosome by chromosome by defining bins of 2 Mb. We found seven chromosome ends showing a significant positive enrichment of upregulated genes at the first 2 Mb from the telomeres (chromosome arms 8q, 9q, 11p, 16p, 16q, 19p and 22q, Figure 1C and S2A). Only one chromosome arm (5p) was enriched for downregulated genes (Figure S2B). Of note, the enrichment in upregulated genes was not influence by the subtelomeric gene density (represented by the grey dots, Figure 1C). Downregulated genes, on the other hand, did not show a particular pattern of enrichment (Figure S3). These results demonstrate that transcriptional upregulation at senescence is specific of a subset of subtelomeres.

**Increase expression of subtelomeric genes is not restricted to replicative senescence**

To study the transcription of subtelomeric genes in other cells and types of senescence, we analyzed published data from Hernandez-Segura *et al*. (Hernandez-Segura et al., 2017). In that study, the authors compiled RNA-seq data from different cell types and defined a set of DEG specific of replicative senescence (RS; including BJ, HFF, MRC-5, WI-38 and IMR-90 cells), oncogene-induced senescence (OIS; IMR-90 cells) and ionizing radiation-induced senescence (IRIS; HCA-2 cells) eliminating DEG shared between those senescence inducers. We performed the same enrichment analysis as shown in Figure 1A and we observed that upregulated genes from RS cells also exhibited a significant enrichment in the first 2 Mb from the telomere (fold enrichment = 1.5 and 1.4 with $p$ = 0.015 and 0.028 respectively, Figure S1A). Again, this enrichment was restricted to upregulated genes since no enrichment was identified for downregulated genes. In addition, DEG of RS cells from Hernandez-Segura *et al* significantly overlapped with the DEG identified in this work (fold enrichment = 3.3 with $p = 1.26e^{-82}$, hypergeometric test, Figure S1B) and a significant portion of upregulated subtelomeric genes was also shared between the two datasets (fold enrichment = 4.4 with $p = 4.54e^{-11}$; Figure S1C).

Interestingly, subtelomeric enrichment in upregulated genes was not restricted to replicative senescence. OIS cells also showed an enrichment of upregulated genes in the first Mb from the telomeres (fold enrichment = 1.5 with $p$ = 0.0017; Figure S1A). , OIS-specific DEG were not represented in the replicative senescent DEG identified in this work (Figure S1B) even for the genes located in subtelomeric regions (Figure S1C). Finally, for the IRIS group we could not found gene expression enrichment at subtelomeres (Figure S1A). Next, we examined the distribution of upregulated genes from published datasets (Figure 1C). We plotted the upregulated genes from different senescence types (Hernandez-Segura et al., 2017) as well as upregulated genes

identified in aged compared to young tissues from the GTEx database (Dong et al., 2021). Similar to our work, published data showed that upregulated genes are also located in a subset of subtelomeres regardless of the senescence inducer (RS, OIS or aged tissue) (Table 1). Overall, these results show that transcriptional changes at subtelomeres are not restricted to replicative senescence but are shared between other senescence stressors.

**The shelterin protein TRF2 influences subtelomeric transcription**

We, and others, had previously showed that the expression of the shelterin protein TRF2 decreases as cells approach senescence in a p53 dependent manner (Fujita et al., 2010; Mendez-Bermudez et al., 2021). However, restoring the levels of TRF2 in pre-senescent cells leads to a higher stability of their heterochromatin as seen by higher compaction, less accumulation of DNA breaks, fewer recombination events and attenuation of DNA loss. Additionally, TRF2 restoration changes the transcriptomic of senescent cells as see by an underrepresentation of pathways related to the immune system and overrepresentation of cell-cycle pathways (Mendez-Bermudez et al., 2021). Therefore, we decided to investigate whether TRF2 could also be involved in the transcription of subtelomeres. For that, we infected pre-senescent MRC-5 cells with a TRF2 expressing lentivirus or an empty control and extracted RNA when the cells reached senescence. In the case of the TRF2-expressing cells, senescence was reached after 75 PD in culture, while the control cells transduced with an empty vector stopped dividing at PD 71. In parallel, we over expressed TRF2 in young MRC-5 cells (PD 30) for six days before RNA extraction. In both, young and senescent cells, the levels of TRF2 were high but constant (Figure S4A). After RNA sequencing and differential expression analysis, we identified 1,366 DEG when we compared

senescent vs young MRC-5 over-expressing TRF2 and 1051 DEG in senescent vs young MRC-5 transduced with and empty vector (Figure 2A). By crossing the two datasets, we obtained three groups of DEG (Figure 2A) which are dependent upon senescence but that behaved differently according to the TRF2 protein levels. In the intersection between the two datasets, we identified 856 genes that are not influenced by the TRF2 protein levels. We called them TRF2 independent genes. As expected, Reactom pathway analysis revealed pathways related to cell cycle arrest and senescence. We found that 510 genes were differentially expressed in senescent compared to young cells only when TRF2 is overexpressed, thus we named this group as high TRF2-dependent genes. This time, we could not find a representative pathway enrichment. Finally, we found 195 genes differentially expressed between senescent and young MRC-5 cells when they were transduced with an empty control vector. We assumed that the regulation of this group of genes is influenced by the natural decrease of TRF2 protein levels seen in senescent cells and we named this group as low TRF2-dependent genes. We performed qPCR to confirm the results obtained in some of the DEG found by RNA-seq (Figure S4C).

Similar to what we have shown in Figure 1A, the upregulated genes of the TRF2 independent gene category were enriched in subtelomeric regions (fold enrichment = 3.0 and 4.3 with $p = 7.57e^{-8}$ and $1.83e^{-13}$ for the first 2 Mb respectively, hypergeometric test) (Figure 2B). Interestingly, similar results were obtained for the low TRF2-dependant genes (fold enrichment = 5.3 and 2.3 with $p = 2.87e^{-9}$ and 0.031 for the first 2 Mb respectively, hypergeometric test) but not for the high TRF2-dependent group. These results show that TRF2 is involved, at least partially, in the derepression of subtelomeric genes at senescence.

**Decreased TRF2 expression disrupts 3D chromatin conformation at subtelomeric regions and leads to upregulation of subtelomeric genes**

To go deeper into the role of TRF2 at subtelomere transcriptional regulation, we downregulated TRF2 in young MRC-5 cells to mimic the TRF2 protein decrease observed at senescence. First, we showed that TRF2 downregulation causes increased expression of the low TRF2-dependent genes described in figure 2A (Figures 3A and 3C). We then conducted 3D FISH experiments on those cells using a telomeric PNA probe and a subtelomeric FISH probe labelled with different fluorophores (Figure 3B and 3D). To produce FISH probes, we used BACs covering TRF2-regulated genes defined by RNA-seq and found at subtelomeres 9q and 14q (Figure 3A and 3C). As control, we used BACs covering more internal regions of chromosome 16p (25.6 Mb from telomere) and 4p (6.2 Mb from telomere) (Figure S5A and S5C). In the 9q chromosome arm, we observed that TRF2 downregulation decreases the number of merged telomeric-subtelomeric signal from about 36 % to 22 % ($p$ = 0.0035, Chi-square test, n = 3; Figure 3C). A similar decrease was observed at the 14q arm with a decrease from 39 % to 23 % when TRF2 is downregulated ($p$ = 0.0008, Chi-square test, n = 3; Figure 4C). We did not find a significant difference with the control probes at chromosomes 16p and 4p (Figure S5B and D).

To investigate whether subtelomeric chromatin organization was also affected in replicative senescence, we cultured MRC-5 cells at 5 % oxygen until they almost reached senescence (10 % were still incorporating EdU while 98 % of the culture was positive for SA-$\beta$-Gal) (Figure S6A and S6B). We performed 3D FISH with the 14q subtelomeric probe described above in young and pre-senescent cells and found that 34 % of subtelomeric probe colocalized with telomeres. This proportion decreased in pre-senescent cells where only 18 % of the subtelomeric probe colocalized with

telomeres (n = 2, *p* = 0.0027, Chi-square test; Figure S6D). At the 3D 9q subtelomere, FISH experiments showed a trend to decrease the telomeric-subtelomeric merged signal (25 % in young cells compared to 19 % in pre-senescent cells (n = 2, *p* = 0.21, Chi-square test, Figure S6D).

In summary, we demonstrated that pre-senescence cells or knockdown of TRF2 in young cells disrupts the 3D chromatin conformation at subtelomeres 9q and 14q, and this was associated with an upregulation of some genes in that area. Thus, we hypothesized that subtelomeric gene expression in senescent cells could be in part due to the 3D chromatin conformation alterations caused by the decrease of TRF2 protein levels.

**Lamin B1 regulates transcription at subtelomeres**

It is known that Lamin B1 is an important regulator of chromatin architecture and a component of the nuclear lamina whose expression is highly decrease upon senescence (Freund et al., 2012; Shimi et al., 2011). Even more, we had shown that Lamin B1 interacts with TRF2, and the decrease during senescence can be partially prevented by overexpression of TRF2 (Mendez-Bermudez et al., 2021). Thus, we examined whether Lamin B1 could be involved in the transcriptional regulation of subtelomeric genes during senescence. For that, we mimicked the loss of Lamin B1 occurring in senescence by knocking down its expression in young (PD 30) MRC-5 cells using siRNAs and we performed RNA-seq. As shown previously in Figure 1A, we performed an enrichment analysis on upregulated genes when Lamin B1 is downregulated. As seen in senescent cells, we observed that the first 2 Mb from a telomere were positively enriched in upregulated genes (fold enrichment = 1.7 and 1.5 with $p = 3.01e^{-4}$ and 0.0085 respectively, hypergeometric test, Figure 4A) with no

enrichment observed with downregulated genes (Figure 4A). Moreover, the distribution of upregulated genes is not homogenous among subtelomeres (Figure S7C). We found that chromosomes 8q and 22q were significantly enriched in upregulated genes in their first 2 Mb (Figure S7C and S7D) with other chromosome ends being enriched in senescent cells also containing upregulated genes under these conditions (Figure S7C compared with Figure 1C). Thus, Lamin B1 downregulation in young fibroblasts recapitulated the phenotype we observed in senescent cells, *i.e.*, subtelomeric gene upregulation. We also confirmed Lamin B1 downregulation and some DEG by qPCR (Figure S7A and B).

Finally, we crossed DEG from senescent cells (with or without TRF2 over expression) with DEG from Lamin B1 downregulation in young cells and observed a significant overlap between these datasets (Figure 4B). Interestingly, when we crossed only the upregulated genes found in the first 2 Mb, we found a significant enrichment between the genes regulated by Lamin B1 downregulation in young MRC-5 with the low TRF2-dependent and TRF2 independent genes (fold enrichment = 8.7 and 4.8 with $p = 8.69e^{-10}$ and $p = 1.26e^{-12}$, respectively, Figure 4C) but not with the high TRF2-dependent genes.

Overall, we concluded that Lamin B1 downregulation was in part responsible for the transcriptional changes observed in senescent cells.

## Discussion

Here we studied the transcriptional changes of replicative senescent in human cells. We found 1051 genes whose expression was altered upon senescence with an enrichment of upregulated genes at the first 2 Mb from the telomeres. Surprisingly this enrichment occurred only at a subset of subtelomeres. When we performed the same

enrichment analysis using public datasets determining OIS-specific DEG, and RS-specific DEG, we observed an enrichment of upregulated genes in subtelomeric regions. These results suggest that the derepression of subtelomeric genes is not specific to replicative senescence. Because the enrichment was found using lists of DEG specific of a senescence-inducers, one can hypothesise that the alleviation of the repression in subtelomere allows a transcriptional response specific of stress, or at least a senescence inducer. Nontheless we cannot exclude that these differences were due to the differences in cell lines or the RNA-seq methodology used.

Surprisingly, upregulated genes of the different datasets analysed here were located only in a subset of subtelomeres, that in many cases were shared among them (see Table 1). It rise the question whether this subset of subtelomeres have particular chromatin composition that make them prone to transcriptional upregulation upon different senescence stressors. It is also possible that proteins associated to telomeres is not the same amongst the chromosome ends creating different compartments that modify the transcriptional pattern of these regions. Evidence of that was obtained by keeping the levels of TRF2 high as the cells reached replicative senescence (see Figure 2). In this experimental setting, TRF2 altered subtelomeric transcription in the same subset of subtelomeres, thus could explain the transcriptional changes observed during senescence, were the levels of TRF2 are normally reduced. We also found that downregulation of TRF2 disrupts the 3D chromatin conformation suggesting, at least part of the transcriptional changes observed during senescence, could be mediated by long range chromatin loops potentially mediated by ITS located close to those regions. We were able to recapitulate the transcriptional changes seen during replicative senescence by knocking down Lamin B1 reinforcing the importance of a higher order organization in the control of subtelomeric transcription.

We, as others (Dong et al., 2021), did not find a specific functional pathway upregulation using Reactome or KEGG analysis from the DEG found at 2 Mb from the telomeres. However, that does not exclude a biological significance for the upregulation of subtelomeric regions upon senescence. Apart from the upregulation of genes, non-coding RNA could also be elevated contributing to the aging process. A strong candidate for that, are noncoding RNA telomeric molecules (TERRA) which are transcribed from subtelomere regions and are known to participate in TPE (Arnoult et al., 2012), to protect telomeres and regulate telomere length (Deng et al., 2009; Redon et al., 2010) to accumulate upon stress (Koskas et al., 2017), and at old age (Balan et al., 2021). Thus, further investigation is needed to reveal the functional contribution of increase transcription at subtelomeres.

Interestingly, TPE, the epigenetic mechanism by which telomeres mediate regulation of transcription in neigbouring regions, is due to chromatin loops between telomeres and subtelmoeres mediated in part by TRF2 (Kim et al., 2016; Robin et al., 2014, 2020). Altogether, we observed, in replicative senescent cells, that upregulated genes were enriched in subtelomeric regions. This enrichment is caused by a derepression of subtelomeric regions and, is partially dependent of TRF2 and Lamin B1 protein level and the disruption of 3D chromatin conformation between telomeres and subtelomeres. Therefore, it strongly supports that this peculiar senescence phenotype is due to an alleviation of TPE.

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## Author Contributions

Methodology, M.R-M.; M.P.; A.M-B.; Software, M.P.; Formal Analysis, M.R-M.; M.P.; A.M-B.; E.G. Conceptualization, A.M-B., J.Y., E.G.; Writing-Original Draft, M.R-M.; A.M-B., E.G.; Funding Acquisition, J.Y., E.G.

## Declaration of Interests

The authors declare no competing interests.

## Figure legends

### Figure 1. Distribution of subtelomeric genes

(A) Distribution of differentially expressed genes (DEG) in senescent versus young MRC-5 cells. Upregulated (left) and downregulated (right) genes are shown. The line plots illustrate the number of DEG related to their distance to telomeres in 1 Mb intervals. Fold enrichment (right Y-axis) is only shown if there is a significant *p* value and the size of the circles corresponds to the *p* value of the enrichment obtained by a hypergeometric test.

(B) Box plot of DESeq2 normalized counts from the first 2 Mb of telomeres. Counts from non-differentially expressed genes (no DEG) and from differentially expressed genes (DEG) in the two conditions (young and senescent) are plotted in different whisker plots. Upregulated DEG counts are plotted in left panel and downregulated DEG counts are plotted in right panel. Data represents median with interquartile range of three biological replicates. Statistical analyses were performed using the Kruskal-Wallis test, (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$; ****$P < 0.00001$). (C) Upregulated genes in the first 2 Mb from telomeres. Each dot represents a gene while red arrows mark the start of the telomere. Grey dots represent all protein coding genes and pseudogenes. Empty dots represent upregulated genes (senescent versus young MRC-5 cells) from this work, while black dots depict upregulated genes in replicative senescence identified in published datasets (Hernandez-Segura et al., 2017). Green and red dots are upregulated genes found in oncogene induced senescent and ionizing radiation-induced senescence respectively (Hernandez-Segura et al., 2017). Turquoise dots correspond to upregulated genes in aged versus young tissues (Dong

et al., 2021). Gray boxes delimitate the subtelomeres that are significantly enriched in upregulated genes in this work.

**Figure 2. Distribution of differentially expressed genes influenced by TRF2**

(A) Venn diagram of DEG found in senescent (sen) versus young MRC-5 cells infected with an empty (E, blue) or a TRF2 expressing lentivirus vector (TRF2, red) respectively. Cells transduced with an empty vector senesce at a population doubling (PD) of 71, while cells expressing TRF2 at a PD of 75. Young cells were transduced at a PD of 30. (B) Distribution of DEG expressing TRF2 in the first 15 Mb from telomeres are shown. Upregulated (upper panels) and downregulated (lower panels) genes for the 3 categories of genes described in A are presented. Empty circles correspond to the *p* value of the enrichment obtained by a hypergeometric test.

**Figure 3. TRF2 downregulation disrupt 3D chromatin conformation at subtelomeres**

(A and C) Schematic representation of chromosome 9q and 14q subtelomere. Telomeres are marked in red, gene location found in RNA-seq is shown with a grey rectangle. The name of the gene is differentiated by low TRF2 dependent (blue color), high TRF2 dependent (red color) and TRF2 independent (purple color). Interstitial telomeric sequences are represented by a vertical red bar while the location of the BAC clone used to produce subtelomeric DNA probe is represented by a green bar. Below the scheme of subtelomeres, gene expression of by RT-qPCR in young MRC-5 + siRNA control (young+sictrl) and in young MRC-5 + siRNA TRF2 (young+siTRF2) normalized to two housekeeping genes (GAPDH and VAMP7) are shown. Data represent mean ± SD of three biological replicates. Statistical analyses were performed

using either t-test or Mann-Whitney test (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$; ****$P < 0.00001$).

(B and D) Representative images of 3D FISH of cells with TRF2 downregulation or controls. Telomeric probe (in red) and subtelomeric probe (in green) are shown. Colocalization was scored as positive when at least one pixel was in common between the telomeric and subtelomeric probes. There biological replicates were performed with at least 30 nuclei analyzed in each of them. Statistics were performed with the Chi-square test, **$P < 0.001$; ***$P < 0.0001$.

**Figure 4. Subtelomeric gene expression is affected by Lamin B1**

(A) Distribution of differentially expressed genes (DEG) in young MRC-5 cells transfected with siLMNB1 or siControl. The first 15 Mb from the telomere are shown. The line charts illustrate the number of DEG related to their distance to telomeres in 1 Mb bins. Significant fold enrichment (right Y-axis) is marked with open circles. The *p* value of the enrichment was obtained by a hypergeometric test.

(B) Venn diagram of differentially expressed genes found between senescent cells and young MRC-5 cells transduced with and empty or TRF2 vector (as shown in Figure 2A) and young MRC-5 cells with downregulation of Lamin B1 (brown circle). Fold enrichment and *p*-value was estimated using a hypergeometric test.

(C) Similar comparison as in B but only upregulated genes present at the first 2 Mb from the telomeres were used for the analysis. Fold enrichment and *p*-value was estimated using a hypergeometric test.

**Figure S1. Enrichment of upregulated DEG obtained with different senescence inducers**

(A) Distribution of upregulated genes (left) and downregulated genes (right) specific of a senescence stimulus identified by Hernandez-Segura *et al.* 2017, along the first 15 Mb from telomeres (RS, Replicative Senescence; OIS, Oncogene Induced-Senescence; IRIS, Ionizing Radiation-Induced Senescence). Plot chart shows the number of senescence specific genes from each stimulus in function of their distance to telomeres. Fold enrichment of genes in each interval is plot along the right Y-axis with the bubble size corresponding to the *p*-value of the enrichment obtained with a hypergeometric test.

(B) Venn diagram showing the cross between the genes identified in this work and the ones described in A. In (C) is shown only the upregulated genes in the first 2 Mb from telomeres. Fold enrichment and *p*-value was calculated using a hypergeometric distribution test.

**Figure S2. Distribution of the enrichment of differentially expressed genes (DEG) in senescence versus young cells**

(A) Enrichment of upregulated and (B) downregulated DEG in MRC-5 cells (senescence vs young). DEG were grouped in 2 Mb non-overlapping intervals (x axis) and enrichment analysis was performed for each interval. Positive fold enrichment ($p < 0.01$, hypergeometric test) is shown in the y axis. Vertical dash lines delimit centromeric regions.

**Figure S3. Distribution of downregulated genes at the first 2 Mb from telomeres**

(A) Downregulated genes in the first 2 Mb from telomeres are depicted. Each dot represents a gene while red arrows mark the start of the telomere. All protein coding genes and pseudogenes are represented by grey dots. Empty dots represent upregulated genes (senescent versus young MRC-5 cells) from this work, while black dots represent upregulated genes in replicative senescence identified in published datasets (Hernandez-Segura et al., 2017). Green and red dots are upregulated genes found in oncogene induced senescent and ionizing radiation-induced senescence respectively (Hernandez-Segura et al., 2017). Turquoise dots relate to upregulated genes in aged versus young tissues (Dong et al., 2021).

**Figure S4. Characteristics of MRC-5 cells used to identify TRF2-dependent genes in senescent cells.**

(A) Western blotting of young and senescent MRC-5 cells transduced with an empty or a TRF2-expressing vector. Young cells were transduced for 6 days while senescent cells were transduced for 25 population doublings before they senesce.

(B) Schematic representation of chromosome 9q subtelomere. Expressed genes found by RNA-seq are shown by a grey rectangle. The name of the gene is differentiated by low TRF2 dependent (blue color), high TRF2 dependent (red color) and TRF2 independent (purple color). Interstitial telomeric sequences are represented by vertical red bars. Gene expression by RT-qPCR in young and senescent (MRC-5, infected with an empty (E) or a TRF2-expressing vector (TRF2), normalized to two housekeeping genes (GAPDH and VAMP7) are shown. Bars represent the mean ± SD of three biological replicates. Statistics were performed using a t-test or Mann-Whitney test, $^{**}P < 0.001$; $^{***}P < 0.0001$; $^{****}P < 0.00001$.

**Figure S5. 3D FISH analysis of young MRC-5 cells with TRF2 downregulation**

(A and C) Schematic representation of chromosome 16p and 4p regions respectively. Location of DEG found by RNA-seq are marked with grey rectangles. Interstitial telomeric sequences are represented by vertical red bars and telomeres with red lines. The location of BAC clones used to produce subtelomeric DNA probes are represented by a green bar.

(B and D) Representative images of intra-chromosomal DNA probe regarding telomeric PNA probe. Representative images of young MRC-5 cells transfected with either an siRNA control (siCtrl, upper images) or an siRNA TRF2 (siTRF2, lower images) hybridized with a BAC probe targeting the subtelomeric region (green) and a telomeric PNA probe (red). Arrows show overlapping signals. Pie charts show the quantification of three biological replicates (Chi-square test).

**Figure S6. 3D FISH of young and senescent MRC-5 cells**

(A) Dividing cells (magenta) were identified by EdU staining (1 µM for 14 h) in young and senescent MRC-5 cells. Approximatively 100 cells analyzed per replicate. Bars represent mean ± SD of three biological replicates. Scale bar = 10 µm.

(B) Senescence-associated $\beta$-galactosidase (SA-$\beta$-Gal) staining is shown in young and senescent MRC-5 cells. Bars show the mean ± SD of three biological replicates (approximatively 500 cells were analyzed per replicate). Scale bar = 100 µm.

(C) Representative 3D FISH images of young and senescent cells using a subtelomeric probe at chromosome 9 (as described in Figure 3A) and (D) chromosome 14 (as described in Figure 3C). Telomeric probe (in red) and subtelomeric probe (in green) are shown. Colocalization was scored as positive when at least one pixel was in common between the telomeric and subtelomeric probes. Two biological replicates

were performed with at least 30 nuclei analyzed in each of them. Statistics were performed with the Chi-square test, ** $P < 0.001$; ns = non-significant.

**Figure S7. Lamin B1 downregulation influence subtelomeric gene expression**

(A) Verification of LMNB1 expression in young MRC-5 after 3 days incubation of siLMNB1 or controls (sictrl).

(B) Schematic representation of the chromosome 9q subtelomere and expression of some subtelomeric genes by RT-qPCR in young (PD 30) MRC-5 transfected with an siRNA control or siRNA LMNB1, normalized to two housekeeping genes (GAPDH and VAMP7). Data show mean ± SD of three biological replicates. Statistics were performed using either t-test or Mann-Whitney test; **** $P < 0.0001$.

(C) Visualization of upregulated and (D) downregulated genes in young MRC-5 cells transfected with an siLMNB1 versus controls in the first 2 Mb from all telomeres (brown dots). Each dot represents a gene and is plotted on the x axis according to its location (length in base pairs). Grey dots show all protein coding genes and pseudogenes.

## Materials et Methods

### Cell Lines

MRC-5 human primary lung fibroblasts were obtained from ATCC. MRC-5 cells were grown in DMEM supplemented with 10 % fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100µg/mL). Cells were cultured at 37°C, 5 % $CO_2$ and 5 % $O_2$.

### Lentivirus infection and siRNA transfection

Lentiviral particles were produced with 293T cells transiently transfected with the virus packaging plasmids p8.91, VSVg and the lentiviral expression vector (empty: pWPIR-GFP or TRF2: pWPIR-GFP-TRF2) by calcium phosphate precipitation. Viral supernatants were collected 48 h after transfection and concentrated by overnight centrifugation at 4°C.

siRNAs (Control, TRF2 and LMNB1) were purchased from Dharmacon (On-Target Plus SMARTpool) and transfected with DharmaFECT1 transfection reagent (Dharmacon) for 3 days. Efficiency of siRNA was assessed by RT-qPCR.

### RNA extraction and RT-qPCR

RNA extractions were performed accordingly to manufacturer's instruction using the RNeasy Plus Mini Kit (Qiagen). Concentration and quality of the extracted RNA was assayed using the Nanodrop 2000 (Thermo scientific) and/or Bioanalyzer instrument (Agilent). One µg of RNA was reverse-transcribed using High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific) in a T100 thermal cycler (Bio-Rad). Quantitative PCR were performed using 10 ng of cDNA with 0.6 µM primers (see Table below) and SYBR Green Master Mix (Roche) on a StepOne plus thermocycler (Applied Biosystems).

Data were analyzed using the Pfaffl method after calculation of primer efficiency. VAMP7 and GAPDH were used as endogenous controls. All reactions were performed in triplicates and at least 3 biological replicates were used to generate each dataset.

| | Forward | Reverse |
|---|---|---|
| *PTGDS* | CTCTACAGCCGAACCCAGAC | CAGAGACATCCAGAGCGTGG |
| *ABCA2* | AAGAACGTGACGCTCAAACG | AAGGAGACTTCCTTCACGGAG |
| *NPDC1* | CTGGACTGTGCCCTGAAGAG | TGGGCCAGGAAGTCAATCTC |
| *SAPCD2* | ATGCTGAAGGAGCAGAACCG | GCTTAATGAGCGCCGACTTC |
| *DPP7* | CTGGTGTCGGACAGGTTCTG | GACTTCCCGTAGTAGCGGTG |
| *RNF208* | TGGCTGTCAACACGTCCATC | TACTACATGATGGAGCAGGCG |
| *CYSRT1* | GAGGACAGACTGCCGTGTTG | GCATCTCCGAGGATGAGGAAC |
| *PNPLA7* | GTACCCACAGGTGGTGACTC | CTTCCTCTGACACGGGCATC |
| *KLC1* | CACTCGTGCACATGAAAGGG | GTACCAGCCGCCATACTCTC |
| *ADSSL1* | TCTGACAGAGCCCACCTTG | AGGTTGGTCCGATTCCCTTC |
| *CEP170B* | CTCGGGAGCTCATCTTCGTG | TGGTCGTAGTTGATGACGGC |
| *LMNB1* | ACATGGAAATCAGTGCTTACAGG | GGGATACTGTCACACGGGA |
| *TERF2* | GCCCATCCTGTTATCCAGAA | CAAAGCCTTTTTGGCCATC |
| *VAMP7* | TTGCCATGTGAGTACGTTAGT | CCGGACAGACTGAAGCCAT |
| *GAPDH* | CTCCTCCACCTTTGACGCTG | TCCTCTTGTGCTCTTGCTGG |

**EdU proliferation assay**

To measure proliferation of MRC-5 cells, click-iT EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher Scientific) was used. EdU was incubated for 14 h at a final concentration of 10 µM. Cells were imaged using a fluorescence microscopy.

**RNA sequencing**

Young MRC-5 cells were infected with empty (pWPIR-GFP) or TRF2 expressing (pWPIR-GFP-TRF2) virus at a multiplicity of infection (MOI) of one. Two days after infection, cells were transfected with an siRNA control or siLMNB1 and collected for RNA extraction four days after, at PD 30. For senescent cells, MRC-5 cells were transduced with a TRF2-expressing vector about 25 PD before they senesce. Total

RNA was extracted at the senescent point (for control cells at PD 71 and PD 75 for TRF2-expressing cells). Each condition was done in triplicates. The proliferative and senescence status of cells were assessed constantly with EdU proliferation assay and Senescence-Associated $\beta$-Galactosidase staining.

Paired-end sequencing (read length: 2 x 150 bp) was performed by NovoGene using an Illumina sequencer. Between 30 and 50 million reads were obtained per sample. Raw reads were trimmed using Trimmomatic (v0.39) with a minimal read length of 35 bp. Trimmed reads were mapped to the human genome assembly (GRCh38) using STAR (v2.6.1d) with the -quantMode GeneCounts (Dobin et al., 2013). Differential expressed genes (DEG) analysis was performed using DESeq2 R package (Love et al., 2014). *P*-values were corrected for multiple testing using the Benjamini and Hochberg default method. The list of differentially expressed genes was obtained with a cutoff of 0.05 on adjusted p-values. A log2 fold-change value of 1 (for DEG analysis between senescent and young MRC-5 cells) and 0.58 (for DEG analysis between young MRC-5 transfected with siLMNB1 and siRNA control) was used. Protein coding genes and pseudogenes were kept for subsequent analysis.

**Senescence-associated $\beta$-galactosidase (SA-$\beta$-Gal) assay**

SA-$\beta$-Gal staining was performed using the Senescence Detection Kit (Abcam, ab65351) according to the manufacturer's instructions. Cells were visualized using a phase-contrast microscopy and the percentage of SA-$\beta$-Gal positive cells was calculated.

**3D FISH experiment**

Coverslip preparation: around 300,000 cells (young MRC-5) or 150,000 cells (senescent MRC-5) were seeded in 6 well plates containing 22 mm coverslips, in parallel their proliferative and senescence status were assessed with EdU proliferation assay and SA-$\beta$-Gal staining. The next day young MRC-5 were transfected with siRNA and incubated for 3 more days. Coverslips were fixed with 4% formaldehyde for 10 min at room temperature followed by 3 washes of 1X PBS 5 min each. Next, cells were permeabilized in 0.5 % Triton-X100 in 1X PBS for 20 min. Coverslips were then incubated in 20 % glycerol in 1X PBS during 30 min followed by six cycles of freezing and thawing by dipping the coverslips in liquid nitrogen followed by 20 % glycerol in 1X PBS. Next, the cells were washed 3 times in 1X PBS for 5 min and incubated in a 0.1 M HCl solution for 10 min. Finally, cells were rinsed with 2X SSC and stored at least one week at 4°C in 50 % formamide dissolved in 2X SSC (pH 7) protected from light.

DNA probe preparation: bacterial artificial chromosome (BAC) clones were purchased from BACPAC genomics (CH17-441B21, CH17-385I3, CH17-64K15) or Thermo Fisher Scientific (RP11-673E5, RP11-10I16, RP11-714N16 and RP11-372E5). Probes for FISH were labelled with digoxigenin (DIG) using DIG Nick Translation Mix (Roche) using 1 µg of BAC DNA. The probe size was assessed on agarose gel (smear around 500 bp) prior to ethanol precipitation. Labelled probes were then resuspended in hybridization buffer (50 % formamide, 10 % dextran sulphate, 1 % Tween, in 2X SSC) and verified on metaphase spreads.

Hybridization: coverslips were incubated with 10 µL of hybridization mix (100 ng of labelled DNA probe and 0.66 µM of telomeric PNA probe Cy3-OO-CCCTAACCCTAACCCTAA, in hybridization buffer), sealed with rubber cement and denatured for 5 min at 80 °C. Slides were incubated 48 h at 37 °C in a humid chamber.

Coverslips were then washed 3 times, 5 min each in 50 % formamide in 2X SSC (pH 7) at 45 °C followed by 3 times for 5 min in 2X SSC (pH 7) at 45 °C followed by a rinse in 1X PBS. Coverslips were then incubated in blocking solution (3 % BSA, 0.3 % Tween in 1X PBS) for at least 1 h at room temperature. Next, cells were incubated with sheep anti-DIG antibody (Roche, 11 333 089 001) diluted in blocking solution at 1/400 for 1 h at 37 °C and then washed 3 times for 5 min in 0.1 % Tween in 1X PBS. Cells were incubated with a mouse anti-sheep Alexa Fluor 488 antibody (Molecular Probes, A11015) diluted 1/500 in blocking buffer at room temperature for 1 h. Finally, coverslips were washed in 0.1 % Tween in 1X PBS 3 times for 5 min and mounted with vectashield with DAPI (Vector labs).

Image processing: Images were acquired with a LSM 880 confocal microscope equipped with a 63X Plan APOCHROMAT, NA=1.4 Oil objective (Zeiss). Images were processed and analyzed with FIJI software. Briefly, two spots were scored as colocalizing when at least one pixel was in common between the two signals in each optical section. For young MRC-5 cells with siRNA control or siTRF2 three biological replicates were used with at least 30 nuclei each. For young and senescent MRC-5 cells, two biological replicates were imaged with at least 30 nuclei each.

**Statistical analysis**

Statistical analysis was performed using the GraphPad Prism v7 software. Student's t-test and the corresponding nonparametric Mann-Whitney test were used for qPCR analysis. Chi-square analysis was used for 3D FISH experiment. Kruskal-Wallis test was used for DESeq2 counts analysis. Differences were considered statistically significant when the *p*-value was $< 0.05$.

The p-value of the enrichment analysis was based on the cumulative distribution function of the hypergeometric distribution. Fold enrichment (FE) was calculated using the following equation:

$$FE = \frac{k}{\frac{s * M}{N}}$$

where *k* represents the number of successes, *s* the sample size, *M* the number of successes in the population and *N* the size of the population.

Concerning the enrichment analysis in the distribution of the DEG in subtelomeres, the distance to the closest telomere were calculated for each gene. Chromosomes were divided in 1 Mb non-overlapping intervals according to the distance from a telomere and DEG were grouped in those intervals. The enrichment analysis was calculated as described above.

**Data and software availability**

RNA-seq data was deposited in the Gene Expression Omnibus database under accession number GSE180406.

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Figure 1

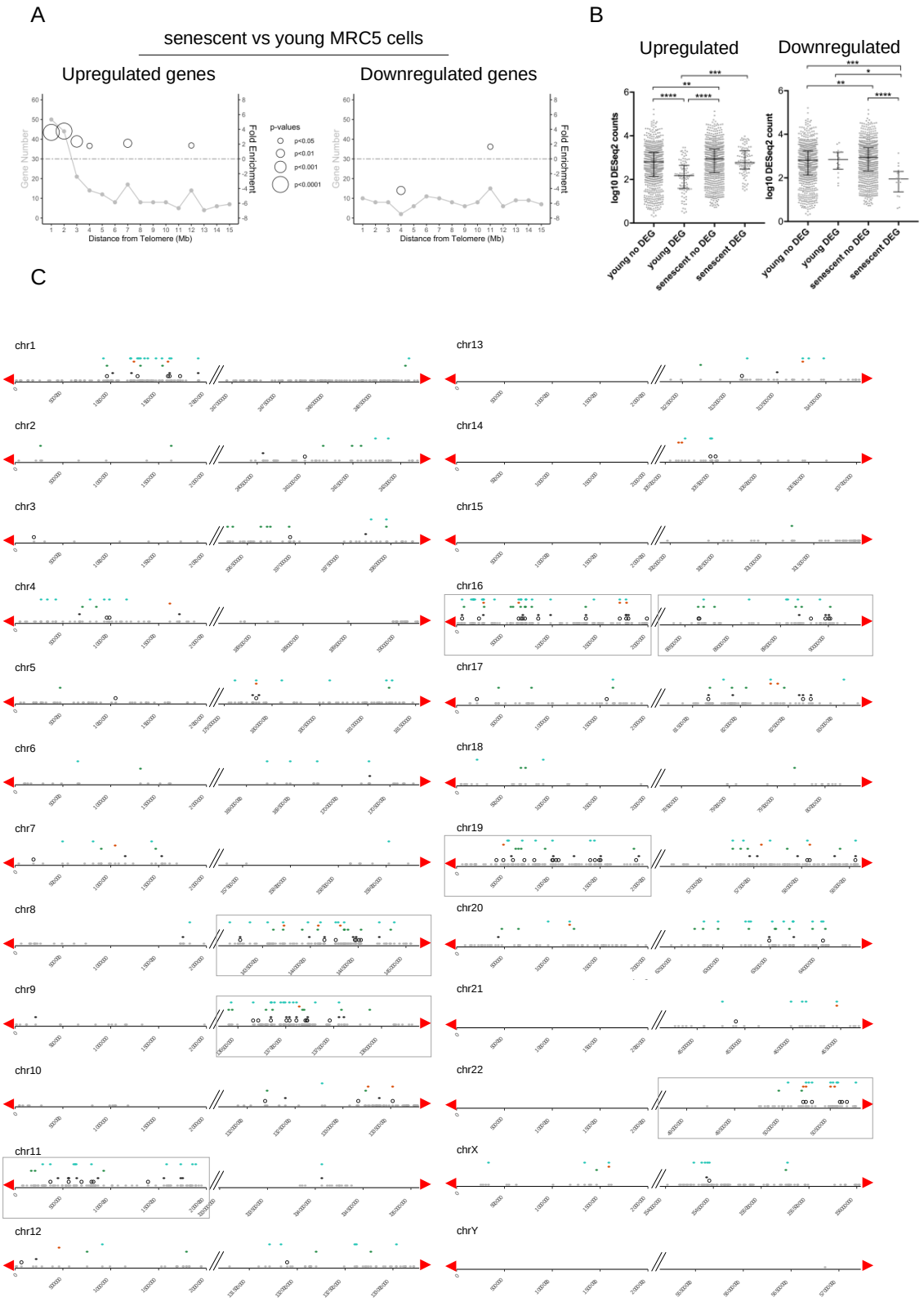
A
senescent vs young MRC5 cells
Upregulated genes
Downregulated genes
Gene Number
Fold Enrichment
Distance from Telomere (Mb)
p-values
p<0.05
p<0.01
p<0.001
p<0.0001
B
Upregulated
Downregulated
log10 DESeq2 counts
young no DEG
young DEG
senescent no DEG
senescent DEG
C
chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr19
chr20
chr21
chr22
chrX
chrY

Figure 2

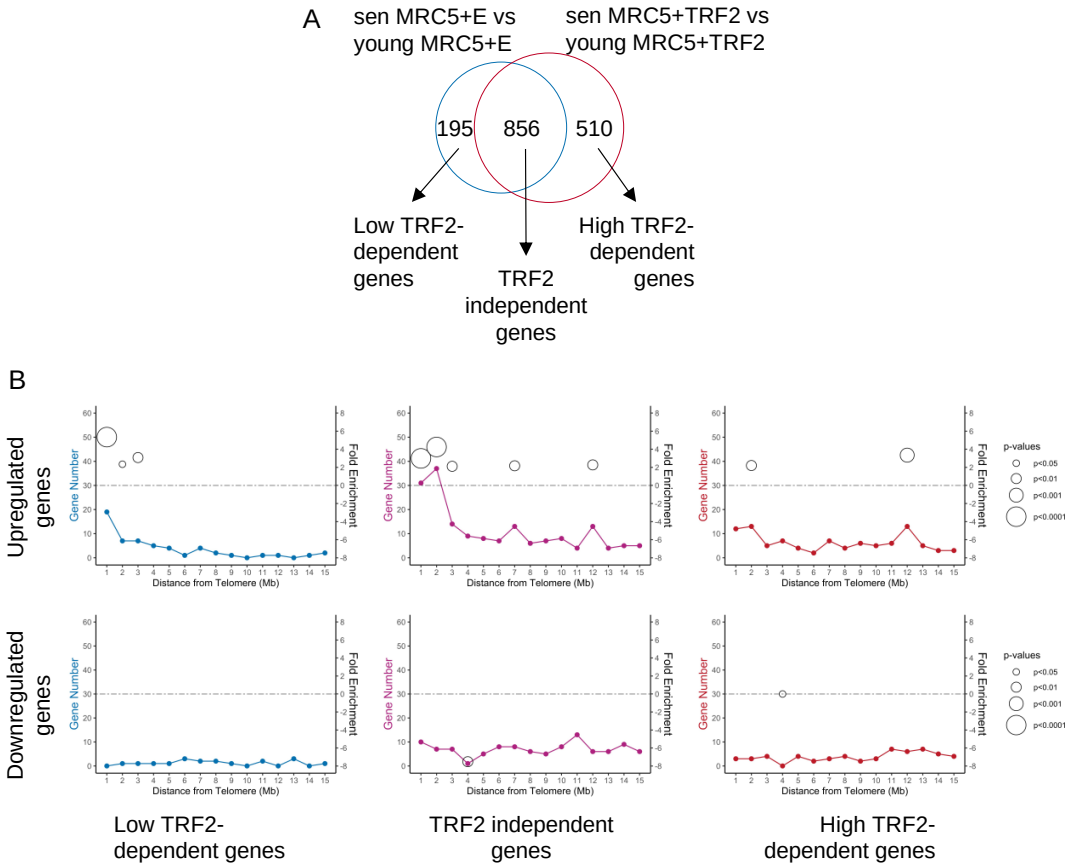
A
sen MRC5+E vs young MRC5+E
sen MRC5+TRF2 vs young MRC5+TRF2
195
856
510
Low TRF2-dependent genes
TRF2 independent genes
High TRF2-dependent genes
B
Upregulated genes
Downregulated genes
Gene Number
Fold Enrichment
Distance from Telomere (Mb)
p-values
p<0.05
p<0.01
p<0.001
p<0.0001
Low TRF2-dependent genes
TRF2 independent genes
High TRF2-dependent genes

Figure 3

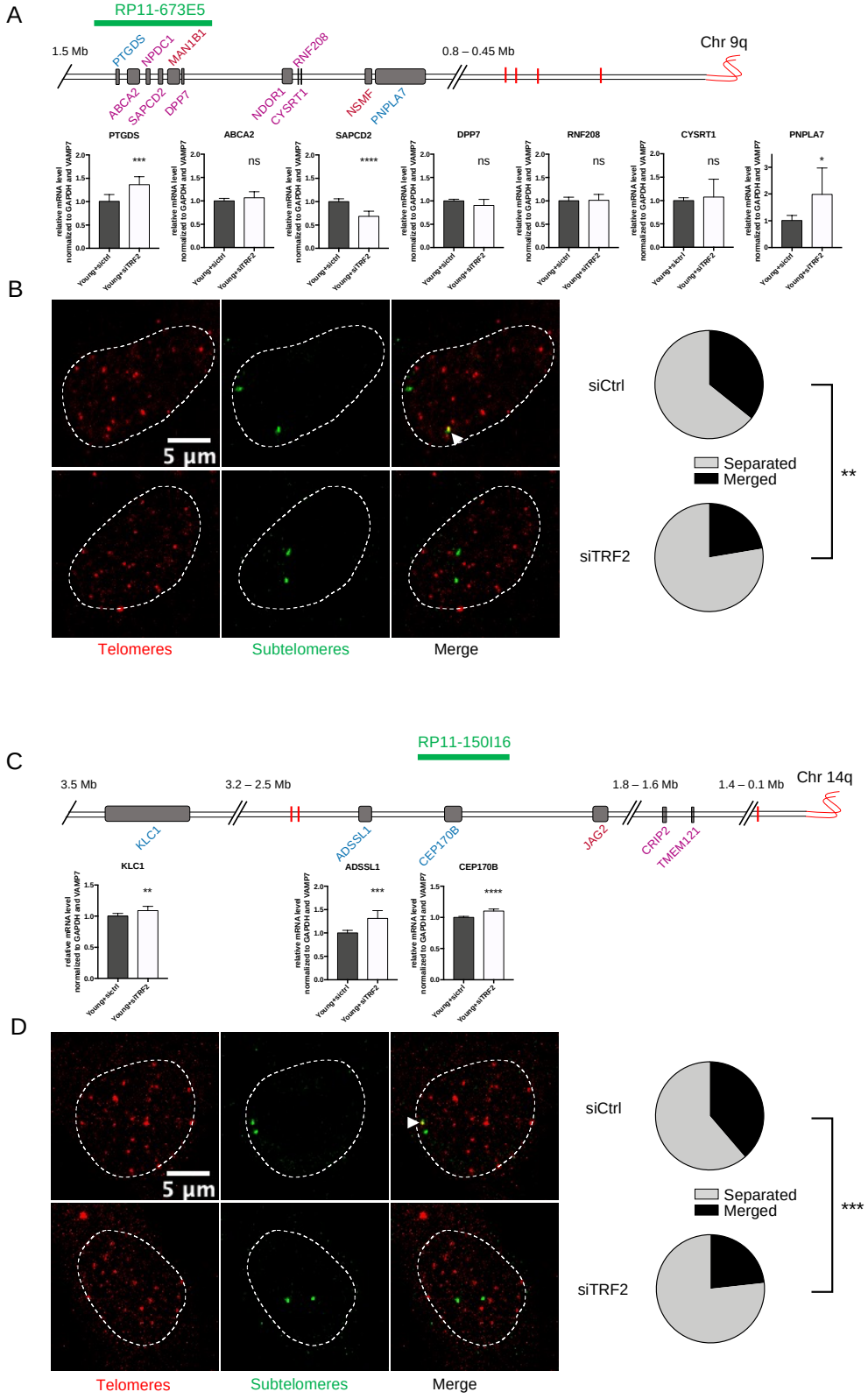
A
RP11-673E5
1.5 Mb
PTGDS
NPDC1
MAN1B1
RNF208
0.8 – 0.45 Mb
Chr 9q
ABCA2
SAPCD2
DPP7
NDOR1
CYSRT1
NSMF
PNPLA7
PTGDS
ABCA2
SAPCD2
DPP7
RNF208
CYSRT1
PNPLA7
relative mRNA level normalized to GAPDH and VAMP7
Young+siCtrl
Young+siTRF2
***
ns
****
ns
ns
ns
*
B
5 µm
siCtrl
Separated
Merged
siTRF2
**
Telomeres
Subtelomeres
Merge
C
RP11-150I16
3.5 Mb
3.2 – 2.5 Mb
1.8 – 1.6 Mb
1.4 – 0.1 Mb
Chr 14q
KLC1
ADSSL1
CEP170B
JAG2
CRIP2
TMEM121
KLC1
ADSSL1
CEP170B
**
***
****
D
5 µm
siCtrl
Separated
Merged
siTRF2
***
Telomeres
Subtelomeres
Merge

Figure 4

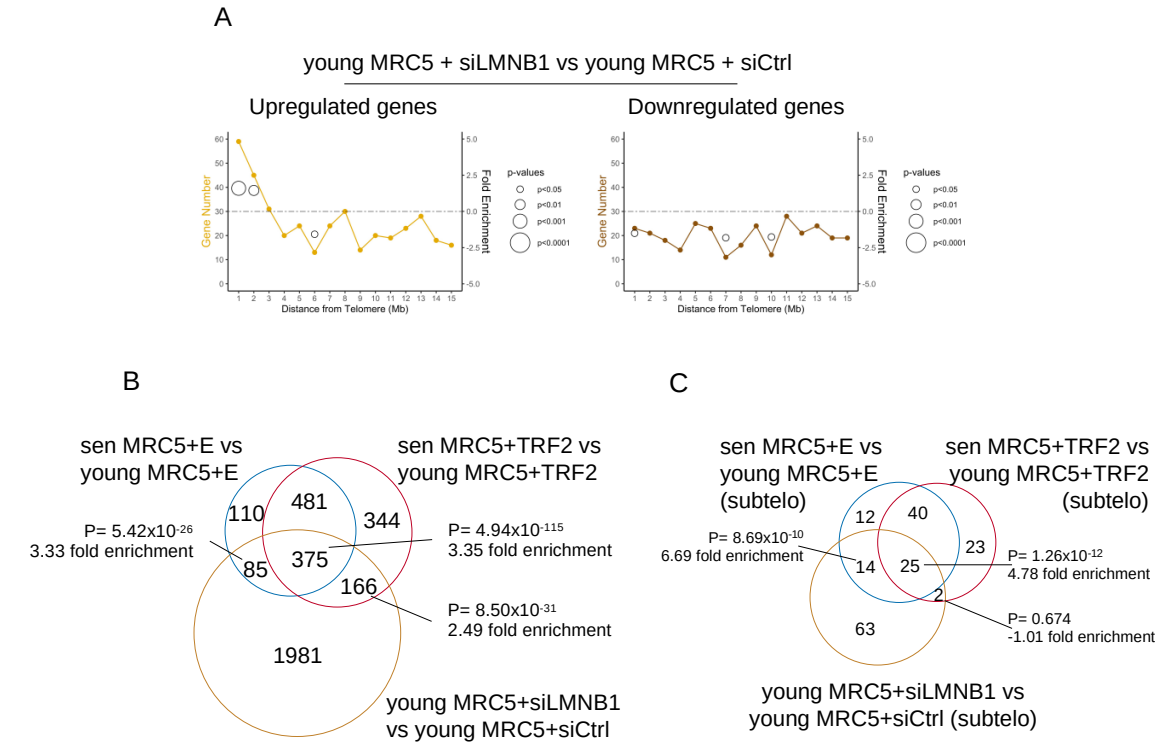
A
young MRC5 + siLMNB1 vs young MRC5 + siCtrl
Upregulated genes
Downregulated genes
Gene Number
Fold Enrichment
p-values
p<0.05
p<0.01
p<0.001
p<0.0001
Distance from Telomere (Mb)
B
sen MRC5+E vs young MRC5+E
sen MRC5+TRF2 vs young MRC5+TRF2
110
481
344
375
85
166
1981
P= 5.42x10-26
3.33 fold enrichment
P= 4.94x10-115
3.35 fold enrichment
P= 8.50x10-31
2.49 fold enrichment
young MRC5+siLMNB1 vs young MRC5+siCtrl
C
sen MRC5+E vs young MRC5+E (subtelo)
sen MRC5+TRF2 vs young MRC5+TRF2 (subtelo)
12
40
23
14
25
2
63
P= 8.69x10-10
6.69 fold enrichment
P= 1.26x10-12
4.78 fold enrichment
P= 0.674
-1.01 fold enrichment
young MRC5+siLMNB1 vs young MRC5+siCtrl (subtelo)

Figure S1 related to Figure 1

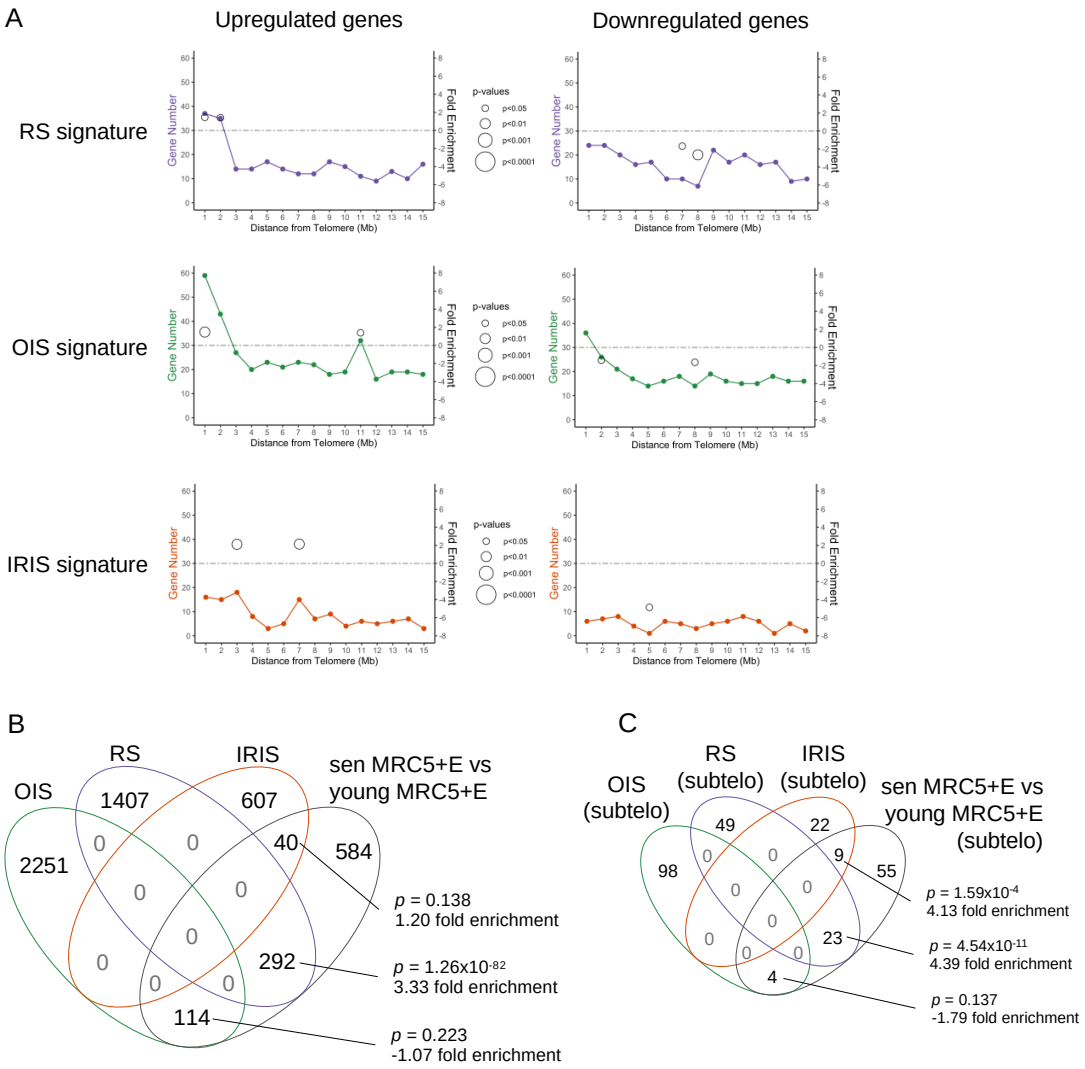

Figure S2 related to Figure 1

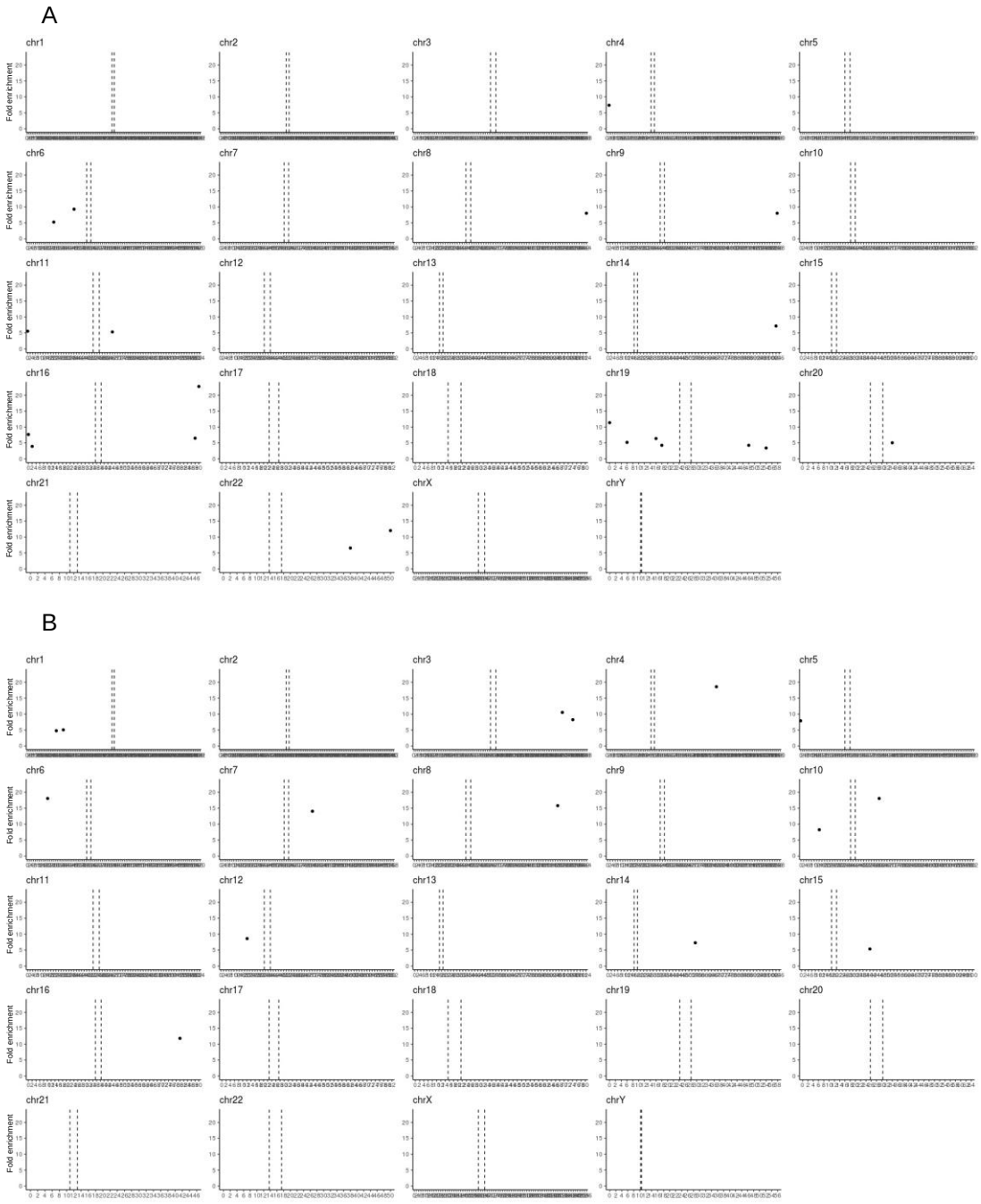

Figure S3 related to Figure 1

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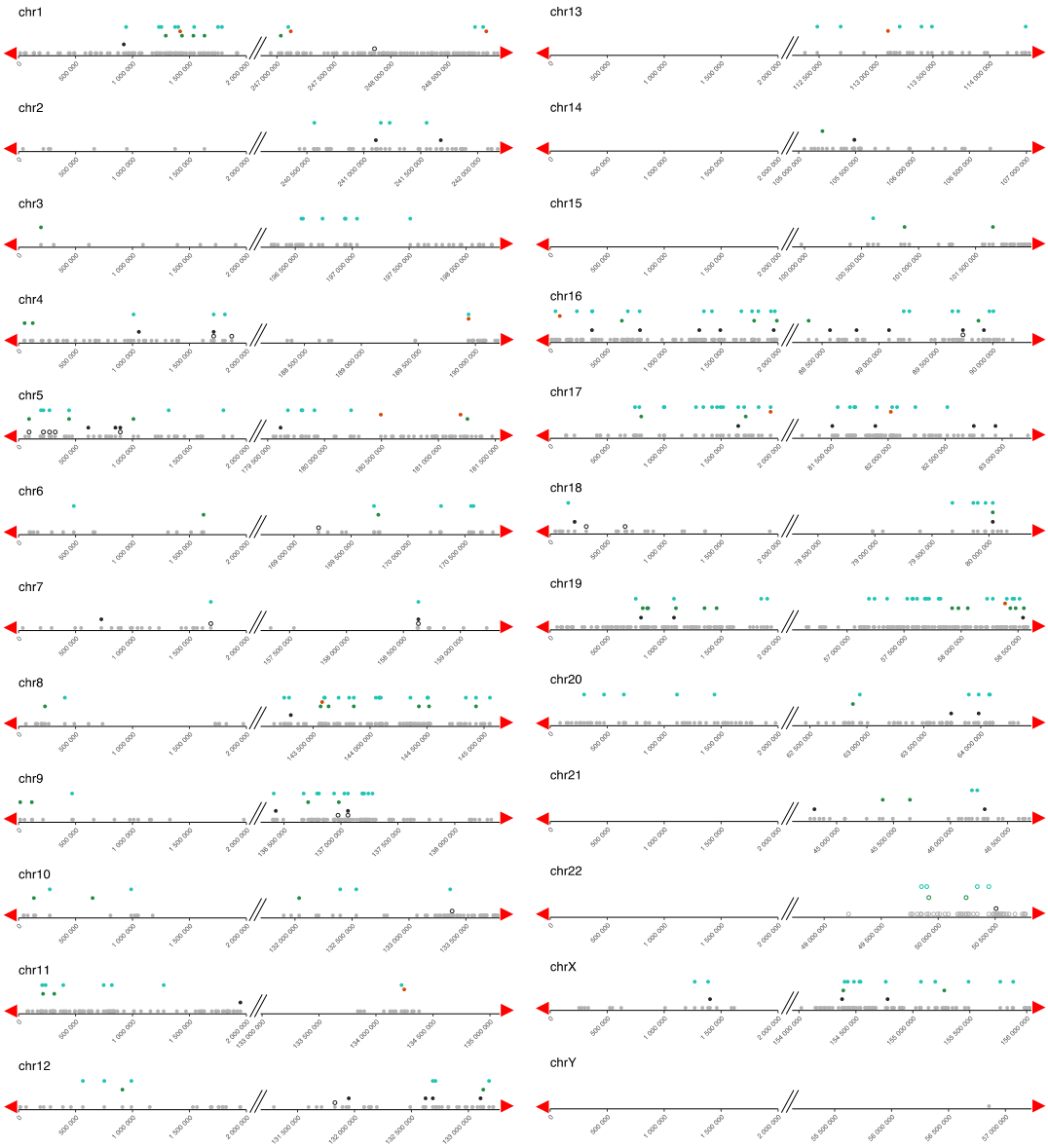

Figure S4 related to Figure 2

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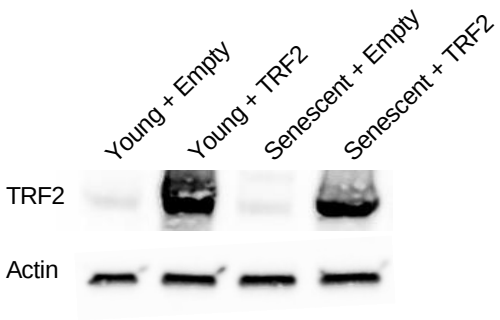


B

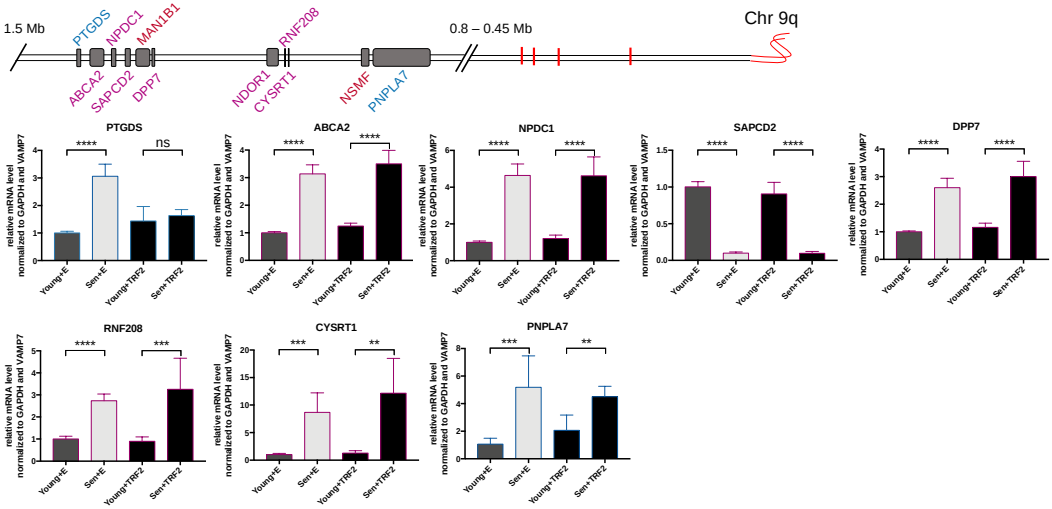

Figure S5 related to Figure 3

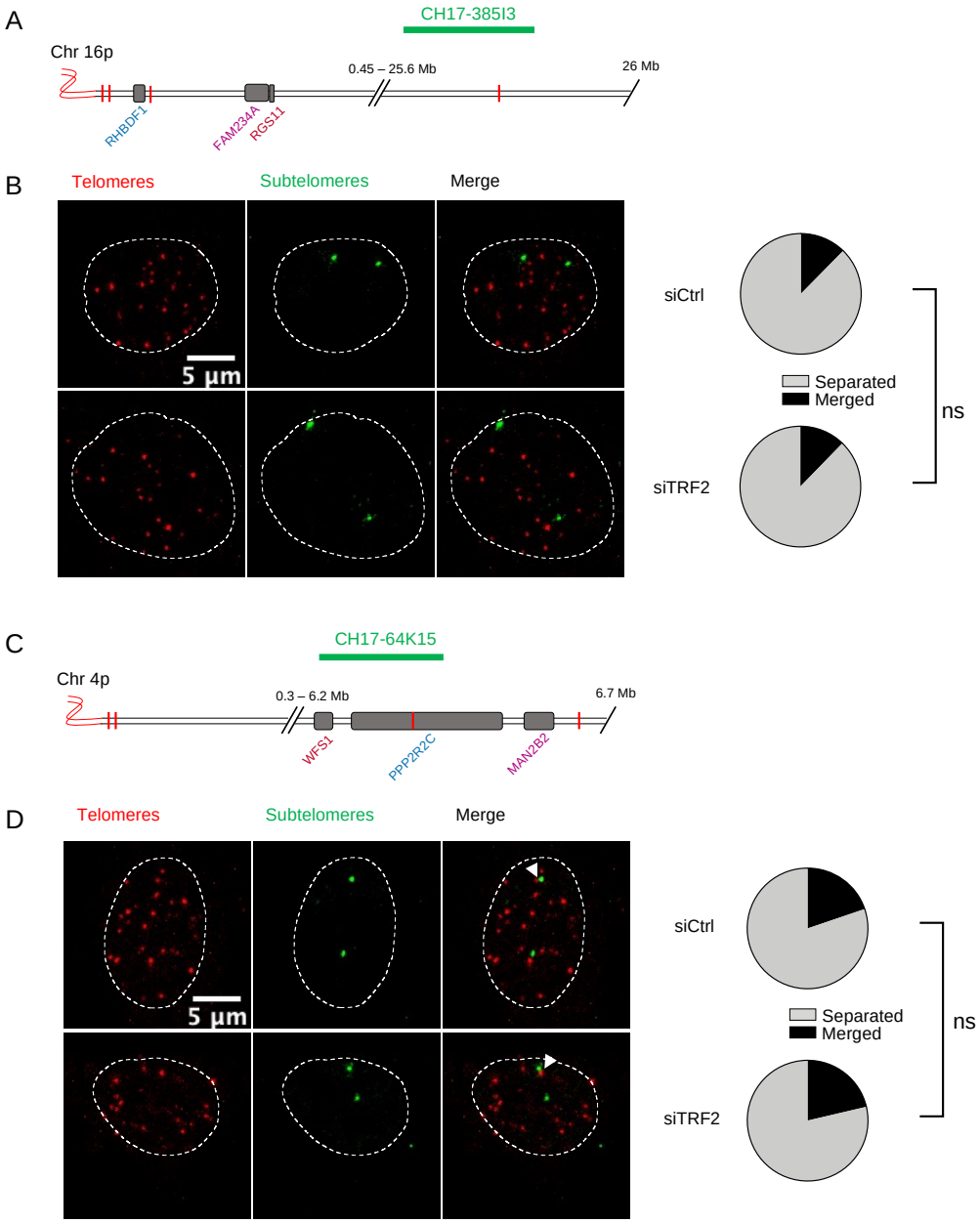

Figure S6 related to Figure 3

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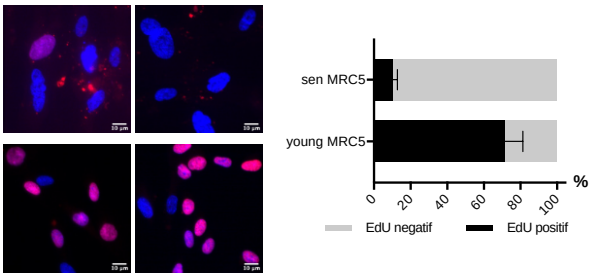


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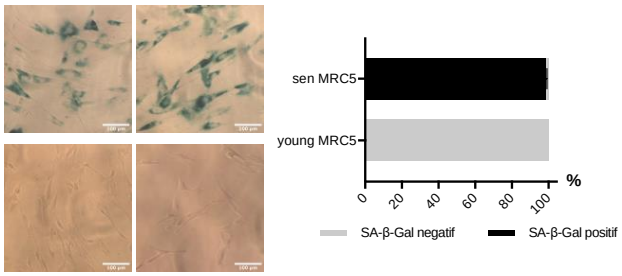
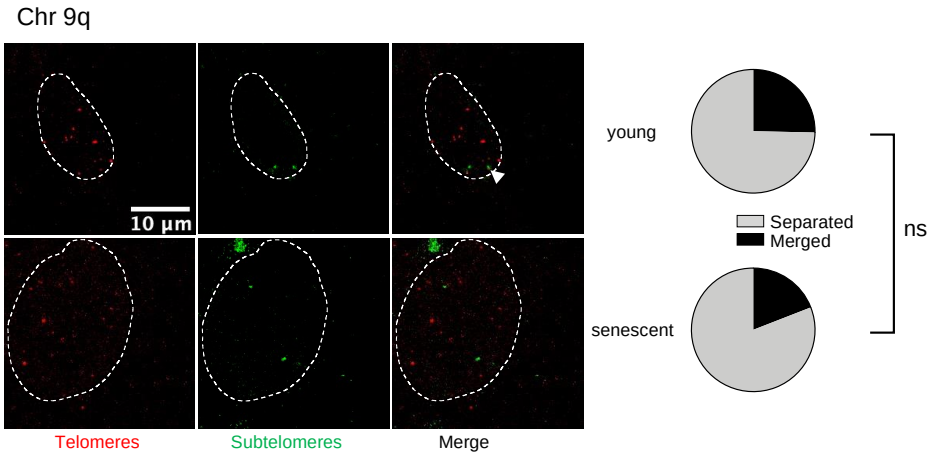



C

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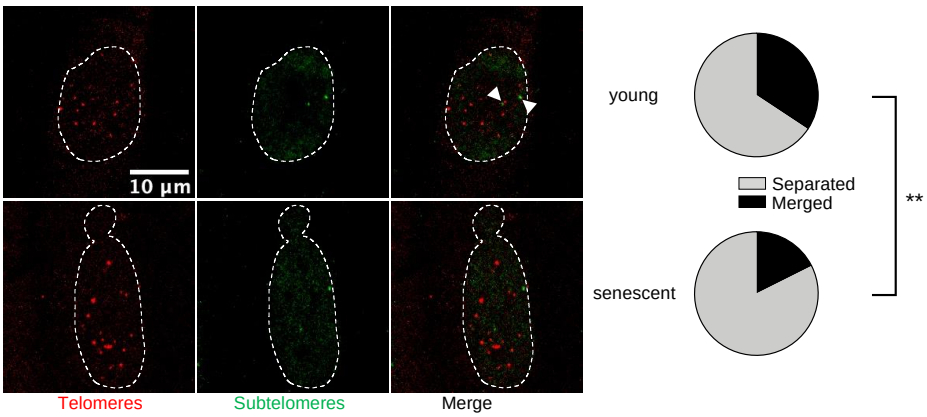
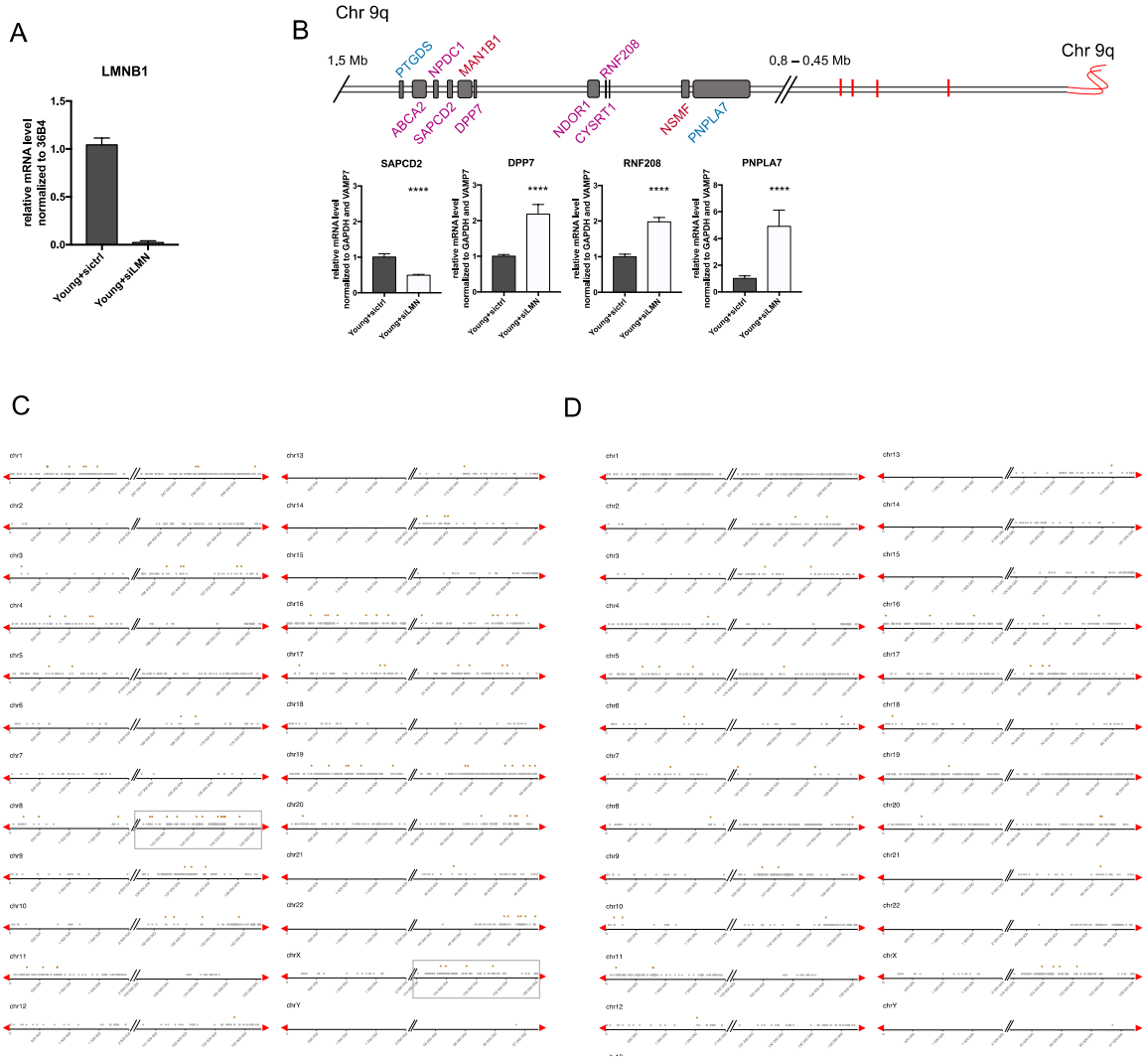


Figure S7 related to Figure 4



Tables

| | 1p | 3q | 8q | 9q | 11p | 16p | 16q | 19p | 20q | 22q |
|---|---|---|---|---|---|---|---|---|---|---|
| **RS sen vs young MRC5** [a] | | | 1.91e-4 | 4.40e-7 | 7.69e-4 | 6.09e-8 | 6.22e-5 | 2.21e-11 | | 3.71e-5 |
| **RS signature** [b] | | | | 2.92e-3 | 2.63e-4 | 7.25e-4 | | | | |
| **OIS signature** [b] | | 6.11e-4 | 2.20e-3 | 9.60e-3 | | | 5.94e-3 | | 1.85e-4 | |
| **IRIS signature** [b] | | | | | | | | | | 1.34e-3 |
| **GTEx dataset** [c] | 8.74e-4 | | 3.80e-3 | 4.98e-4 | 5.86e-3 | 8.87e-7 | | | 4.69e-5 | 3.76e-4 |

**Table 1. Subtelomeric enrichment of upregulated genes**

Enrichment of DEG at subtelomeres in replicative senescence (RS) vs young MRC-5 cells from this work (a), from RS, oncogene-induced senescence (OIS), ionizing radiation-induced senescence from Hernandez-Segura (b) and from aged tissues from the GTEx database (Dong *et al*., 2021) (c). The *p* value of the enrichment where a significance was obtained using a hypergeometric test is shown.

| Upregulated DEG in the first 2Mb from a telomere | | |
|---|---|---|
| Low TRF2-dependent | TRF2 independent | High TRF2-dependent |
| FAM20C | CHL1 | FNDC11 |
| RPH3AL | RASSF7 | CPLX1 |
| RHBDF1 | RHBDL1 | TMEM175 |
| IQSEC3 | B4GALNT4 | RGS11 |
| TUBGCP6 | FBXL16 | GAS6 |
| HDAC10 | METRN | ZNF497 |
| MZF1 | EPS8L2 | MAPK11 |
| MADCAM1 | PLPPR3 | BSG |
| ARHGAP39 | TCEA2 | CRACR2B |
| PNPLA7 | DGKQ | NSMF |
| ZNF135 | IDUA | CD151 |
| KIFC2 | FAM234A | PCSK6 |
| PPP1R16A | PIDD1 | BAIAP3 |
| KLHL17 | SHC2 | TMEM129 |
| MROH1 | DENND6B | DUSP8 |
| SCX | CYP2E1 | IFITM10 |
| SPIRE2 | PNPLA2 | NME3 |
| WDR90 | HCN2 | SIRPA |
| DBNDD1 | ZSCAN18 | JAG2 |
| MAPK8IP3 | MAPK8IP2 | SCAMP4 |
| COL18A1 | MFSD3 | AGPAT2 |
| EME2 | ARSA | PCYT2 |
| GLI4 | SHANK3 | SDF4 |
| MRNIP | PALM | MAN1B1 |
| SCNN1D | EEF1A2 | SNAI3 |
| OXLD1 | LMF1 | |
| | KNDC1 | |
| | CENPBD1 | |
| | GAS8 | |
| | RAB40B | |
| | FN3K | |
| | SLC43A2 | |
| | SLC12A7 | |
| | GNPTG | |
| | ABCA7 | |
| | SYNGR3 | |
| | ARHGAP45 | |
| | F10 | |
| | APC2 | |
| | REEP6 | |
| | ADAMTSL5 | |
| | BRSK2 | |
| | FNDC10 | |
| | MIB2 | |
| | TMEM259 | |
| | GRIN3B | |
| | GAMT | |
| | NDOR1 | |
| | RNF208 | |
| | CYSRT1 | |
| | HAGH | |
| | ABHD17A | |
| | SNED1 | |
| | CRIP2 | |
| | PLXNA3 | |
| | EGFL7 | |
| | DIPK1B | |
| | PIGZ | |
| | ULK1 | |
| | AJM1 | |
| | DPYSL4 | |
| | TMEM121 | |
| | NPDC1 | |
| | ABCA2 | |
| | CYBA | |
| | MVD | |
| | SLC35E2A | |
| | DPP7 | |

**Table 2. List of the upregulated genes found in the first 2Mb from a telomere.**

Gene symbols of the upregulated genes located in the first 2Mb from a telomere and found in the three groups of DEG identify in Figure 2A.

# DISCUSSION

Telomeres are the protective nucleoprotein complex capping chromosomal termini and protecting them from unwanted DNA damage response activation and recombinational repair. Telomeres are also a signalling complex. Via the regulation of subtelomeric chromatin, known as TPE, or the redistribution of transcription factors, telomeres are thought to regulate gene expression in response to diverse stimuli.
Among the diversity of hallmarks regulating aging, telomeres shortening is clearly demonstrated to be developmentally regulated in human. Inhibition of telomerase in somatic cells, during development, leads to a loss of telomeric sequences at each cell division, eventually causing telomeres deprotection, triggering DDR and precipitating replicative senescence. Because accumulation of senescent cells was shown to be a major contributor of aging; telomeres shortening, and the loss of their protective functions, are seen as drivers of human aging. However, it is still puzzling whether telomeres signalling properties are involved in aging.

In this study, by analysing the transcriptome of replicative senescence cells, we observed an enrichment of upregulated genes specifically in the first 2Mb from a telomere, *i.e.* in subtelomeric regions. These upregulated subtelomeric genes were sensitive to the levels of TRF2 and Lamin B1 and their upregulation correlates with a disruption of a physical interaction between a telomere and their subtelomeric loci. Additionally, we observed that the upregulated genes were enriched only in some subtelomeric regions. Using RNAseq public data of senescence cells and aged tissues, we found that the same subset of subtelomeres contained genes specifically derepressed during senescence and aging.

## Alleviation of repression in subtelomeric regions, to which end?

In this study, we analysed the transcriptome of replicative senescence MRC-5 lung fibroblasts. We found 1,051 differentially expressed genes (DEG) in old cells with short telomeres (senescent) compared to young cells with long telomeres (proliferative). Interestingly, we found that the first 2Mb from telomeres were enriched in upregulated genes in replicative senescent cells (paper's figure 1A). We then performed an analysis of the RNAseq read count distribution from the genes located in the first 2Mb from a telomere (paper's figure 1B). It suggests that the upregulated DEG in subtelomeric regions were repressed in young cells, and this effect was

alleviated in senescent cells, resulting in an upregulation of gene expression at senescence. Additionally, we observed that the total level of read counts between young and senescent cells, in subtelomeric regions, globally increased. Therefore, additionally to a significant upregulation of a subset of genes, subtelomeric gene transcription globally changed at senescence.

The subtelomeric enrichment in upregulated genes is in agreement with a recent study published this year by Dong *et al*. 2021. Taking advantage of the transcriptomic data from GTEx, a database aggregating samples from 54 non-diseased tissues collected on nearly 1000 individuals with different ages, they also observed that genes upregulated with age were enriched in subtelomeric regions. Therefore, this specific enrichment, according to our observations could be due to the accumulation of senescent cells (Muñoz-Espín and Serrano, 2014), and the decrease in telomere length (Demanelis et al., 2020) in aged tissues.

Intriguingly, we did not identify specific functional pathways in the subset of upregulated genes located within the 2Mb from a telomere, using several tools such as KEGG, Reactome or GO term analysis. One simple explanation is that overall, 94 genes match these criteria, which could be not enough to perform a comprehensive functional analysis. But when we performed a similar analysis on all the genes located in the first 2Mb from a telomere (1,293 protein coding genes), only one pathway was found enriched: “Olfactory Signalling Pathway”. The 55 subtelomeric genes associated to this pathway belong to the Olfactory Receptor family, a dynamic family of more than 900 genes, arranged in clusters and dispersed in numerous genomic location including subtelomeric positions (Trask et al., 1998; Young and Trask, 2002). Therefore, contrary to yeasts, or plasmodium, subtelomeres in human are not enriched in peculiar gene families. It suggests that subtelomeres are less specialized, but contains genes associated with various pathways. Nonetheless, even if no enriched pathways arise from the upregulated genes found in subtelomeres in senescent cells, it does not exclude a biological significance related to this upregulation.

To understand the biological relevance of the enrichment in upregulated genes in subtelomeric regions, we took advantage of a published senescence signature identified by Hernandez-Segura *et al.* In that study, the authors performed a meta-analysis of different RNA sequencing data from different fibroblasts submitted to

various senescence inducers. Thanks to this analysis, they identified a fibroblast senescence signature, *i.e.* a set of DEG, common among fibroblast cell lines and specific to senescence but independent from the senescence inducer (**Figure 16**). In parallel, they identified sets of DEG specific to a senescence stimulus. They identified 1,699 DEG specific to replicative senescence (RS; obtained by the meta-analysis of RNAseq coming from BJ, HFF, MRC-5, IMR90 and WI-38 fibroblasts), 2,365 genes specific to oncogene-induced senescence (OIS; obtained from IMR90 fibroblasts over-expressing Ras), and 647 DEG specific of ionizing radiation-induced senescence (IRIS; obtained from HCA-2 fibroblasts treated with y-radiation) (Hernandez-Segura et al., 2017).

Using these specific senescence signatures, we performed an enrichment

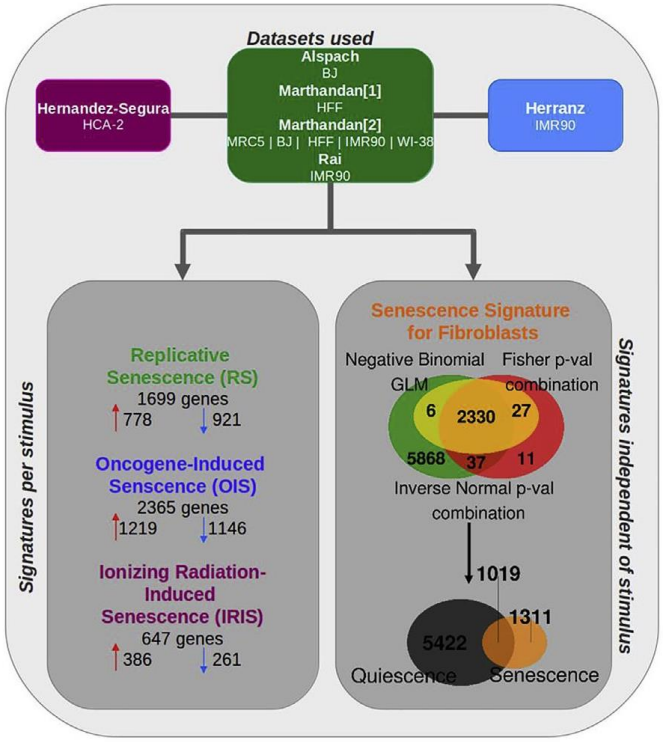



**Figure 16 : Experimental design of Hernandez-Segura *et al.* used to identify senescence signatures:**
Seven RNA-seq datasets obtained from the indicated studies were used to build a stimulus-specific signature and general signature of senescent fibroblasts, regardless of the stimulus. Only genes with a p % 0.01, calculated by the three methods and with expression unchanged or in the opposite direction in quiescence, were included in each signature. The number of genes constituting each signature is displayed in the flower plot. From Hernandez-Segura *et al.*, 2017

analysis of the DEG in subtelomeric regions. Interestingly, we noticed that, not only the RS signature but also the OIS signature, are enriched in upregulated gene in subtelomeric regions (paper's figure S1A). This result suggests that different stresses impact differently the regulation of subtelomeric genes. Of note, we did not identify a significant enrichment in cells treated with ionizing radiation. However, we only analysed the DEG specific to this signature, therefore we cannot exclude that the analysis of the global HCA-2 transcriptome could reveal a subtelomeric enrichment of

upregulated genes. We crossed the upregulated genes, found in the first 2Mb from a telomere, from the different senescence signatures with the RS transcriptome performed here. We noticed a significant overlap between RS signature from Hernandez-Segura and the RS DEG of this work. However, the subtelomeric DEG from the OIS signature were not represented in the RS DEG of this work (paper's figure S1C). This observation was expected because we were using an OIS signature, which was described as OIS specific. Importantly, only 8 upregulated subtelomeric genes of this work were found in common with the fibroblast senescence signature (data not shown). Additionally, we also crossed the age-dependent upregulated genes found in the first 2Mb from a telomere in different tissues identified by Dong *et al.* We did not find either a strong overlap between tissues, suggesting a tissue specific signature of the subtelomeric transcriptome in aged tissues (**Figure 17**).

Several conclusion and hypothesis can be drawn from those data. First, transcriptional changes in subtelomeric regions are not restricted to replicative senescence as we and others observed a similar pattern in OIS and aged tissues. Second, the subtelomeric upregulated genes in senescent fibroblasts are not part of


**Figure 17 : Venn diagram of subtelomeric genes upregulated with aging in different tissues:**
Venn diagram of genes upregulated with age and located at less than 2Mb from a telomeres in three tissues. Whole Blood, Skeletal muscle and Tibial artery. Genes identified by Dong *et al.* 2021.

the universal senescence signature. Third, the subtelomeric DEG seem specific to a particular stressor or a particular tissue. This last two observations suggest that the upregulation in senescent cells of subtelomeric genes could be part of an adaptative stress response that is dependent of an initial stressor or a cell type/tissue. But

differences could also be explained by the RNAseq methodology. It has also been suggested that the upregulation of subtelomeric genes consequently to telomere shortening could be the illustration of an increase in transcriptional noise, *i.e.* an increase in cell-to-cell transcriptional heterogeneity (Vijg, 2020; Vijg and Dong, 2020) leading to aging. This stochastic effect could explain the poor overlap between RS and OIS subtelomeric DEG. However, it is in contradiction with the significant overlap that we found between the two RS transcriptomic analyses. Additionally, we also found that subtelomeric DEG, following a downregulation of LMNB1 in young cells, significantly overlap the subtelomeric DEG in RS (paper's figure 4C). Therefore, LMNB1 downregulation is in part responsible for the reproducible transcriptional change observed in senescent cells. These data, additionally to what have been described in *S. cerevisiae*, *Candida* spp. and *Trypanosoma*, argue in favour of a regulated process over a stochastic effect.

In order to better understand the function of subtelomeric genes derepression, in line with a stress response mechanism, it could be of interest to analyse already published transcriptomes of stressed cells, related or not to telomere, and to decipher whether we could observe a subtelomeric enrichment in upregulated genes.

Taken together, we suggest that the derepression of subtelomeric genes occurs in response to a stress and participate to a cellular stress response.

## A particular nature of the subtelomeres responding to repression alleviation?

Compiling different transcriptomic data with ours, we noticed that subtelomeres enriched in upregulated DEG were shared among datasets (paper's figure 1C). Interestingly, the observation that not all subtelomeres were subject to a similar level of gene derepression was also noticed when telomere position effect was assessed in fibroblasts and myoblasts (Robin et al., 2014). It raises the question whether these subtelomeres share a peculiar chromatin feature that makes them prone to transcriptional regulation upon different stressors. To explore this idea, we correlated different epigenetic marks obtained from the ENCODE database (performed in proliferative fibroblasts), with the number of upregulated genes in each subtelomere

(**Figure 18**). We observed a positive correlation with the CCCTC-binding factor (CTCF), suggesting that a 3D chromatin conformation could be important specifically in those subtelomeric regions. We also observed a positive correlation with H3K27me3, a mark of polycomb repression, as well as with EZH2, the catalytic component of Polycomb Repressive Complex 2, PRC2. Surprisingly, we did not observe a correlation with the constitutive heterochromatin mark H3K9me3. These data suggest that subtelomeres prone to upregulation at senescence are actively repressed by the polycomb in proliferative fibroblasts. Interestingly, EZH2 protein level was shown to gradually decreased as fibroblasts approach replicative and oncogene-induced senescence, until a complete depletion, due to ATM signalling (Ito et al., 2018). One could ask whether this repression of the catalytic subunit of PRC2 could be responsible for this subtelomere specific derepression. Reversely, one can wonder whether this repression by PRC2 in subtelomeric region is governed by telomere and is part of TPE. In line with this idea, TERRA has recently been shown to recruit PRC2 at ALT telomeres to favour the deposition of heterochromatin marks (Montero et al., 2018). It should be interesting to see if this recruitment of PRC2 at telomeres is conserved in somatic and telomerase positive cells and if it could participate to the TPE in human cells.

These correlative data argue in favour of a specific chromatin environment in the subtelomere subject to an enrichment in upregulated genes at senescence. However, these analyses were performed with ChIPseq data obtained in proliferative fibroblasts. Additional analysis in senescence fibroblasts should be performed to see how the correlative pattern is affected. Because we suspected changes in chromatin conformation, it could be of interest to explore datasets assessing chromatin accessibility (DNase I hypersensitive site sequencing (DNase-seq), Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) (Minnoye et al., 2021)) in our context. Because telomere shortening is heterogeneous in cells (Baird et al., 2003), and because telomere shortening was shown to regulate subtelomeric gene expression, subtelomeres enriched in upregulated genes at senescence could probably be adjacent to shorter telomeres. However, the telomeres commonly reported as short telomeres, respectively the 17p (Britt-Compton et al., 2006; Martens et al., 1998) and the 6p (Meier et al., 2007; Zou et al., 2004), were not found enriched in the analysed datasets (see paper's table 1).

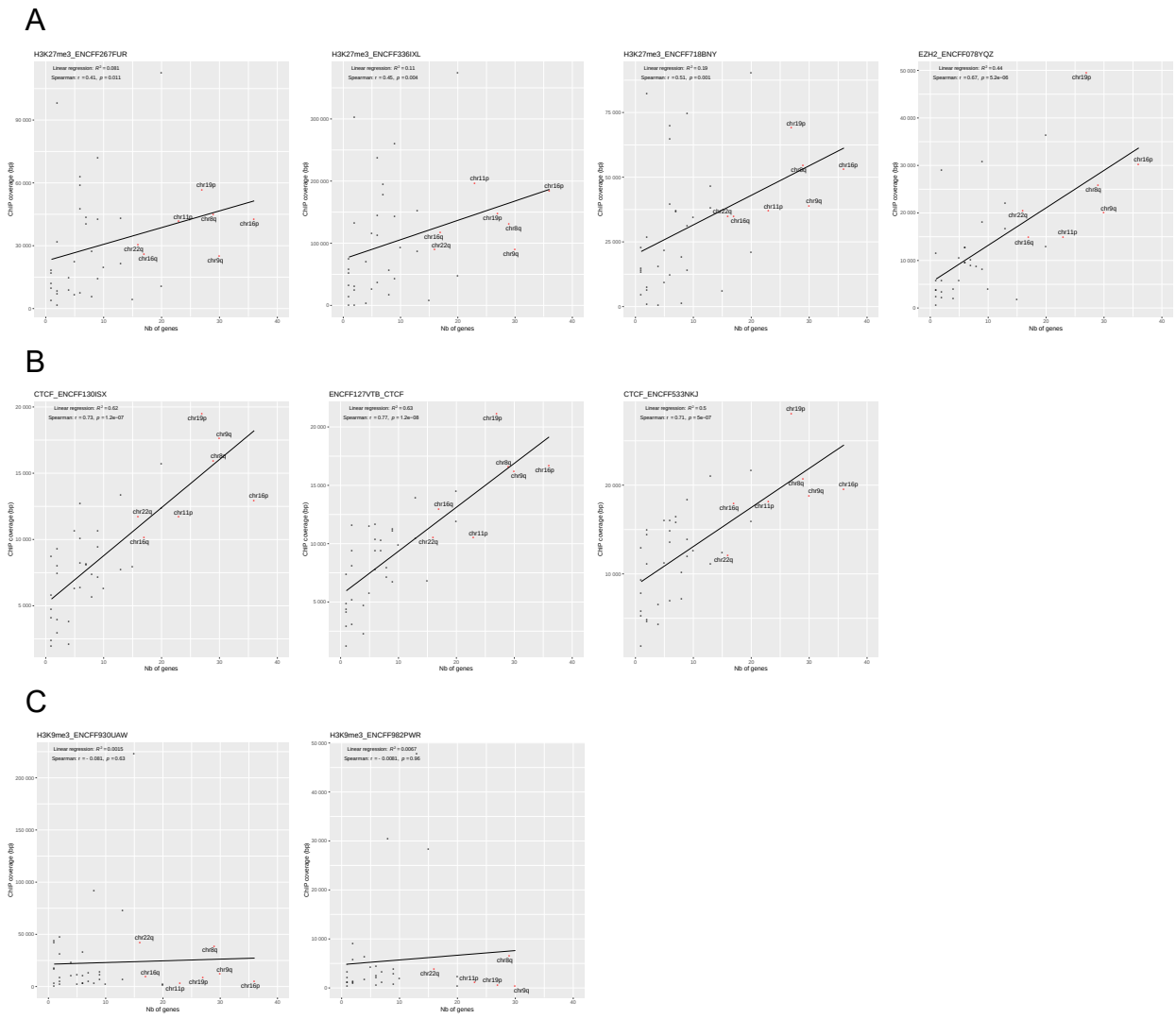



**Figure 18 : Some epigenetic marks correlate with the number of upregulated genes founds at each telomere.**
(A) H3K27me3 and EZH2 ChIP coverage are plotted against the number of upregulated genes (presented in Figure 1C) in each subtelomeres.
(B) CTCF ChIP coverage are plotted against the number of upregulated genes (presented in Figure 1C) in each subtelomeres.
(C) H3K9me3 ChIP coverage are plotted against the number of upregulated genes (presented in Figure 1C) in each subtelomeres.
Correlation calculations were performed for each subtelomeric arm. The linear regression and the Spearman correlation test were performed using the *lm* and the *cor.test* functions from the stats R package

Importantly, we observed that same subtelomeric regions are prone to derepression consequently to RS, OIS and aging. However, different genes are upregulated depending on the initial stressor or the tissue. It suggests a tight regulation of the derepression and the observed effect is probably not the sole consequence of

the loss of EZH2 and H3K27me3 at senescence. Several layers of regulation including transcription factors and 3D chromatin conformation regulation, probably act together to regulate these subtelomeric transcriptional changes. Cooperation between subtelomeric silencing alleviation and transcription factors for instance, are in agreement with what was observed in different model organisms to ensure an appropriate transcriptional response (described in the chapter 3 of the introduction).

## 3D chromatin conformation and telomeres?

In this work, we also highlighted the importance of 3D chromatin conformation in the regulation of subtelomeric genes. Using 3D FISH, we observed that subtelomeric loci containing repressed genes in young cells were in physical proximity with telomeres. In pre-senescent cells or upon TRF2 downregulation, the percentage of interaction between telomeres and subtelomeres was shown to decrease compared to young cells, suggesting a disruption of chromatin organisation. Importantly, TRF2 downregulation in young cells resulted in an increase in gene expression in the studied loci concomitantly with the observed change in chromatin organisation (paper's figure 3). These data support the hypothesis that changes in chromatin conformation in senescent cells, *i.e.* the reduction of an interaction between telomeres and subtelomeres, result in a derepression of subtelomeric genes.

Additionally, we also observed that downregulation of Lamin B1 in young cells impact the expression of subtelomeric genes similarly to what was observed in senescent cells. It is known that Lamin B1 is a major component of the lamina network and actively participates to the chromatin organization and consequently gene regulation. Notably, Lamin B1 participates to the establishment and maintenance of silenced chromatin compartment named Lamina-associated domains (LAD) ((Guelen et al., 2008). In senescent cells, Lamin B1 expression is strongly decreased and was shown to trigger changes in chromatin architecture and in gene expression (Freund et al., 2012; Shah et al., 2013; Shimi et al., 2011). Additionally, it has been recently observed in the team, that TRF2 and Lamin B1 could interact and the overexpression of TRF2 in senescent cells could partially prevent Lamin B1 decrease (Mendez-Bermudez et al., 2021). It suggests that Lamin B1 is in part responsible for the

subtelomeric silencing. Whether it is through its chromatin compartmentalization properties, in interaction with TRF2 or not, still need to be verified. Of note, both TRF2-independent and low TRF2-dependent subtelomeric genes were similarly affected by a decrease of Lamin B1. It suggests that Lamin B1, in subtelomeric silencing, could act both with and without TRF2. At senescence, both TRF2 and Lamin B1 decreases participate to the derepression of subtelomeric genes probably by disrupting chromatin organisation.

In senescent cells, chromatin organisation undergoes a profound remodelling. Using chromosome conformation capture based-methods (Hi-C, chromosome capture followed by high-throughput sequencing), several teams observed changes in long-range chromatin interactions, switches between active (A) and repressive (B) compartments, and topological associated domains (TADs) remodelling (Chandra et al., 2015; Criscione et al., 2016; Sati et al., 2020; Zirkel et al., 2018). However, because of their repetitive natures, telomeric sequences are difficult to map and are usually not taken into account in sequencing-based techniques such as Hi-C. Consequently, the involvement of telomeres in higher-order chromatin organization at a genomic scale was left behind.

Nonetheless, telomeric loops were observed to mediate transcriptional regulation in subtelomeric regions (Kim et al., 2016; Robin et al., 2014, 2020). Notably, it has been proposed that TRF2 could mediate long range chromatin loops between telomeres and subtelomeric ITS with the involvement of Lamin A/C (Kim et al., 2016; Wood et al., 2014). Additionally, we and others observed that telomere shortening and/or senescence disrupts colocalization between subtelomeric and telomeric probes in association with gene expression changes. All these results suggest the importance of telomeric loops in the transcriptional regulation of subtelomeric genes. In order to decipher the higher order of telomeric chromatin organisation, a study using atomic force microscopy (AFM) was initiated in the team. Preliminary work coupling AFM with fluorescence microscopy revealed that telomeres, in native condition, were recurrently associated with a globular chromatin object. This object, with a volume of 70nm$^3$, could contain 13Mb of chromatin (nucleosome + DNA). This calculation, based on the volume of one nucleosome, is an overestimation since it does not consider other chromatin-bound proteins (like the shelterin complex, for instance). However,

compared to the 15kb of a telomeric sequences, these objects cannot be composed solely of telomeric chromatin. A proposed hypothesis is that these objects contain telomeric chromatin interconnected with sub-telomeric chromatin (Lakhal and Pisano, unpublished results). Deciphering if subtelomeric region and telomeres physically interact to form a higher order chromatin structure could be promising to understand the relationship of those two regions. To go further, it could be interesting to ask if these chromatin objects participate to the transcriptional regulation mediated by telomeres.

## Is Telomere Position Effect at play?

Little is known about TPE in human cells however we can still draw some guidelines. First, TPE has been shown to be an epigenetic phenomenon implicating the histone deacetylase SIRT6 and regulated by telomere length (Baur et al., 2001; Koering et al., 2002; Tennen et al., 2011). Second, TPE has been shown to be discontinuous at natural subtelomeres (Lou et al., 2009). Third, TPE has been shown to involve chromatin loops between telomeres and subtelomeric regions mediated by TRF2 to regulate gene expression up to 10 Mb away from a telomere (Kim et al., 2016; Robin et al., 2014, 2020).

By exploring the transcriptome of replicative senescent cells, we identified for the first time that subtelomeric regions were enriched in upregulating genes compared to young cells. Moreover, an analysis of the RNAseq read counts suggests that upregulated genes corresponded in fact to derepressed genes. Therefore, we could hypothesise that short telomeres in senescent cells trigger a TPE alleviation, resulting in derepression of subtelomeric genes. We also observed that depressed genes often formed little clusters in subtelomeric regions, interspaced with non-affected genes, illustrating a discontinuous effect. The overexpression of TRF2 in senescent cells allowed us to identify three groups of DEG, *i.e.* TRF2 independent, high TFR2-dependent and low TRF2-dependent DEG. Interestingly, low TRF2-dependent DEG, additionally to the TRF2 independent DEG, exhibited an enrichment in upregulated genes in subtelomeric regions. Because TRF2 protein level decreases at senescence (Fujita et al., 2010), it suggests that some subtelomeric genes were repressed in a

TRF2 dependent manner. We further confirmed this regulation by downregulating TRF2 in young cells and observed similar subtelomeric genes being overexpressed. Therefore, as in TPE, TRF2 regulates subtelomeric gene repression in young cells, a phenomenon alleviated in replicative senescent cells. Using 3D FISH, we observed changes in chromatin conformation between telomeres and subtelomeres both in a TRF2-dependent and a senescent-dependent manner. These changes correlate with changes in gene expressions. Consequently, we could hypothesise that, as in TPE, chromatin loops are disrupted upon TRF2 downregulation and telomere shortening.

Altogether, our data in senescent cells strongly suggested that in senescent cells, the observed enrichment in upregulated genes in subtelomeric regions is a consequence of the TPE alleviation.

However, we did not assess whether these observed changes in gene expression were associated with epigenetic changes. Because SIRT6 is for now the only histone deacetylase known to be implicating in TPE in human (Tennen et al., 2011), it could be of interest to analyse its genome-wide distribution in young and senescent cells by ChIPseq in association with changes in epigenetic marks.

To better understand the involvement of TPE genome-wide, both in term of transcriptomic changes, epigenetic regulation and chromatin conformation, it is necessary, in my point of view, to enter into the “seq” era. Studying telomeres and subtelomeres using “seq” techniques will be challenging because of their repetitive and polymorphic natures. Because of that, telomeric proximal regions were, for long, not very well map in the reference genome. Recently, thanks to long-read sequencing, the Telomere-to-Telomere (T2T) consortium, released a complete sequence of the human genome (Nurk et al., 2021). Another team also improved our understanding of subtelomeric regions by analysing their polymorphic nature (Young et al., 2020). These recent improvements will help up to analyse new sequencing data to investigate the TPE.

Nonetheless, we will also need to adapt sequencing techniques to the repetitive nature of telomeres. In my opinion, a big challenge with this, is to study the long chromatin range and the impact of telomere on chromatin organisation. Indeed, until now telomere implication in chromatin organisation was mostly studied using 3C techniques, using as a probe, a telomere-proximal, but still subtelomeric, probe (Robin

et al., 2014, 2020). With 3D-FISH, also, subtelomeric probes are often used to infer chromatin changes involving telomeres (Zhang et al., 2021). Therefore, in order to study telomeric chromatin interactions genome-wide, I would like to propose the use of a ChIA-PET (Chromatin Interaction Analysis by Paired-End Tag sequencing) based technique (Fullwood et al., 2009). By combining ChIP-seq and 3Cs methodology, it allows the determination of long-range chromatin loops in association with a specific protein. Therefore, I hypothesise that by determining the chromatin loops associated with several shelterin proteins (TRF2 and TRF1, for instance), we could succeed to determine which chromatin regions are physically interacting with telomeres. Because TRF2 and TRF1 can also binds ITS (Simonet et al., 2011), a stringent sorting of the reads should probably be applied, as proposed for ChIP-seq analysis (Vaquero-Sedas and Vega-Palas, 2019). However, because telomeric sequences are all the same among chromosomes, it will be impossible to decipher if telomeres interact in *cis* or in *trans* with subtelomeric regions. Nonetheless, this coupling of shelterin ChIP with Hi-C, could provide some insight on the telomeric chromatin interactome. Testing this idea in cells with different telomere length or with the downregulation of key player of chromatin organization such as Lamin B1 or CTCF, could provide a lot of information on how telomeres interact with intrachromosomal chromatin.

Taken together, our results suggest that TPE is implicated in the transcriptional changes observed in senescent cells. If the implication of TPE in senescence is confirmed by furthers studies, we could make several assumptions. First, assessing the subtelomeric enrichment in upregulated genes could be a first step to decipher whether a protein, a growth condition, or any other type of variable, could impact TPE. In this respect, we identified Lamin B1 as a putative player in TPE since its downregulation increased subtelomeric gene expression. Second, we observed that, not only replicative senescence, but also oncogene-induced senescence exhibits subtelomeric gene upregulation. These results are in line with the idea that not only telomere shortening but also damages sensed by telomeres could impact TPE (Boussouar et al., 2013). Third, the transcriptional consequences of TPE alleviation in senescent cells are still elusive. However, based on what has been observed in several model organisms, and the fact that different stresses trigger different transcriptional responses presented here, we suggest that TPE could participate to a stress response. Finally, we observed that not all subtelomeres are equal when it comes to the proposed

TPE alleviation in senescent cells. Preliminary analysis of ChIP-seq data highlight differences in chromatin composition between subtelomeres that correlate with the number of upregulated genes. Notably, we identified a strong correlation between EZH2 and its chromatin mark H3K27me3, with the number of upregulated genes. Therefore, we proposed that the Polycomb Repressive Complex 2 could participate to TPE mechanism.

To summarize the current findings on TPE, I would like to propose a model (**Figure 19**). In brief, telomeres, via chromatin loops, silence epigenetically genes located in subtelomeric regions containing a distinctive chromatin environment. This epigenetic silencing is mediated in part by TRF2, Lamin B1 and SIRT6. Whether these proteins interact together to mediate this silencing is still unknown. At senescence, telomere shortening associated with a decrease in TRF2, Lamin B1 and SIRT6, impact heterochromatin modifications and chromatin loops which, consequently, alleviate TPE and derepressed subtelomeric genes.

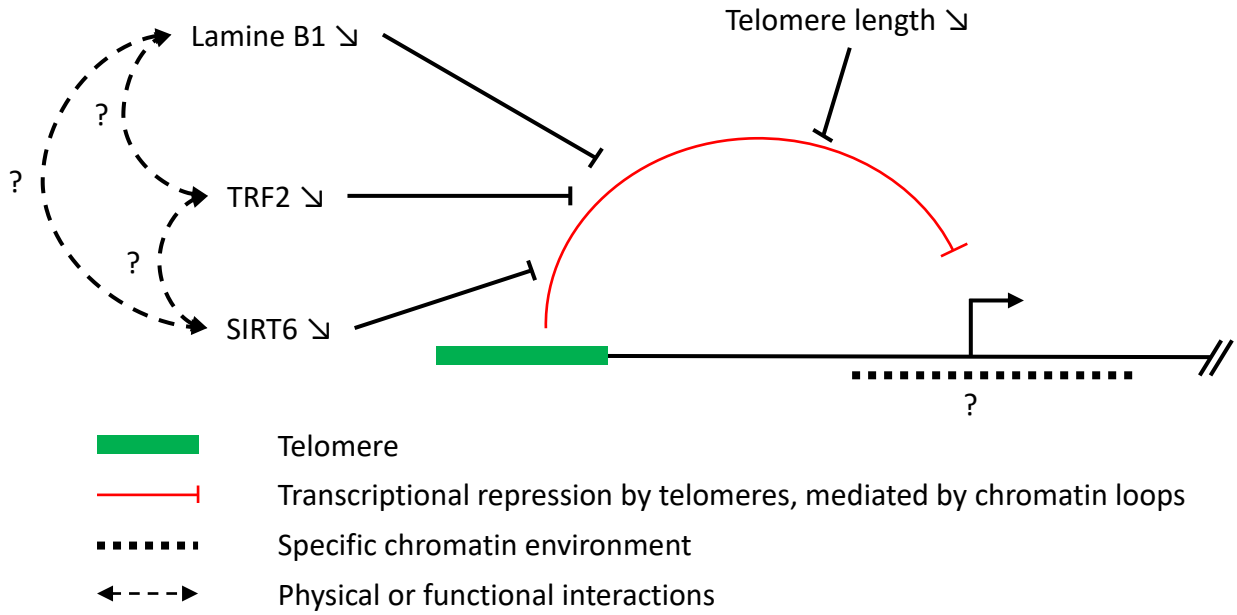



**Figure 19: Model for Telomere Position Effect in human:**
See discussion for details.

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Les télomères, des éléments de régulation de la transcription dans les cellules sénescentes.

Les télomères sont des structures nucléoprotéiques, localisés à l'extrémité de l'ADN des cellules eucaryotes, qui préservent l'intégrité des chromosomes. Ces structures subissent des changements au cours du développement et du vieillissement, comme un raccourcissement de leurs tailles ou une altération dans leur composition en protéines (notamment le complexe shelterin). Cependant, les télomères n'ont pas qu'une fonction protectrice et peuvent aussi réguler l'expression des gènes présent dans les régions adjacentes, *i.e*. subtélomères, voir plus distantes. Ce processus est connu comme l'Effet de Position Télomérique (TPE), découvert initialement chez la levure *Saccaromyces cerevisiae*, puis chez la drosophile et *Schizosaccharomyces pombe* et plus récemment chez l'humain. Dans ces organismes, les gènes situés en position subtélomérique sont réprimés par un mécanisme épigénétique qui est dépendant de la taille des télomères et des protéines les composant ainsi que des structures tridimensionnelles adoptées par la chromatine télomérique et subtélomérique. Le TPE peut être décrit comme un mécanisme de propagation de l'hétérochromatine, du télomère vers le centromère, accompagné de boucle de chromatine permettant d'étendre la répression de la transcription dans des régions plus interne.

Dans ce contexte, le but de ma thèse est de déterminer l'impact des télomères sur les changements transcriptionnels observés lors de la sénescence cellulaire. A cette fin, nous avons séquencé les ARNm de fibroblastes de poumon (MRC-5) jeunes et sénescents (RNAseq). Nous avons observé un enrichissement subtelomérique des gènes dont l'expression est augmentée en sénescence. Ce résultat suggère une levée de la répression induite par le TPE à la sénescence. Cette dérépression ne concerne que certains gènes et certains subtélomères.

Nous avons aussi testé l'hypothèse que les protéines shelterin puissent prendre part au TPE, notamment TRF2 (Telomeric Repeat Binding Factor 2) dont l'expression est diminuée à la senescence. Nous avons donc augmenté le niveau d'expression de TRF2 dans les cellules sénescentes. Ainsi, nous avons pu observer que TRF2 modulait l'expression de certains gènes subtélomériques dans les cellules sénescentes. Par 3D-FISH, nous avons montré que cet effet de TRF2 s'accompagnait d'un remodelage spatial des subtélomères.

Dans son ensemble, ce travail révèle la contribution des télomères au programme transcriptionnel des cellules sénescentes et jette ainsi les bases de l'importance du TPE dans le processus de la senescence.