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# Telomere integrity in post-mitotic skeletal muscle cells : tissue homeostasis and role of FOXO3a on telomere protection

Maria Sol Jacome Burbano

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# THÈSE DE DOCTORAT

Intégrité télomérique dans des  
cellules musculaires post-mitotiques :  
homéostasie tissulaire et  
rôle de FOXO3a dans la protection  
télomérique

**Maria Sol JACOME BURBANO**

Institut de Recherche sur le Cancer et le Vieillessement, Nice – IRCAN  
CNRS UMR 7284 / INSERM U1081 / UCA

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et cellulaires  
d'Université Côte d'Azur

Dirigée par : Pr Eric Gilson

Soutenue le: 25 Novembre 2021

Devant le jury, composé de :

Dr Vincent Mouly, DR1, Sorbonne  
Université

Dr Joao Passos, DR, Clinique Mayo

Pr Björn Schumacher, PU, Université de  
Cologne

Dr Marina Shkreli, CR, Université Côte  
d'Azur

Dr Marie-Noëlle Simon, DR2, Aix  
Marseille Université



# Intégrité télomérique dans des cellules musculaires post-mitotiques : homéostasie tissulaire et rôle de FOXO3a dans la protection télomérique

## Composition du jury

### Présidente du jury

**Dr. Marie-Noëlle Simon**, DR2, INSERM, Aix Marseille Université

### Rapporteurs

**Dr. Vincent Mouly**, DR1, CNRS, Sorbonne Université

**Dr. Marie-Noëlle Simon**, DR2, INSERM, Aix Marseille Université

### Examineurs

**Dr. Joao Passos**, DR, Clinique Mayo

**Pr. Björn Schumacher**, PU, Université de Cologne

**Dr. Marina Shkreli**, CR, INSERM, HDR Université Côte d'Azur

## **Intégrité télomérique dans des cellules musculaires striées squelettiques post-mitotiques : homéostasie tissulaire et rôle de FOXO3a dans la protection télomérique**

Parmi les processus conduisant au vieillissement, le raccourcissement des télomères est le seul clairement programmé au cours du développement dans de nombreux vertébrés. Ceci est dû au problème de réplication observé à chaque division cellulaire. Les télomères sont composés par des répétitions d'ADN (T2AG3), d'un complexe de protéines spécifique (shelterin), d'un ARN non-codant (TERRA) et d'un ensemble de facteurs ayant des fonctions dans les mécanismes de réparation et de réplication. Chez l'humain, le complexe shelterin est composé de six protéines dont TRF2 (telomere repeat-binding factor 2) qui permet la formation d'une boucle d'ADN (t-loop), qui cache l'extrémité des chromosomes d'une activation non voulue de la réponse à l'ADN endommagé. Étant donné que les cellules post-mitotiques différenciées ne se divisent pas, le rôle des télomères dans ces cellules a été longtemps négligé. Donc pendant ma thèse, je me suis intéressée au rôle des télomères dans les cellules musculaires squelettiques différenciées.

Dans la première partie de mon projet, j'ai contribué à un travail déterminant que l'expression de TRF2 diminue dans le muscle squelettique humain, cette diminution commençant chez le jeune adulte. Par la suite, j'ai étudié les conséquences d'une inhibition de l'expression du gène codant pour TRF2 (*TERF2*) dans les cellules musculaires squelettiques différenciées. Contrairement à ce que l'on aurait pu s'attendre, l'inhibition de TRF2 n'expose pas les télomères aux facteurs de reconnaissance et de réparation de l'ADN endommagé. Ceci est observé dans des cellules musculaires humaines en cultures (myotube) et dans les cellules musculaires d'un modèle de souris où le gène *TERF2* est invalidé spécifiquement dans les cellules musculaires squelettiques différenciées. Nonobstant, ces cellules musculaires privées de TRF2 développent un phénotype de dysfonctionnement mitochondrial avec une augmentation du contexte oxydatif qui s'explique, au moins en partie, par un rôle de TRF2 dans l'expression de la sirtuine mitochondriale SIRT3.

Dans la deuxième partie de mon travail de Thèse, j'ai étudié les mécanismes de protection des télomères dans les myotubes privés de TRF2. J'ai pu mettre en évidence un rôle non-canonique du facteur de longévité FOXO3a dans la protection de ces télomères dépourvus de TRF2.

Enfin, dans la troisième partie de mes études, j'ai caractérisé le modèle de souris d'inhibition de *Terf2* spécifiquement dans la fibre musculaire mature. Cette inhibition entraîne, chez les femelles, un retard dans le vieillissement des cellules musculaires squelettiques associé à une légère augmentation de la force musculaire et un rallongement de leur longévité.

En résumé, mes résultats ont révélé dans les cellules musculaires squelettiques différenciées un mécanisme inédit de protection des télomères impliquant un rôle non-canonique de FOXO3a, une connexion entre le facteur shelterin TRF2 et le fonctionnement des mitochondries ainsi qu'une fonction paradoxale de TRF2 dans le

vieillesse musculaire et la longévité. Je discute ces résultats dans le contexte d'un continuum développement-vieillesse.

**Mots clés :** Télomères, muscle strié squelettique, résistance au stress, dommages à l'ADN, FOXO3a, SIRT3, vieillissement

**Telomere integrity in post-mitotic skeletal muscle cells:  
Tissue homeostasis and role of FOXO3a on telomere protection.**

Among the processes leading to aging, telomere shortening is the only one normally programmed during development in many vertebrates. This is due to the replication problem observed at each round of replication. Telomeres are composed of DNA repeats (T2AG3), a specific protein complex (shelterin), a non-coding RNA (TERRA) and a set of factors having functions in DNA repair and of replication. In humans, the shelterin complex is composed of six proteins including TRF2 (telomere repeat-binding factor 2) which allows the formation of a DNA loop (t-loop), hiding the ends of the DNA from unwanted activation of the DNA damage response (DDR). Since differentiated post-mitotic cells do not divide, the role of telomeres in these cells has long been overlooked. So during my thesis, I was interested in the role of telomeres in differentiated skeletal muscle cells.

In the first part of my PhD project, I contributed to a work showing that the expression of TRF2 decreased in human skeletal muscle cells, this decrease starting in young adults. Subsequently, I studied the consequences of inhibiting the expression of the gene encoding TRF2 (*TERF2*) in differentiated skeletal muscle cells. Contrary to what one might have expected, inhibition of TRF2 does not expose telomeres to a deprotection toward DDR. This is observed in cultured human muscle cells (myotube) and in muscle cells from a mouse model where the *TERF2* gene is specifically invalidated in differentiated skeletal muscle cells. Notwithstanding, these TRF2-deprived muscle cells develop a mitochondrial dysfunction phenotype with an increase in oxidative context, which is explained, at least in part, by a role of TRF2 in the expression of the mitochondrial sirtuin SIRT3.

In the second part of my PhD work, I studied the mechanisms of telomere protection in myotubes deprived of TRF2. I was able to demonstrate a non-canonical role of the longevity factor FOXO3a in the protection of muscle cell telomeres lacking in TRF2.

Finally, in the third part of my PhD work, I characterized the mouse model of inhibition of *Terf2* specifically in mature muscle fiber. This inhibition causes, in females,

a delay in the aging of skeletal muscle cells associated with a slight increase in muscle strength and a lengthening of their longevity.

In summary, my results revealed in differentiated skeletal muscle cells a telomere protection mechanism involving a non-canonical role of FOXO3a, a connection between the shelterin factor TRF2 and the functioning of the mitochondria as well as a paradoxical function of TRF2 in muscle aging and mouse longevity. I discuss these findings by putting them in the context of a development-aging continuum.

**Key words:** Telomeres, skeletal muscle, stress resistance, SIRT3, DNA damage, FOXO3a, aging



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*"I am among those who think that science has great beauty.*

*A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. We should not allow it to be believed that all scientific progress can be reduced to mechanisms, machines, gearings, even though such machinery has its own beauty".*

*Marie Curie, 1933*

*"Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth."*

*Jules Verne*

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## *INTRODUCTION*

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# 1 Telomeres: Basic Molecular Mechanisms

## 1.1 Telomeres: the origin of research

Nowadays, the telomere research on aging related topics and others is spread all over the world. According to the PubMed data base, only in 2020, there were 746 published papers related to the Mesh-term “telomeres”, among them 135 reviews. Nonetheless, the very first insights of telomere existence appeared during the 30’s, thanks to the work of Barbara McClintock and Hermann Muller on maize and *Drosophila melanogaster* (Creighton and McClintock, 1931; McClintock, 1931; Muller Hermann, 1938), respectively, in which they noticed that induced intrachromosomal breaks are different from natural chromosome ends. Although intrachromosomal breaks frequently induced over-crossing or chromosome fusion, normal chromosome ends fusions were not observed in any of those research models. In 1938, Muller name this natural chromosome ends “telomeres” from the Greek “telos” and “meros”, meaning “end” and “part” respectively. But it is only 40 years after that the first telomeric sequence is described in *Tetrahymena thermophila* as DNA 3'-TTGGGG-5' tandem repeats (Blackburn and Gall, 1978) and 50 years for the human telomeric sequence 3'-TTAGGG-5' (Moyzis et al., 1988). Interestingly, in a letter from Barbara McClintock to Elizabeth Blackburn in 1983, McClintock described her previous work on maize chromosomes during meiosis and her unpublished observations on telomeres. Strikingly, she mentions for the first time “an enzyme required for formation of new telomeres” and that this enzyme is not produced in the gametophyte and endosperm but are produced by embryonic cells”. This is an amazing example of scientific discussion and science shared beautifully, as this enzyme correspond to the now known telomerase, characterized in 1985, and this research was rewarded by a Nobel Price on Physiology and Medicine in 2009 (Greider and Blackburn, 1985). Next, in 1990, Calvin Harley and colleagues observed progressive telomere shortening in several human fibroblasts, correlated with replicative passages (Harley et al., 1990). Hence, these results suggested that telomeres length maintenance is associated with the previously hypothesized Hayflick limit and replicative senescence, thus, it placed an emphasis on cellular aging (Hayflick, 1965). Thereafter, an outbreak of data on telomeres, revealed it function, mechanisms, partners and other details that some of them will be, in part, explained in this section.

A part of this section has already been published in 2021 in a review in *Cells*: “The Power of Stress: The Telo-Hormesis Hypothesis” (Jacome Burbano and Gilson, 2021). Nevertheless, it has been completed with new bibliography, comments and further details.

## 1.2 Telomere regulation

Telomeres are nucleoprotein structures at the ends of linear chromosomes that are required to maintain genome stability by providing a terminal cap (Giraud-Panis et al., 2010). Their function and replication require a complex interplay between specialized and general factors involved in replication and the DNA damage response. Strikingly, their structures change during an organism’s lifetime, both as a result of developmentally regulated programs and external factors. This, together with significant variability among organisms in telomere structure and regulation, raises several fundamental questions regarding telomere evolution and their contribution to development, life history, aging, disease susceptibility and the stress response. To understand fully the “telomere logic” in the stress response, it is necessary to understand the molecular challenges that these structures must face, and the evolutionary solutions adopted by different organisms.

### 1.2.1 Challenge 1: The End-Stability Problem

In eukaryotes, the linearity of chromosomes requires the need to distinguish natural DNA termini from accidental breaks, to avoid unwanted recombination, karyotype instability and unwanted cell cycle arrest. The solution to this stability problem is the specific association of chromosome termini with protective factors including non-coding RNA (Telomeric Repeat-containing RNA or TERRA), and specialized proteins, for which the shelterin complex represents the paradigm (Ghilain et al., 2021; Giraud-Panis et al., 2010).

In most eukaryotes, the telomeric DNA sequence is composed of G-rich DNA repeats ending at chromosome termini with a single-stranded DNA G-rich 3'-overhang (or G-tail) (Gomes et al., 2011; Meyne et al., 1989; Moyzis et al., 1988). This 3' overhang allows the formation of a telomeric DNA loop (t-loop) resulting from the invasion of the 3' overhang into the internal duplex DNA (Figure 1). The formation of this t-loop is required to maintain telomere stability in a large range of organisms and

in many different types of cells. Its formation is assisted by the shelterin subunit TRF2 (Benarroch-Popivker et al., 2016).

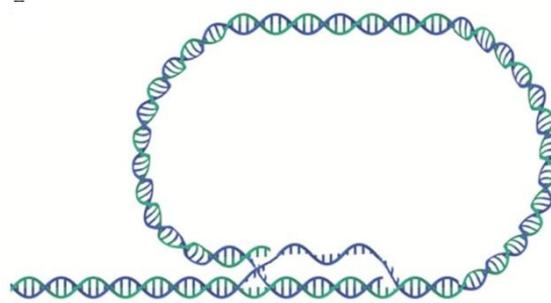


Figure 1: T-loop structure formed by the invasion of the 3'-single stranded overhang into the double stranded telomeric DNA. Image taken from (Giraud-Panis et al., 2013)

The shelterin complex consists of proteins bound to the double-stranded part of telomeric DNA (e.g., TRF1 and TRF2 in chordates; Rap1 in *Saccharomyces cerevisiae*; DNT-1 and DNT-2 in *Caenorhabditis elegans*) (Giraud-Panis et al., 2010; Yamamoto et al., 2021) and single-stranded telomeric DNA (e.g., POT1 in humans; POT-1/POT-2 in nematodes). Finally, some shelterin subunits create a bridge between the duplex and the 3' overhang nucleoprotein shelterin complexes (e.g., TPP1/ACD and TIN2 in mammals), or simply bind to specific other subunits (for example, mammalian RAP1 binds to TRF2) (Figure 2). One notable exception to this scheme is *Drosophila melanogaster* telomeric DNA, which is composed of long arrays of the non-long-terminal-repeat retrotransposons HeT-A, TART and TAHRE, bound by the Terminin protein complex that has no clear evolutionary relationship with shelterin and is elongated by retrotransposition, but also by recombination and gene conversion (Figure 2) (Cacchione et al., 2020; Mason et al., 2008).

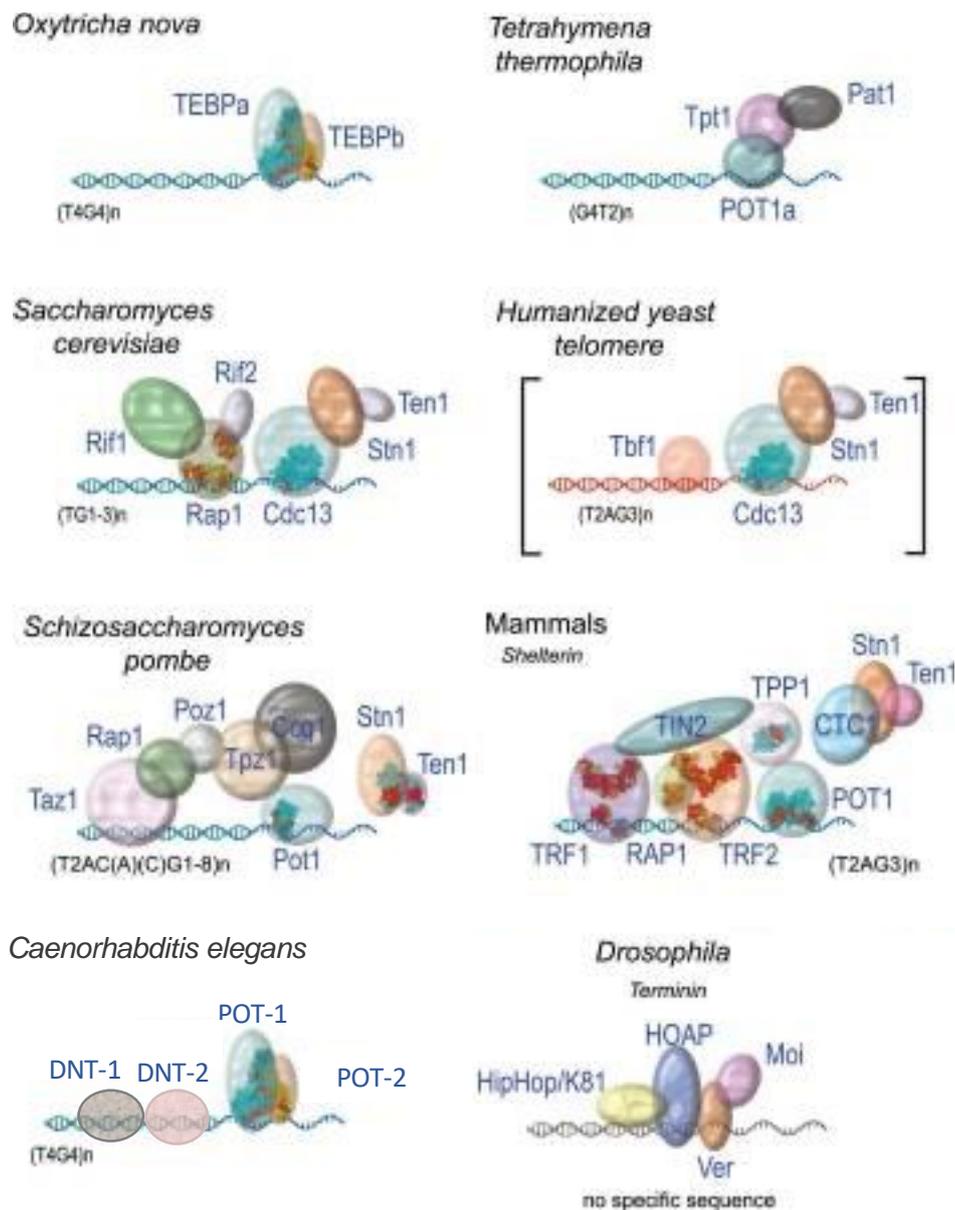


Figure 2: Telomere capping proteins in different species.

Protecting chromosome ends by protein complexes is conserved across evolution. Nonetheless, telomeric proteins evolve rapidly, thus, they are unique for a group of living being or even unique for each specie. Image adapted from (Giraud-Panis et al., 2013).

The termini of linear chromosomes must be protected in order to avoid being recognize as double strand or single strand DNA breaks. Therefore, telomeres require tools to guarantee the termini protection, such as proteins or the t-loop structure, described above, which prevents ataxia telangiectasia mutated (ATM) activation and chromosome end-to-end fusions (Benarroch-Popivker et al., 2016; Ghilain et al., 2021; Pickett et al., 2009; Wang et al., 2004). Several DNA damage response proteins have been observed at telomeres, showing that there is a DNA damage activation at

dysfunctional telomeres: 53BP1, Rad17, ATM pS1981,  $\gamma$ H2AX, Mre11 complex, etc (d'Adda di Fagagna et al., 2003; Takai et al., 2003).

Moreover, TRF2 is critical for telomere protection (Figure 3) : i) TRF2 is required for t-loop formation (Benarroch-Popivker et al., 2016), ii) TRF2 is important to inhibit ATM pathway (Karlseder et al., 1999; Okamoto et al., 2013) at different steps of the DDR signaling. First, the TRFH, dimerization domain of TRF2, inhibits ATM activation (Karlseder et al., 1999; Okamoto et al., 2013). Second, a subpart of the TRF2 hinge domain, known as iDDR, blocks ATM signaling propagation downstream ATM activation and prevents telomere fusions (Okamoto et al., 2013). Third, TRF2-Myb domain binds directly to Chk2, a target of ATM kinase, at functional telomeres to interrupt the DDR signaling spread (Buscemi et al., 2009). iii) TRF2 protects from telomere end-to-end fusion via classical nonhomologous end joining (c-NHEJ) (van Steensel et al., 1998). In very short telomeres, TRF2 recruitment of RAP1 counteracts NHEJ (Lototska et al., 2020). iv) TRF2 is required to avoid homologous recombination (HR) at telomeres (Poulet et al., 2009; Saint-Léger et al., 2014; Wang et al., 2004).

The ataxia telangiectasia and Rad3-related kinase (ATR) is inhibited by telomeres in two independent manners. First, at the single stranded telomeric DNA (ssDNA) TPP1-POT1 avoid RPA1 binding and subsequent ATR activation (Denchi and de Lange, 2007; Gong and de Lange, 2010; Kratz and de Lange, 2018) (Figure 3). Second, TRF1 is required for telomere replication fluent movement, by repressing stalled forks, thus, it avoids fragile sites formation and ATR activation (Martínez et al., 2009; Sfeir et al., 2009) (Figure 3). Indeed, it has been proposed that TRF1 recruits BLM helicase, a member of the RecQ DNA helicases family, to release replication fork access (Zimmermann et al., 2014).

Finally, TPP1/POT1 has also been observed in protection against NHEJ and homology-directed DNA repair (HdDr) (Denchi and de Lange, 2007; Glousker et al., 2020) (Figure 3).

Altogether, telomere function of protection to avoid undesired DDR activation relies on telomere capping by the shelterin complex. Relocation of these proteins or decreased expression during could trigger a premature aging phenotype and even genomic instability drive cancer transformation.

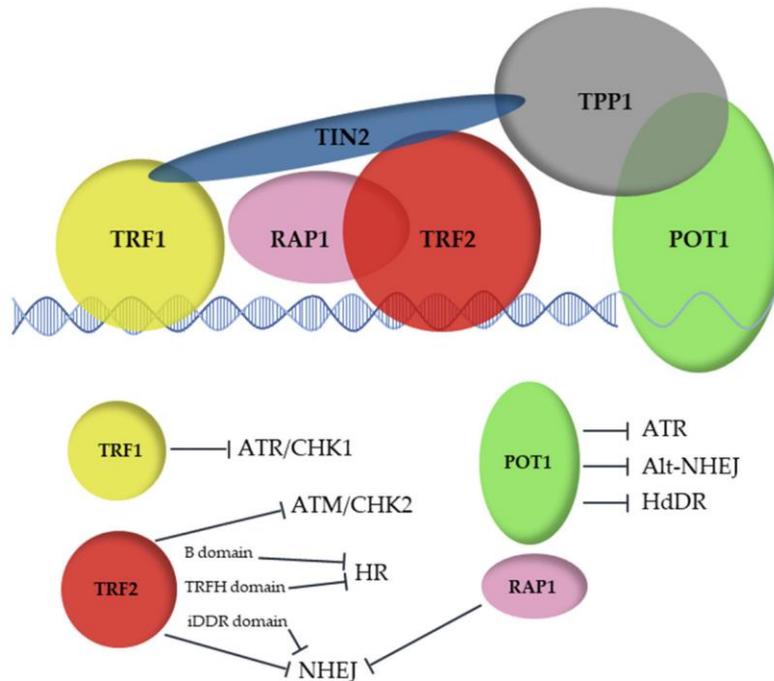


Figure 3: The shelterin complex cap chromosome termini and prevents undesired DNA damage response activation at telomeres. Figure adapted from (Ghilain et al., 2021).

### 1.2.2 Challenge 2: End Replication Problem

The full replication of the terminal DNA cannot be achieved by conventional replication machinery (Gilson and Géli, 2007a; Lingner et al., 1995; Olovnikov, 1973; Watson, 1972). On the lagging telomeres, the G-tail formation may result simply from the removal of the last RNA primer. On the leading telomeres, the end product is either blunt or 5' protruding. Since in many organisms a G-tail can be detected at both daughter telomeres, a 5' resection activity must take place to convert it into a 3' overhang. Indeed, coupled with replication, TRF2 targets the leading telomere with the 5'-exonuclease Apollo, which initiates the 5' resection. At the lagging strand, POT1 inhibits Apollo resection (Wu et al., 2012a). At both ends, the Exo1 nuclease elongates the 3' overhangs by pursuing a 5' resection. It remains unknown how this resection is controlled. Finally, POT1 recruits the single-stranded DNA-binding trimeric complex CST (CTC1-STN1-TEN1) to refill the 5'-strands (Wu et al., 2012a) (Figure 4).

There are several processes that compensate for this unavoidable shortening. One such mechanism relies on telomerase, a specialized reverse transcriptase with an RNA template that elongates the G-rich strand using the 3' overhang as a substrate.

Telomerase is composed of two subunits: TERT, the telomerase reverse transcriptase, and TERC, the telomerase RNA template. Using a single telomere length system and monitoring telomere DNA length (TL) evolution in budding yeast, it was shown that telomerase activity is reduced by over elongated telomeres: excess of Rap1p at the long yeast telomeres triggers a progressive *cis*-inhibition of telomerase (Marcand et al., 1999). This indicates that it is important to keep TL within a regulated range of size.

In mammalian species with large body mass, including humans and some birds, the level of telomerase expression is much higher in germ, stem and progenitor cells than in somatic differentiated cells. In some species, such as laboratory mice, telomerase expression is less repressed and more evenly distributed within cell types than in humans, but still higher in the germ and stem cell compartments (Hausmann et al., 2007; Roake and Artandi, 2020; Wright et al., 1996). Importantly, the amount of telomerase present in adult somatic stem cells and progenitors is generally not enough to replenish telomeric DNA fully when these cells divide to regenerate the corresponding tissue or to respond to external stimuli.

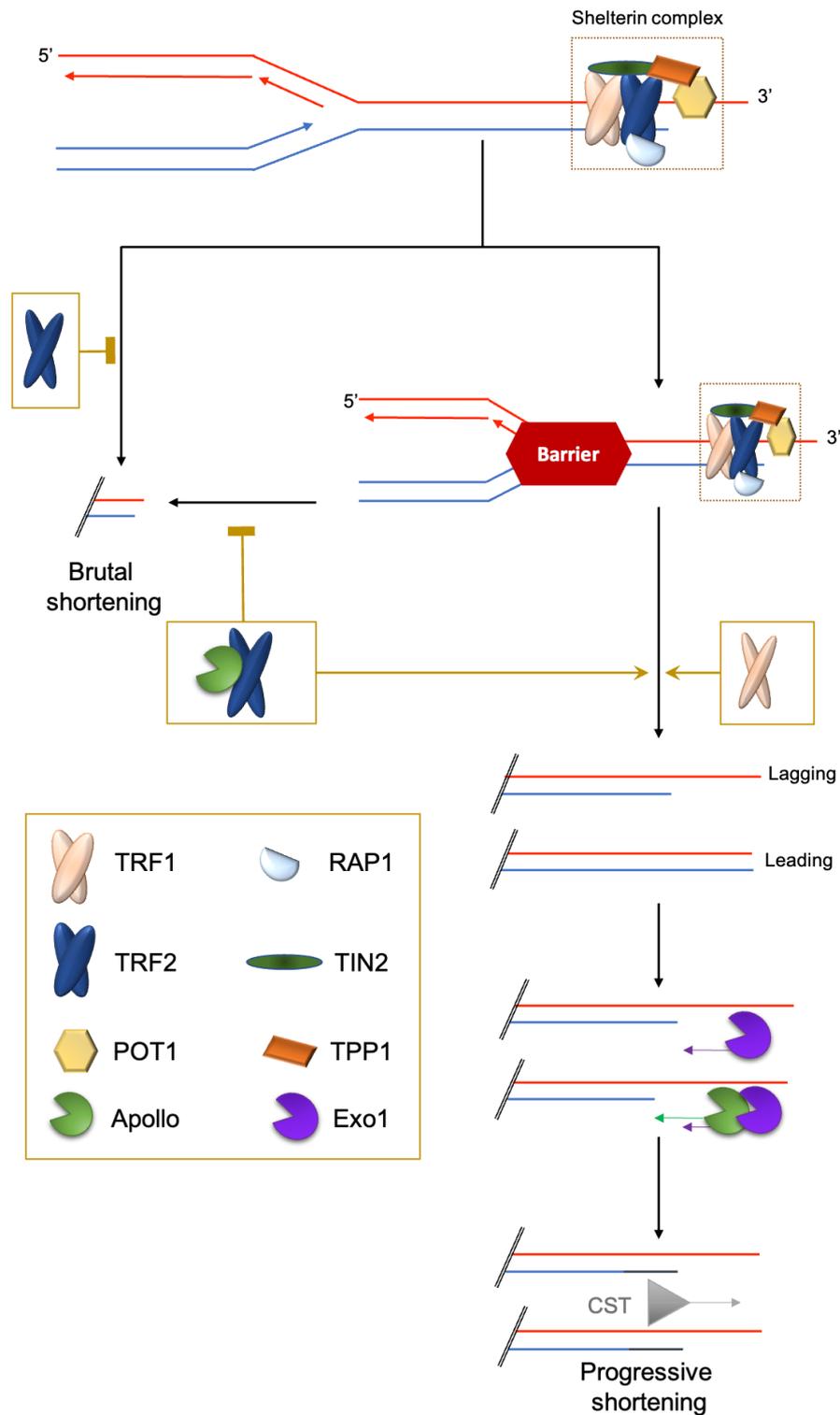


Figure 4. Brutal and progressive telomere DNA shortening.

Image taken from (Jacome Burbano and Gilson, 2021). Telomere replication at the leading or lagging strands is not similarly regulated. At the G'-rich lagging strand, due to the end-replication problem, the parental strand is not completely replicated, triggering a 3'-overhang. At the leading strand, there is a blunt end or 5' protruding that is eroded in a manner coupled to replication by the TRF2-dependent recruitment of the 5'-exonuclease Apollo. Next, at both strands, the Exo1 exonuclease prolongs the 3'-

overhang. Finally, POT1 recruits the CST complex (CTC1-STN1-TEN1) to replenish the 5'-strands. The brutal appearance of critically short telomeres that cannot be explained by the progressive telomere shortening was first observed in yeast (Chang et al., 2007). They are likely to stem from intra-telomeric recombination (Ancelin et al., 2002; Pickett et al., 2009) or the collapse of DNA replication forks. Indeed, it is known that telomeric repeats can form recombinogenic t-loops (Saint-Léger et al., 2014; Wang et al., 2004) and are difficult to replicate (Miller et al., 2006; Sfeir et al., 2009; Ye et al., 2010a).

In many vertebrates, TL is subjected to a progressive erosion cadenced by cell divisions and the residual level of telomerase activity. There are also exceptions to this scheme; for instance, in the long-lived bat species *Myotis myotis*, mean TL does not shorten with age but changes with the environment (Foley et al., 2018). In fact, the rate of telomere shortening in blood cells during development and aging is not strictly related to the level of telomerase activity (Gomes et al., 2011; Whitemore et al., 2019). This suggests that telomerase may have functions other than regulating telomere length and that TL might be regulated independently of telomerase. Indeed, the mean TL is the net result of shortening and elongation rates, rendering it difficult to interpret TL changes only in terms of telomerase-dependent elongation (Marcand et al., 1999). One hypothesis is that the rate of telomere DNA shortening, but not the absolute length, controls the aging process (Whitemore et al., 2019). It is not yet known whether there exist developmentally regulated mechanisms, independently of telomerase activity, that modulate the rate of TL shortening.

Interestingly, eukaryotic cells are able to use a telomerase-independent mechanism, ALT (alternative lengthening of telomeres), which is based on homologous recombination (Bryan et al., 1997). It is activated in a number of human tumors, in human cells immortalized in culture, and also in normal somatic tissues (Neumann et al., 2013). In plants, the ALT mechanism is activated upon telomerase dysfunction and possibly also during the earliest stages of normal plant development (Růcková et al., 2008). At the organismal level, ALT was suggested by Gomes and colleagues (Gomes et al., 2011) for koala and wombat telomeres, based on the presence of long and interrupted telomere repeats and the absence of telomerase expression. However, it is not known whether these ALT-like telomeres use the same process of maintenance as the ALT + cancer cells. Along this line, a recent work that revealed the existence of an ALT-like mechanism in *Terc* knock-out (KO) mouse stem cells, with a unique non-telomeric sequence (mTALT) (Kim et al., 2021). This process might be related to the one used in wombat. Hence, ALT or ALT-like mechanisms could

give an advantage in the context of species with no telomerase. In order to avoid cancer development, we can imagine ALT process is finely regulated in species using only this pathway to preserve their telomeres.

### 1.2.3 Challenge 3: The Hard-to-Replicate Problem

In addition to the end-replication problem, the progression of the replication fork through telomeric chromatin is difficult and may result in fork collapses leading to the loss of a large part of the telomeric DNA. This is prevented by specific mechanisms involving the shelterin subunits TRF1 and TRF2: TRF1 manages classical stalled forks leading to ATR activation and TRF2 manages topologically-constrained stalled forks (Ohki and Ishikawa, 2004; Sfeir et al., 2009; Ye et al., 2010a) (Figures 3 and 4).

Interestingly, the function of TRF2 in this process requires the same factor, Apollo, as the one favoring the progressive shortening of the leading strand (Ye et al., 2010a). Inhibiting TRF2-Apollo interaction is associated with severe progeroid syndrome in humans (Touzot et al., 2010). The long-lived giant tortoises *Chelonoidis abingdonii* and *Aldabrachelys gigantea* carry an Apollo variation within the TRF2 binding domain that is not conserved in other reptiles, fishes or mammals and, thus, could contribute to the extreme longevity of these organisms (Quesada et al., 2019). Besides, among the positively selected mutations within the naked mole rat (NMR, *Heterocephalus glaber*) genome, at least three are related to telomeres (Kim et al., 2011). NMR is the longest living rodent with a lifespan estimated at more than 35 years. Among these telomere-related proteins the NMR TRF1 protein induces telomere shortening, better binding to telomeres, enhanced glycolytic capacity and telomere protection upon hypoxia when it is overexpressed in mouse embryonic fibroblasts (MEFs) (Augereau et al., 2021). Hence, these findings suggest that the NMR developed an improved stress resistance strategy that includes shorter telomeres. Altogether, these findings suggest an evolutionary advantage, at least in some organisms, to control progressive shortening due to the end replication problem. This hypothesis seems at odds with the wealth of studies showing that TL is positively associated with improved healthspan and lifespan in vertebrates (Aviv, 2006; Heidinger et al., 2012). Usually, short telomeres are associated with chronological aging, cardiovascular and age-related diseases and poor prognosis (Aviv and Shay,

2018). However, long telomeres are not as beneficial as was initially thought: several studies have shown an association between long telomeres and some types of cancer. Actually, in a genome-wide association study, nine cancers out of 35 (including glioma, neuroblastoma and endometrial cancer) were associated with increased TL (The Telomeres Mendelian Randomization Collaboration et al., 2017). To understand the “raison d’être” of these different telomere trajectories between organisms and individuals, and their consequences for health, we need to understand more fully the complex interplay between genetic and environmental determinants regulating the response of telomeres to stress during development and aging.

### 1.3 Telomere Response to Stress

A stressor can be defined as a factor that leads to a rupture in homeostasis, whether it be at the cellular, systemic or organismal level. Thus, the environment can be defined as the combination of stressful situations to which individuals are exposed during their lifetime, including lifestyle, climate, pollutants, pathogens and social relationships. Organisms have evolved mechanisms to cope with their environments and predictable environmental fluctuations. Abnormal exposure to stressors is a well-known risk of age-related disease and premature aging (de Magalhães and Passos, 2018). Different types of stressors also lead to an acceleration of TL shortening, leading to critically short telomeres and cellular senescence, a mechanism that could explain the link between stress and aging. To give an example, the telomere shortening rate can increase by roughly five times in fibroblast cultured in hyperoxia compared to normoxia (von Zglinicki et al., 1995). The rapidity by which these changes are signaled to the cells is not well understood. One can imagine two non-mutually exclusive modes of signaling. On one hand, shortened telomeres are phenotypically “silent” until they reach a critically short length that triggers senescence. On the other hand, even small changes in telomere length variation can have an impact, even before cells reach senescence. In the following paragraphs, I discuss how various stresses can modulate telomere structure and function (Figure 5).

### 1.3.1 Inflammation

During aging there is an increase of circulating inflammatory markers and a decrease in immunological capacities, leading to a systemic chronic inflammatory state, also known as “inflammaging” (Kale et al., 2020). Chronic inflammation is often associated with diseases such as rheumatoid arthritis or chronic obstructive pulmonary disease (COPD). Noteworthy, liver TL attrition is significantly exacerbated in cirrhosis or chronic hepatic livers (Aikata et al., 2000). In mouse, repeated *Salmonella* exposure decreases TL in leukocytes, but not in hepatocytes or splenocytes (Ilmonen et al., 2008). If these results indicate that inflammation can drive TL shortening, the reverse can also occur and a wealth of data present telomere dysfunctions as drivers of chronic inflammation (see e.g., (Chakravarti et al., 2020).) Nonetheless, I will focus on the processes of telomere shortening triggered by inflammation.

Inflammation-induced TL shortening may be due to the inhibition of telomerase (Figure 5). In support of this hypothesis,  $TNF\alpha$  treatment shortens telomeres via ATF7-dependent telomerase disruption (Maekawa et al., 2018). However, infection is capable to trigger accelerated TL attrition in telomerase-deficient mice (Herrera et al., 2000), revealing the existence of telomerase-independent mechanisms linking inflammation to TL shortening. These mechanisms might include the production of ROS during inflammation; for example, via secretion by immune cells such as neutrophils (Lagnado et al., 2021). Indeed, the rate of telomere shortening increases under oxidative stress, and the G-richness of telomeric DNA renders it highly susceptible to oxidization, leading to the accumulation of 8-oxo-guanine (explained below, Figure 10). This disrupts the binding of telomere protective factors and can induce DNA breaks and prevent extension by telomerase (Figure 5 and 10) (Barnes et al., 2019a; Saretzki et al., 2003).

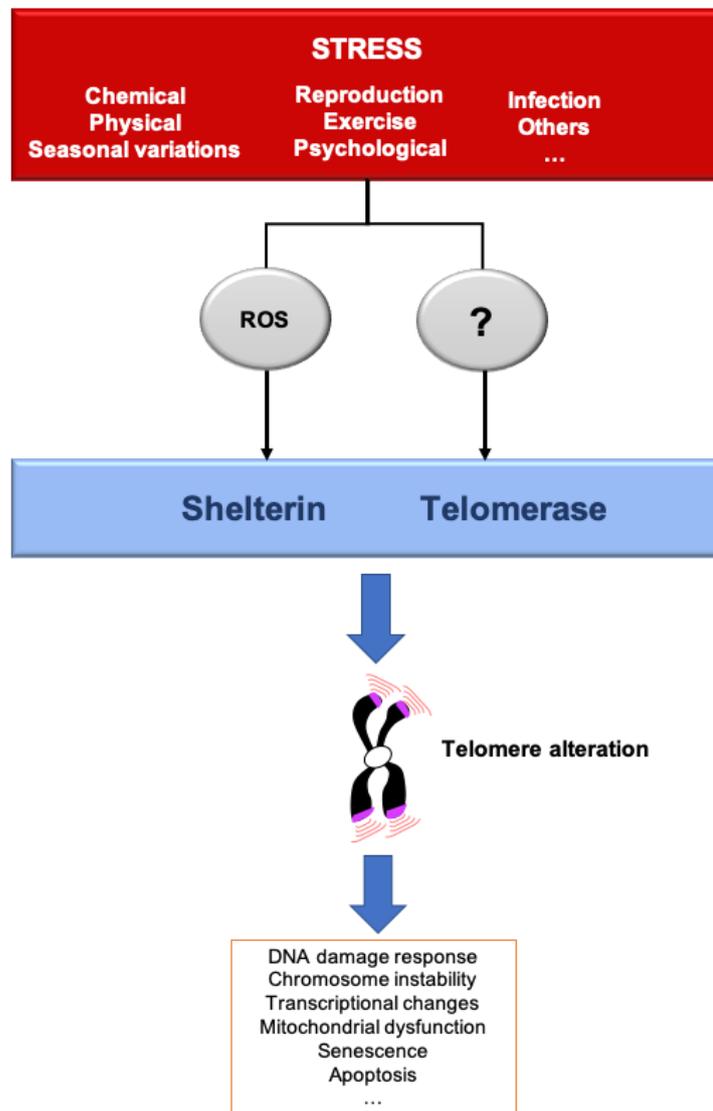


Figure 5. Stressors affect telomere homeostasis.

Figure taken from (Jacome Burbano and Gilson, 2021). Several stresses such as changes in pH or temperature, exercise, irradiation, pathogens infection, reproduction/breeding efforts, exposure to chemicals or psychological pressure, among others, can affect telomere integrity. The most described mechanisms induced by several of these stressors include telomerase inhibition and an increase of ROS, that preferentially oxidizes the telomeric DNA, a process that we dubbed teloxidation (Jacome Burbano et al., 2021a). Upon challenge, telomeric alterations induce the DNA damage response activation (DDR), senescence, transcriptional changes and mitochondrial dysfunction.

### 1.3.2 Life Factors

Under psychological stress, the Hypothalamic-Pituitary-Adrenal (HPA) axis secretes high levels of glucocorticoids (GC): cortisol and corticosterone. Their release allows an immediate response to stress, but chronic exposure may be detrimental (Monaghan and Spencer, 2014). GC block certain non-essential processes to store

energy during periods of stress (Hausmann and Marchetto, 2010) but are also associated with behavioral response. There are several examples of telomere changes due to psychological stress (Epel and Prather, 2018; Rentscher et al., 2020). For instance, TL shortens in children who experience a violent childhood (Shalev et al., 2013). Acute psychological stress also increases telomerase activity for short periods, correlated with cortisol/corticosterone levels in humans and rats (Beery et al., 2012; Epel et al., 2010). In fact, glucocorticoid accumulation seems to modify telomere homeostasis, including TL, although the mechanisms involved remain elusive (Angelier et al., 2018). One possible mechanism links psychological stress and oxidative stress, which is one of the consequences of GC activity (Costantini et al., 2011). Indeed, GC regulating genes and downstream pathways are involved in ROS production (Figure 5) (Hausmann and Marchetto, 2010) and/or mitochondrial metabolism (Casagrande et al., 2020). Highly stressed women have shorter TL, decreased telomerase activity and increased oxidative stress signatures (Epel et al., 2004). Thus, oxidative stress may be a mechanism for the effects of psychological stress and GC on telomeres, as it is for inflammation.

Other daily way of life decisions, such as diet, smoking and physical activity have been linked to telomeric changes. To give an example, the Mediterranean diet has been highlighted for its anti-aging capacities and cohort studies suggest that it deaccelerates telomere shortening in humans (Crous-Bou et al., 2014). Indeed, individuals suggested to a diet rich in fibers, vitamins and unsaturated fatty acids tend to have longer telomeres and lower oxidative stress. Caffeine consumption, another strong antioxidant agent, has also been correlated with longer leukocytes TL (Liu et al., 2016). Food can modulate TL but also telomeric protein state. For example, Chinese obese children had shorter TL but higher TERT promoter methylation (Liu et al., 2021). Additional to

food intake, several studies have analyzed an association between exercise and telomere length. To our knowledge, most of the published results show a positive correlation between TL and training, as compared to sedentary people (Arsenis et al., 2017; Brandao et al., 2020). Nevertheless, physical exercise might also lead to a certain level of telomere dysfunction: occasional intense exercise would tend to produce an excessive amount of ROS leading to higher DNA damage, and thus, telomere damage while low intensity but regular activity would lead to lower oxidative stress and better adaptation (He et al., 2016). Physical exercise can also modulate

telomere structure and function by increasing TERRA transcription (Chang et al., 2020; Diman et al., 2016).

### 1.3.3 Chemical Stress

Exposure to chemical stressors can greatly modulate the health of organisms. The stressors can be toxic/genotoxic compounds or pesticides and airborne pollutants. Several studies report an association between chemical exposure (benzene, polycyclic aromatic hydrocarbons, ethanol, etc.) and telomere attrition in human cells (Harpaz et al., 2018; Zhang et al., 2013). Ethanol triggers TL shortening in human fibroblasts, whereas in budding yeast it has a TL lengthening effect (Harpaz et al., 2018; Romano et al., 2013). In humans, the shortening effect of ethanol appears to be mediated by oxidative stress (Figure 5), mainly induced by alcohol dehydrogenase (Haorah et al., 2008; Harpaz et al., 2018). Nevertheless, zebra finches exposed to trace metal elements exhibit decreased TL with no change in oxidative stress (Saulnier et al., 2020). As well, bleomycin induces telomere instability and aberrations but it has not been determined whether it modifies TL (Al-Wahiby and Slijepcevic, 2005; Benkhaled et al., 2008; Bolzán, 2021). Overall, the effect of chemical stress on TL varies depending upon the nature of the chemical, the mode and duration of exposure and the organism exposed. The mechanisms appear to be quite diverse, but in at least some cases are mediated by oxidative stress.

### 1.3.4 Physical Stress

Physical stress is another source of external tension for telomeres. It is well known that genotoxic insult triggers persistent DNA damage at telomeres (Fumagalli et al., 2012a; Rochette and Brash, 2010). A particular case is UV irradiation, whose main photoproduct is cyclobutane pyrimidine dimers formed between two consecutive pyrimidine bases, which are often present in telomeric repeat DNA sequences. Thus, telomeres are a preferential target for UV-induced DNA damage (Rochette and Brash, 2010). If the telomeric photoproducts at telomeres remain unrepaired they can interfere with shelterin DNA binding, telomeric DNA replication and transcription, and thereby lead to TL shortening and uncapping (Fouquerel et al., 2019a). Indeed, UV exposure is associated with accelerated TL shortening (Ikeda et al., 2014; Ma et al., 2012; Stout and Blasco, 2013) and prevents TRF1 binding (Parikh et al., 2015). However, another

study did not observe increased TL shortening in human fibroblasts irradiated with UVB rays (Rochette and Brash, 2010). It is possible that the ability of telomeres to repair UV-induced photoproducts varies among cell types and organisms, which could be interpreted as an adaptive strategy of telomeres to repair UV lesions only when needed.

Recently, it was shown in astronaut leukocytes that long-duration spaceflights (6–12 months) lengthened telomeres, but that telomeres were rapidly shortened to a pre-spaceflight length within a few days after the astronauts returned to Earth (Garrett-Bakelman et al., 2019; Luxton et al., 2020). The authors postulated that these effects were caused by space radiation (Figure 5). Curiously, this telomere elongation might not be due to the canonical telomerase activity, which decreases during spaceflight, but to an increased rate of DNA damage and recombination that could favor ALT mechanisms. Indeed, astronaut cells exhibit elevated DNA damage and increased mitochondrial stress and ROS production. One interesting hypothesis is that oxidative stress activates the ALT pathway by triggering replication stress (Fouquerel et al., 2019b) and relocating telomerase to mitochondria (O’Sullivan et al., 2014).

As well, a counterintuitive TL lengthening was observed in human senior after 30 to 60 sessions of repeated exposure to hyperbaric oxygen therapy (HBOT) (Hachmo et al., 2020). B cells showed 37% TL lengthening after HBOT, but natural killers, T-helper and T-cytotoxic TL were also extended in a minor amplitude. Besides, the proportion of T-senescent cells was decreased after HBOT treatment (Hachmo et al., 2020). Thus, HBOT seems to have a senolytic effect playing a role on telomere length and senescent cells clearance.

Exposure to elevated temperatures is another environmental stress that could affect telomere dynamics. Indeed, in *Saccharomyces cerevisiae*, incubation at high temperatures shortens telomeres (Hachmo et al., 2020). Human cells that undergo heat shock accumulate TERRA and disrupt TRF2 from telomeres (Romano et al., 2013).

Besides, pH variation may be a cause of stress within an ecosystem. TL was observed to be reduced in HeLa cells cultured at low pH, but the heterogeneity of individual TL was narrow (Koskas et al., 2017; Petrova et al., 2014). The authors proposed that telomerase elongates short telomeres under low pH, while long telomeres are more strenuously eroded.

Finally, large-scale reef coral bleaching is a major ecological problem. Coral bleaching results in symbionts loss, due to harmful environmental conditions, specially driven by climate-changes, pollutants and other stressors. Coral bleaching can be reproduced in laboratory by housing corals in permanent darkness but normally fed. Recently, it has been published that mean telomeric DNA length and the frequency of long telomeres is reduced in the *Stylophora pistillata* reef coral after six months in permanent darkness (Rouan et al., 2021).

Overall, telomeres are conserved structures across the three of life. Although there are enormous advances on telomere structure and regulation understanding, one can anticipate that other functions and/or interactors might be discovered, as well as telomeric chromatin status and the presence/absence of telomeric proteins in different conditions. In order to protect genome stability, telomeres, their structures and proteins, need to regulate replication stalled forks, cap the chromosome termini to “hide” them from DDR machinery and control their shortening. Apart from the extremity of chromosome protection, the two other challenges are related to cell division. Therefore, I may ask, what is the status of telomeres in post-mitotic cells, such as neurons or skeletal muscle. Are telomeres regulated in a different manner during aging in post-mitotic cells?

## 2 Telomeres in long-lived post-mitotic cells aging

I have addressed the question whether telomeres have a role on long-lived post-mitotic cells aging in a bibliography work published as a review in *Mechanisms of Ageing and Development* in 2020, entitled “Long-lived post-mitotic cell aging: is a telomere clock at play?” (Jacome Burbano and Gilson, 2020). Therefore, this chapter will be based on my previous work structure and content but completed with recent bibliography and comments.

There is a growing consensus that cellular senescence is one of the aging basic processes. Cellular senescence can be defined as a permanent non-dividing cellular state triggered by numerous types of stress and certain physiological signals leading to the activation of the tumor suppressing pathways p53/p21 and p16<sup>INK4a</sup>/pRB, as well

as profound changes in cellular morphology, chromatin structure, metabolism and gene expression, including a senescence-associated secretory phenotype (SASP), the expression and secretion of inflammatory cytokines, growth factors, proteases and other molecules (Gorgoulis et al., 2019). Cellular senescence is distinct from other non-dividing cellular states such as the quiescence that is a reversible absence of proliferation and the terminal differentiation corresponding to the acquisition of a functional specialization by cells that are often irreversibly cell-cycle arrested and therefore named post-mitotic.

An emerging paradigm stipulates that the accumulation of senescent cells is a major contributor to physiological and pathological aging and that their elimination improves health (Anderson et al., 2019; Baker et al., 2011, 2016). Both dividing and non-dividing cells can enter into senescence in response to stress (Anderson et al., 2019; Farr et al., 2016; Jurk et al., 2014; Ohashi et al., 2018; Oubaha et al., 2016; Victorelli et al., 2019) (Figure 6), although most of our knowledge on cellular senescence stems from dividing cells.

As described above, cellular senescence and aging have been essentially described in replicative cells. However, another type of aging process at the cellular level concerns terminally differentiated long-lived post-mitotic cells (LLPMCs), such as myofibers and neurons, whose function can decline with age with no overt sign of cellular senescence. For instance, aging neurons progressively show a decline in axonal transport, focal accumulations of cytoplasmic and membrane proteins, synaptic transmission alteration, impaired synaptic plasticity, and altered calcium homeostasis (Mattson and Arumugam, 2018; Milde et al., 2015; Vagnoni and Bullock, 2018; Vagnoni et al., 2016), which correlates with profound transcriptional changes (Mertens et al., 2015). Also, aging myofibers exhibit progressive dismantlement of neuromuscular synapses, alterations in muscle-specific transcriptional mechanisms, and the progressive loss of the most powerful fast fibers (Fry et al., 2015).

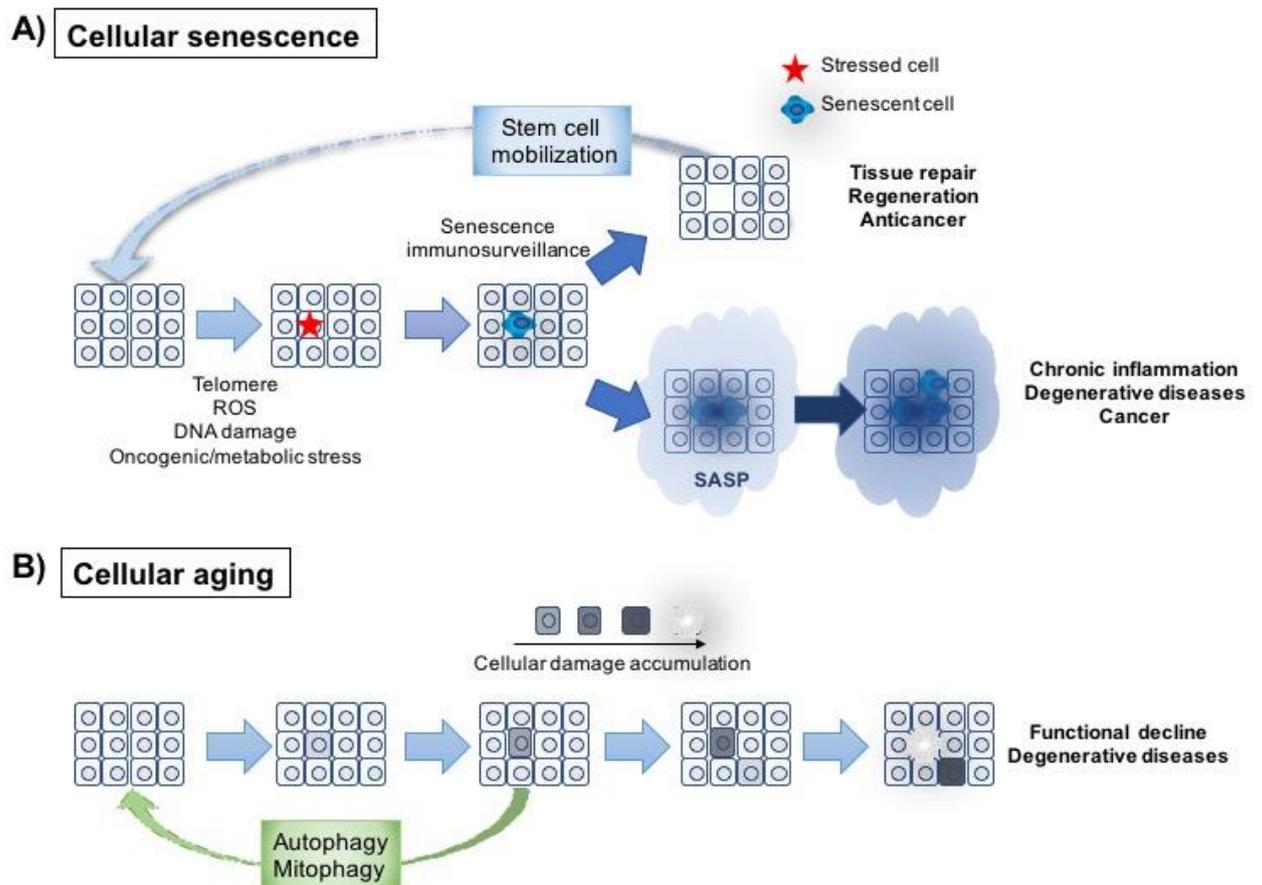


Figure 6. Two mechanisms of cellular aging.

A) Over time, cells are exposed to different types of stress, leading to cellular senescence. The immune system is mobilized to eliminate senescent cells. In order to fill empty spaces left by eliminated senescent cells, stem cells must undergo differentiation to preserve tissue integrity. If senescent cells escape to immunosurveillance and accumulate in tissues, a chronic pro-inflammatory environment is created through SASP. B) In the course of time, the function of long-lived post-mitotic cell (LLPMC) can progressively decline without developing a clear senescence phenotype, most likely because of the progressive inability of these cells to repair and renew damaged molecules. Image taken from (Jacome Burbano and Gilson, 2020)

If stem cell division can be considered a rejuvenation mechanism, the situation is different for LLPMCs, which mostly rely on their intracellular capacities of repair and renewal (e.g., autophagy and mitophagy). It is generally accepted that an increase in reactive oxygen species (ROS) production by dysfunctional mitochondria and the age-dependent decrease in the efficiency of the cellular mechanisms renewing, removing and repairing damaged molecules are responsible for the functional decline observed in LLPMCs during aging (Bua et al., 2006; Fritzen et al., 2016; Kraysberg et al., 2006;

Linnane et al., 1989; Loos et al., 2017; Rubinsztein et al., 2011; Sakuma et al., 2016; Terman et al., 2010; von Zglinicki et al., 2021) (Figure 6B). However, the cascade of age-related events that triggers these changes and its link to LLPMC senescence is unclear.

## 2.1 Skeletal muscle, a model of LLPMC

Skeletal muscle is mainly composed by long-lived post-mitotic myofibers, and it is considered as an example of post-mitotic tissue.

More than 600 skeletal muscles have been counted in the human body. It is the main tissue among the whole muscular mass that also includes cardiac and smooth muscle (Chal and Pourquié, 2017). Skeletal muscles are responsible of volunteer movement and represents around 40% of our body mass. Therefore, its maintenance and good health are critical for daily performance and mobility (Tieland et al., 2018). Although the muscular aging symptoms appear at old age, loss of skeletal muscle mass is detected starting approximately at 40 years old. This process is known as sarcopenia, and it might encompass 30 to 50% of skeletal muscle loss, but also a decline on muscle strength, leading to a decrease in physical activity (sedentary life) and could be a major impediment to accomplish daily life simple tasks or carrying weight. Altogether, sarcopenia represents an increase of frailty and co-morbidity risk. Hence, it is a matter of public health to enhance old people healthy aging and quality of life.

### 2.1.1 Skeletal muscle development and structure

In this section, I will briefly discuss about skeletal muscle formation and its main structure. Information collected here does not go in deep detail. However, it is important to mention that muscle development involves many factors and regulating pathways, some of them remain unknown.

Each step of skeletal muscle development and formation is composed by the emergence of new specifically differentiated cells from fetal stem cell to adult myofiber, with unique markers. Interestingly, during development there are two sequential phases of myogenesis (E10.5-E12.5 and E14.5-E17.5 in mouse embryo) (Biressi et al., 2007). Fetal stem cell are positive for Pax7, Pax3 and Myf5 (Ott et al., 1991). During

first myogenesis, primary fibers or myocytes are formed by muscle cell fusion and expresses specific markers such as Myog, MyoD, desmin, embryonic MyHC (Myosin Heavy Chain), skeletal  $\alpha$ -actin *Acta1* (or *HSA*) (Figure 7) (Babai et al., 1990; Biressi et al., 2007; Murphy and Kardon, 2011; Ontell et al., 1993). Then, another set of myoblasts differentiate during the second myogenesis phase, progenitor cells fuse with each other and with the primary fiber to form myofibers. A pool of Pax7<sup>+</sup> embryonic progenitor cells remains during adulthood, it corresponds to satellite cells (Figure 7) (Snijders et al., 2015). Satellite cells were first observed in frog skeletal muscle by electron microscopy, and defined as “dormant” unidentified cells associated to myofibers (Mauro, 1961).

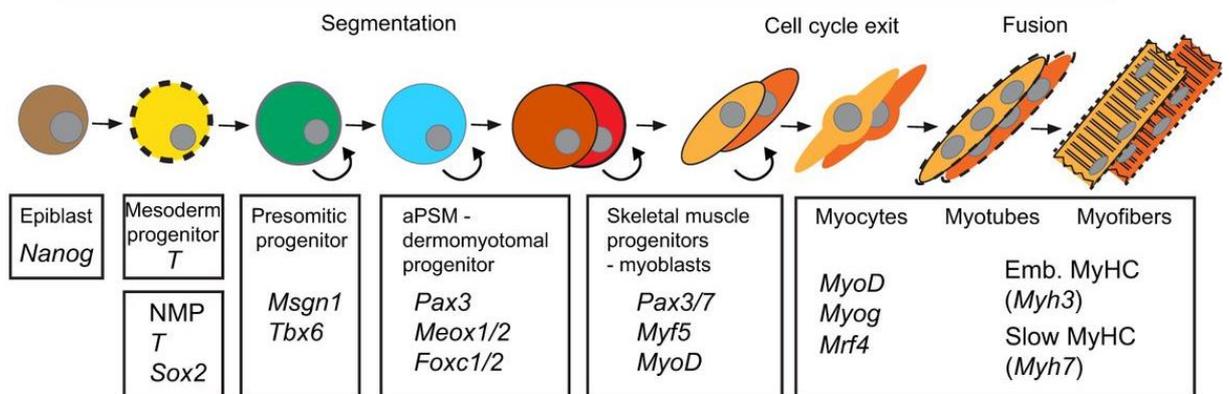


Figure 7: Differentiation from embryonic cell toward skeletal muscle.

At each differentiation step, characteristic cellular markers are shown. Image adapted from (Chal and Pourquié, 2017).

Next, the contractile structure of striated skeletal muscles are multinucleated myofibers, composed by cylindrical myofibrils ordered in an extremely organized structure called sarcomere. It consists of alternate thick myosin with thin actin bands. Each sarcomere structure is delimited by a Z-line at each side (Sweeney and Hammers, 2018) (Figure 8). Myosin heads “walk” through actin filaments by binding, extending and releasing them again in an ATP and Ca<sup>2+</sup> consuming process during muscle contraction (Lehman et al., 1994; Lorand, 1953).

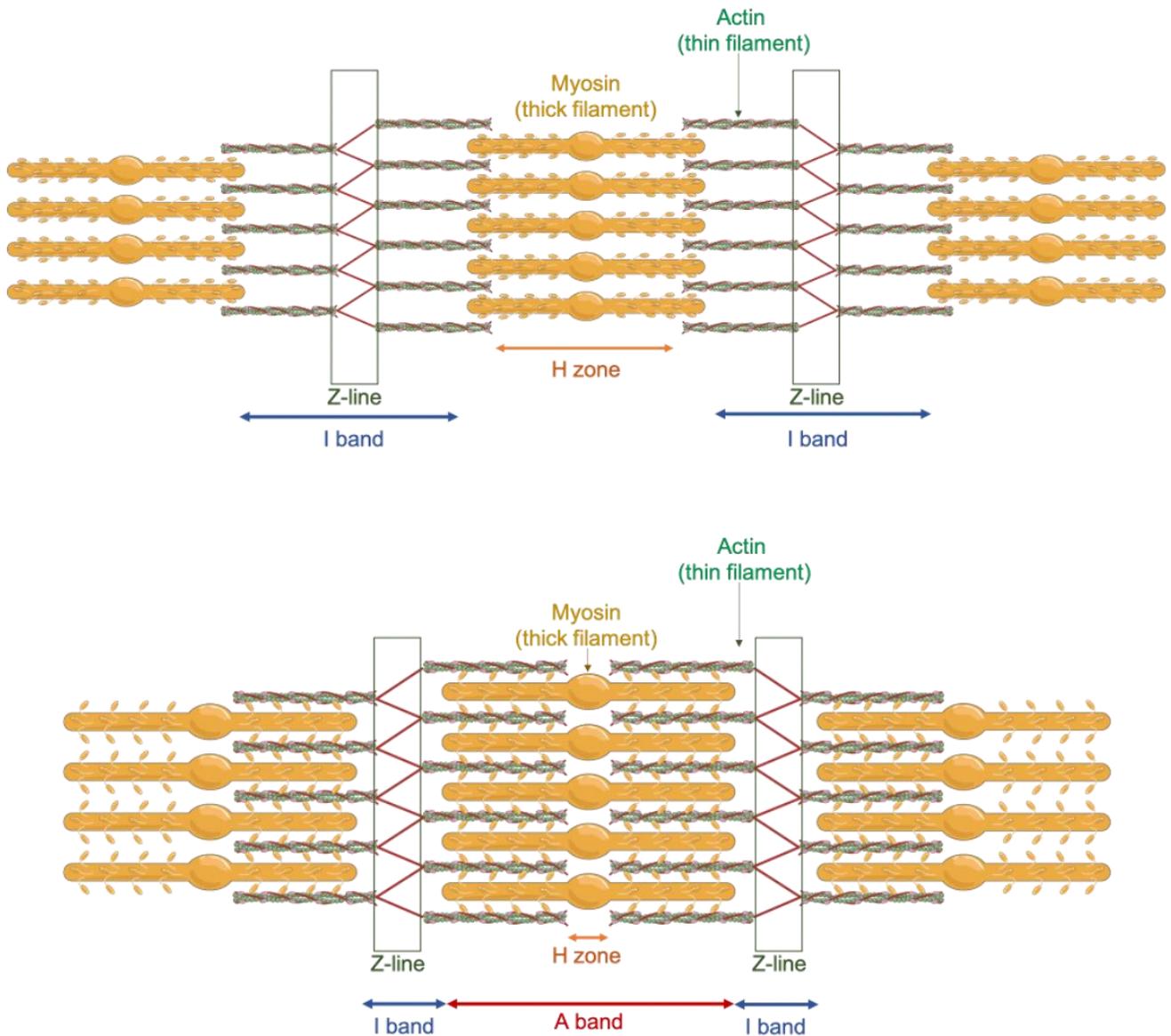


Figure 8: Sarcomere structure and skeletal muscle contraction.

Sarcomere is built by alternate thick myosin and thin actin filaments, delimited by Z lines. The H zone corresponds to myosin filament alone. The A band is the overlapped part between myosin and actin fibers. Finally, the I band is the part on actin fibers alone. Upper panel: relaxed muscle, large H zone and I bands. Lower panel: skeletal muscle contraction, myosin filaments slide along thin filaments using the head part of myosin protein by attaching, extending and relaxing.

Each myofiber is surrounded by the sarcolemma, a membrane that invaginates into the cell forming T-tubules (transverse tubules), responsible for action potential transmission. Nearby T-tubules, the skeletal muscle endoplasmic reticulum, known as sarcoplasmic reticulum, regulates calcium storage, release and uptake during



display a fast and powerful contraction response, but they rapidly reach muscle fatigue (a sprint). According to their function, skeletal muscles are composed by a mixture of type I and type II fibers. The percentage of them would define whether a muscle is oxidative or glycolytic. For example, in mice, *soleus* is an oxidative muscle composed essentially by type I fibers, *gastrocnemius* contains a mixture of type I and type II fibers, whereas *tibia anterioralis* mainly contains type II glycolytic fibers.

	<i>Type I</i>	<i>Type IIa</i>	<i>Type IIb</i>	<i>Type IIx</i>
<i>Metabolism</i>	Oxidative	Glycolitic	Glycolitic	Glycolitic
<i>Contraction time</i>	Slow	Fast	Very fast	Fast
<i>Power strength</i>	Low	Medium	High	High
<i>Fatigue resistance</i>	High	Intermediate	Low	Intermediate
<i>Myosin heavy chain (Human)</i>	MYH7	MYH2	MYH4	MYH1

Table 1. Skeletal muscle fiber types

It is noteworthy that skeletal muscle is not only composed by mature myofibers but also by satellite cells, endothelial, nervous, immune cells and can be infiltrated by adipocytes. Satellite cells are localized at the between the external periphery of sarcolemma and the basal lamina. Among satellite cells there are Pax7<sup>low</sup> cells that are prone to enter cell cycle and Pax7<sup>high</sup> cells that remains in a quiescent state (Rocheteau et al., 2012).

### 2.1.2 Skeletal muscle regeneration and aging

Although skeletal muscle is a post-mitotic tissue, it has a considerable regeneration capacity upon injury, that relies principally on satellite cells (Renault et al., 2002a). The regeneration process is divided in four overlapping and precise steps: degeneration, inflammation, regeneration and remodeling repair (Barberi et al., 2013;

Tidball and Villalta, 2010). In brief, after injury, an abundant amount of calcium becomes extracellular, rapidly targeting mitochondria and sarcoplasmic reticulum (Oberc and Engel, 1977). Subsequently, these two organelles impair proteostasis leading to myofiber necrosis. Then, immune cells, especially neutrophils and M1/M2 macrophages invade muscle, in a time-dependent order, inducing inflammation (Barberi et al., 2013; Domingues-Faria et al., 2016). Next, the regeneration process starts with satellite cell activation and differentiation. Myonuclei involved in this regeneration step, are issued from activated satellite cells and exhibit a central position within myofibers, instead of the classic peripheral myonuclei position (Roman and Gomes, 2018). Importantly, there are evidence in mouse that the central nuclei resulting from a regeneration process are still observed long time after injury (94 weeks) (Meyer, 2018), suggesting that not all nuclei activated during regeneration reach a final peripheral position. Besides, regenerating myofibers express embryonic MyHC (Guiraud et al., 2019). Finally, during the remodeling part, repaired myofiber becomes mature and nervous contractile system is set up by re-innervation (Barberi et al., 2013; Bodine-Fowler, 1994). It is important to note that other cells, mesenchymal stem cells (MSCs), an adult multipotent stem cell population have also been observed on muscle structure and regeneration (Cooper et al., 2006). It has mostly been studied in rodents, that bone-marrow MSCs are capable of myogenic conversion (Corbel et al., 2003; Ferrari et al., 1998; Gussoni et al., 1999; LaBarge and Blau, 2002) but more recently, MSCs from blood vessel wall showed myogenic capacity and are involved in injured muscle regeneration (Lorant et al., 2018). Although it is a groundbreaking field, further research is still required to establish a stem cell therapy to prevent muscle wasting in humans.

A decline on skeletal muscle regeneration and function is observed in humans starting from 40 years old, being evidence of skeletal muscle aging, along with reduced myofiber number and increased atrophy (Lexell et al., 1988; McCormick and Vasilaki, 2018). It is not clear whether skeletal muscle aging in humans is rather a consequence of fiber atrophy than fiber loss (Wilkinson et al., 2018). Sarcopenia is caused by a numerous of processes which includes increased inflammation, fat infiltration, apoptosis, DNA damage, ROS and advanced glycosylated end products (AGEs) accumulation, protein degradation and mitochondrial dysfunction, motor unit reorganization and satellite cells reduced number and lower activation (Barberi et al.,

2013; Chakkalakal et al., 2012; Etienne et al., 2020; Kadi et al., 2004; Larsson et al., 2019; McCormick and Vasilaki, 2018; Renault et al., 2002a; Verdijk et al., 2014).

These diverse processes are interconnected one to each other, and thus, it is difficult to determine which one could be the initial triggering cause of skeletal muscle aging. For instance, it has been shown that loss of satellite cells in mice muscle induces neuromuscular junction degeneration, favoring myofiber atrophy (Liu et al., 2017). It has been also proposed, in mouse and rats, that denervation is an early stage of the skeletal muscle aging, preceding myofiber atrophy (Deschenes et al., 2010; Sheth et al., 2018). Noteworthy, not all skeletal muscle age equally : type II fast-twitch fibers are more prone to atrophy than type I fibers (Lexell et al., 1988; Verdijk et al., 2014). Besides, satellite cells loss is greater at young age in muscle composed mostly by fast fibers (Shefer et al., 2006; Verdijk et al., 2014). Extensor digitorum longus (EDL) displayed a reduced satellite cell number in adult mice (1 year old), whereas in soleus the severe satellite cells loss appears at geriatric age (2,5 years old) (Shefer et al., 2006). However, satellite cells depletion did not affect mice muscle mass or cross-area at different ages, suggesting that age-dependent loss of satellite cells does not directly affects sarcopenia but higher fibrosis was detected (Fry et al., 2015, 2017; Keefe et al., 2015). Skeletal muscle aging is accompanied by changes in the extracellular matrix (ECM), resulting in higher stiffness. This, along with environmental changes might perturb ECM interactions with satellite cells, promoting their reduced proliferation, decreased number and affecting skeletal muscle balance during aging (Boers et al., 2018; Franco et al., 2019; Fry et al., 2017; Thorley et al., 2015).

Although there are some insights on myonuclei aging, the mechanisms remain elusive. Whether myonuclei are eliminated and replace during lifetime, maybe by satellite cells renewing time to time myofiber nuclei, is unclear. Evidence in mice and in tobacco hawkmoth *Manduca sexta* suggest that myonuclei do not undergo apoptosis and are kept despite myofiber atrophy (Bruusgaard et al., 2010; Schwartz, 2019; Schwartz et al., 2016). Besides, within mouse myofibers the number of myonuclei decreases with age, their distribution pattern and shape are not uniform compared to young myonuclei (Bruusgaard et al., 2006). Recently, using a single nucleus RNA-sequencing, it has been found an age-specific myonuclear population, expressing *Nr4a3*, *Ampd3*, and *Enah*, in 30 months old mice, it evidences for a disruption on transcriptional uniformity observed in younger mice (Petraný et al., 2020). Further

studies are required to determine myonuclei specific aging and understand its mechanisms in human skeletal muscle.

Myonuclear turnover is extremely slow and has been estimated at approximately 15 years (Spalding et al., 2005). Therefore, one might ask whether satellite cells are required for healthy skeletal muscle maintenance, in absence of injury or perturbation. Is there myonuclei replacement during aging in an injury-independent manner? For instance, research projects using labeled Pax7<sup>+</sup> cells (total population or using brainbow reporter to follow satellite cells lineage) determine that satellite cells contributed by providing new myonuclei in different mouse muscles during adulthood, even if there was any muscle perturbation (Keefe et al., 2015; Tierney et al., 2018). Hence, the satellite cell pool seems to be sufficient for mouse lifetime, even at old age, under healthy aging condition. Nonetheless, if there are repeated episodes on injury/regeneration, this pool might be drained (Franco et al., 2019).

Moreover, it has been recently observed that human satellite cells undergo around 13 somatic mutations per year. Mutation accumulation increases with time and could affect muscle stem cell functions (Franco et al., 2018). Importantly, during aging, these mutations might be propagated into myofibers resulting in damaged skeletal muscle activity, exacerbating sarcopenia.

Additionally, methylation might be an interesting process in skeletal muscle homeostasis as there are differences regarding the methylation profiles according to fiber-type, sex and aging (Bigot et al., 2015; Davegårdh et al., 2019; Liu et al., 2013; Oe et al., 2021; Thorley et al., 2015). Indeed, DNA and histone methylation increases during aging in human and mouse satellite cells, resulting in worsen capacity to undergo and maintain quiescence (Bigot et al., 2015; Liu et al., 2013). Activated satellite cells undergo an asymmetrical cell division leading to cells that enter differentiation and a proportion of quiescent Pax7<sup>+</sup> satellite cells. Age-dependent amplified methylation downregulates quiescence pathways, forces their differentiation, reducing the reserve satellite-cell pool.

Besides, satellite cells are the mitotic component of skeletal muscles. Thus, one can imagine they are affected by replicative senescence. Do satellite cells senesce?

Whereas some studies found any or little signs of satellite cells senescence (Bigot et al., 2015), others found morphological changes (larger and flatter), altered proliferation capacity and telomere shortening (Decary et al., 1997; Kadi and Ponsot, 2010; Mouly et al., 2005; Wernig et al., 2005). Satellite cells isolated from young donors (newborns, 5 months old children) reach higher population doubling than those isolated from adults (young to old adults: 26 to 86 years old), showing that young cells have a better proliferative capacity. There are opposite opinions regarding telomere length during satellite cells aging. Some results revealed any difference (Renault et al., 2002b; Tichy et al., 2017), whereas others suggest a telomere decreased length corresponding to donor age or population doubling (Decary et al., 1997; Mouly et al., 2005; Wernig et al., 2005). Moreover, satellite cells from old mice or humans showed an increased expression of the senescence markers *p16<sup>INK4a</sup>* and *p21* (Carlson et al., 2009; Sousa-Victor et al., 2014; Suetta et al., 2013).

Overall, satellite cells seem to undergo a classical senescence process, observed by proliferation arrest but we can imagine that it is also accompanied by metabolic, transcriptional, stress response changes. Does senescence-mediated decline satellite cell function affect myogenic differentiation? For instance, old satellite cells do not compromise myotube differentiation. However, at old ages the satellite cell pool and proliferation capacity are reduced. The ratio of nuclei in multinucleated fibers compared to the total number of nuclei (fusion index) was equivalent in differentiation promoted by satellite cells isolated from middle-age or old adults (Mouly et al., 2005). Nevertheless, old myotubes contain less myonuclei, but the fusion index is compensated because the total number of nuclei is also lower.

Skeletal muscle is also a secretory organ producing myokines, namely cytokines and proteins secreted by myofibers (Saini et al., 2021). Myokines have autocrine, paracrine and endocrine effects, regulating muscle mass and function but also influencing other organs: brain, liver. For instance, over 3000 myokines have been described, among them approximately 600 are produced by human myocytes (Lee and Jun, 2019). The first discovered myokine is myostatin, a TGF $\beta$  family member, that inhibits myogenesis and muscle growth (McPherron et al., 1997). Myostatin secretion is inhibited during physical exercise and increased during aging (Mancinelli et al., 2021). Importantly, just a minority of myokines have been studied, among them there

are FGF21, IL-6, IL-15, irisin and BDNF (Lee and Jun, 2019; Mancinelli et al., 2021). They have been implicated in skeletal muscle mass maintenance, fatty oxidation and mitochondrial biogenesis regulation (Lee and Jun, 2019; Quinn et al., 2013; Wang et al., 2016a). Some of them have beneficial effects on muscle growth and metabolism but others induce muscle atrophy, inflammation and fibrosis. Therefore, myokines might have a critical role on skeletal muscle aging but also influence other tissues aging. For instance, IL-6 and TNF $\alpha$  upregulation is associated with chronic inflammation during sarcopenia. Besides, leptin supplementation enhances aged mice skeletal muscle regenerative capacities (Han et al., 2018). Research on uncharacterized myokines and their secretion monitoring during lifetime might be a clue to understand systemic organismal aging and potential therapies.

Altogether, skeletal muscle aging mechanisms have not been fully understood. Most of these studies have been conducted on rodents and some in *Drosophila*, but data on human skeletal muscle aging is limited. Although its structure is well characterized, some factors involved during aging, such as telomere homeostasis, have been disregarded due to myofiber post-mitotic status. Therefore, the mechanisms of skeletal muscle aging and regeneration are still largely elusive.

### 2.1.3 Telomeres and muscular-related diseases

Telomeres have been associated to several diseases, particularly in premature-aging disorders, such as Cockayne Syndrome or Hutchinson-Gilford Progeria Syndrome (Turner et al., 2019). Nevertheless, telomeres, and more precisely telomere length has been described as an important factor in other diseases exhibiting muscular dysfunctions (Chang et al., 2016; Robin et al., 2015; Sacco et al., 2010; Stadler et al., 2013a; Tichy et al., 2017).

First, Fascioscapulohumeral muscular dystrophy (FSHD) is a genetic disorder associated with rearrangements in the telomere proximal locus 4q35. It has been linked to a decreased number of the D4Z4-array, less than 11 copies whereas in healthy patients there up to 150 (Stadler et al., 2013a). D4Z4 encodes *DUX4* gene, that is overexpressed in FSHD muscular cells with short telomeres, suggesting a role of telomeres on *DUX4* expression control (Stadler et al., 2013a, 2013b). Whereas the exact causes of FSHD onset are still in discussion, *DUX4* is considered as a potential

candidate. FSHD patients have progressive increasing muscle atrophy, frailty and muscle degeneration. A long-range chromatin loop has been described between telomeres and the *SORBS2* (Sorbin and SH3 domain-containing protein 2) gene locus in normal myoblasts or in FSHD myoblasts with long telomeres but it is lost in FSHD myoblasts with short telomeres (Robin et al., 2015). The loss of this chromatin loop correlated with higher *SORBS2* expression and with D4Z4 hypomethylation was associated with the FSHD phenotype. *SORBS2* expression might be regulated by a Telomere Position Effect-Over Long Distances (TPE-OLD, over 10 Mb) (Robin et al., 2015). *SORBS2* is normally expressed in differentiated healthy myotubes. Thus, authors postulated that the dismantlement of this telomeric long-range chromatin loop forces FSHD myoblasts with short telomeres to undergo differentiation. Therefore, due to the D4Z4 array localization and telomere length dependent *DUX4* and *SORBS2* expression in FSHD myoblasts, telomeres are a central factor to understand this muscular dystrophy.

Second, the most studied muscular disorder is Duchenne muscular Dystrophy (DMD). This disease is a X-linked recessive pathology, induced by a mutation in the gene coding for *dystrophin*, resulting in progressive muscle degeneration and cardiac hypertrophy, starting during childhood (2-3 years old). A pioneer study determined that in DMD patients telomeres in muscle cells shorten rapidly, compared to aged matched healthy controls (Decary et al., 2000). This telomere accelerated erosion was also observed in another muscular dystrophy: limb girdle muscular dystrophy (LGMD 2C) (Decary et al., 2000). These findings supported the idea that at very young age, skeletal muscle from patients carrying these dystrophies undergo repeated regeneration events, trying to compensate degeneration. It results in muscle stem cell greater proliferation and probably premature replicative senescence due to telomere shortening. The telomere shortening in DMD muscle was confirmed in a single cell scale, focusing in muscular stem cells from mice and DMD teenager patients (Tichy et al., 2017). In contrast, any TL variation was detected in young vs old muscle stem cells from control mice. Strikingly, the dystrophin deficient mouse model: *mdx*, exhibits a mild degenerative phenotype, that does not recapitulate human DMD symptoms, limiting its use for research (Bulfield et al., 1984). Therefore, it was hypothesized that telomerase expression in mice should attenuate the *mdx* mice dystrophy symptoms. Indeed, telomerase is expressed in mouse somatic tissues, thus, it could limit telomere shortening. However in human DMD, previous research detailed above, suggested

that telomere shortening in muscular stem cells might prevent their damage repair/regenerative function by limited proliferation and reduced stem cells pool (Decary et al., 2000). Hence, a mdx mice lacking telomerase (mdx/ mTR) was created and recapitulates the severe human DMD symptoms: progressive muscular dystrophy, cardiomyopathy, shorter telomeres and impaired muscular stem cells proliferation (Chang et al., 2016; Sacco et al., 2010). Moreover, telomere shortening in DMD was associated with TRF1 overexpression (Aguennouz et al., 2011). Interestingly, there is a proliferation-independent progressive telomere shortening in cardiomyocytes from mdx/ mTR<sup>G2</sup>, due to DNA damage response and p53 activation, along with p21 overexpression, PGC1  $\alpha/\beta$  repression leading to dysfunctional mitochondrial biogenesis (Chang et al., 2016).

Altogether, these findings importantly highlight a link between telomeres and muscle homeostasis. Could these progressive degenerative phenotypes be considered as a premature muscular aging?

## 2.2 Telomeric DNA length changes during LLPMC aging

Among the known clocks of aging, only the unrelenting telomeric DNA (TL) shortening is a developmental process that can act as a driver of senescence in dividing cells (Han et al., 2018). The “clock” starts ticking when telomerase is shut down during mid-embryogenesis in most cells of the human body. Therefore, telomeric DNA becomes progressively shorter upon each round of somatic replication, ultimately leading to replicative senescence. Hence, it is believed that telomeric DNA shortening, by nature linked to cell division, is not involved in LLPMC aging.

When age-related TL shortening is accelerated, for instance in rare progeroid syndromes such as Dyskeratosis congenita (Armanios and Blackburn, 2012) but also due to life-style in normal individuals (Ornish et al., 2013; Vyas et al., 2021), It is associated with a broad spectrum of age-related pathologies, including cardiovascular and neurodegenerative diseases, type II diabetes, pulmonary fibrosis, and arthrosis (Martínez and Blasco, 2017; Turner et al., 2019). Thus, it is key to understand how age-related telomere changes contribute to normal and pathological aging.

In this part, I discuss recent reports unveiling telomere changes during LLPMC senescence and aging as well as their links to mitochondrial dysfunction and cell

differentiation. These findings invite us to study the roles for telomeres in LLPMC function and to consider the hypothesis that a telomere clock is at play during development and aging in both dividing and non-dividing cell.

In agreement with the view that TL shortening is intrinsically linked to cell division and therefore mainly concerns tissues with a high level of cell renewal, TL was shown to decrease in peripheral blood leukocytes and no TL change was detected during the aging of low-proliferative tissues, which are mostly composed of LLPDCs, such as skeletal muscle (Decary et al., 2000; Hiam et al., 2020; Ponsot et al., 2008; Renault et al., 2002a) and brain. Accordingly, telomeric DNA is longer in low as compared to highly proliferative tissues (Daniali et al., 2013).

However, this view is challenged by recent reports of age-dependent TL shortening in low proliferative tissues. Carneiro *et al.* showed that TL decreases in several zebrafish tissues, including muscle, but at different attrition rates (Carneiro et al., 2016). A decrease in TL was also observed during the aging of various mouse low-proliferative cell types and tissues, such as the retina (Baek et al., 2019), brain (Ain et al., 2018) and cardiomyocytes (Aix et al., 2016; Anderson et al., 2019). Also in human samples, TL was reported to shorten in adipocytes (Monickaraj et al., 2012, 2013) and in aging fat and skeletal muscle tissues (Chahine et al., 2019; Daniali et al., 2013; Magi et al., 2018; Robin et al., 2020) as well as in cerebral white matter (Nakamura et al., 2007), cortex and hippocampus (Demanelis et al., 2020). There is also evidence of TL shortening in mouse and human oocytes (Ge et al., 2016b; Uysal et al., 2021; Yamada-Fukunaga et al., 2013).

How to explain these seemingly disparate results regarding TL dynamics in post-mitotic tissues?

First, most of these studies were performed at the whole-tissue level, making it difficult to distinguish the TL of LLPDC from those of proliferative cells present in these tissues. For instance, in mammalian cardiac tissues, whereas cardiomyocytes occupy 70-85% of heart volume, they represent only 30-40% of the cardiac cells (Zhou and Pu, 2016). Therefore, the important TL shortening observed in Wistar rat heart during aging, could not be directly attribute to a TL erosion in cardiomyocytes (Hastings et al., 2004).

Second, various methods are used to determine TL (telo-quantitative PCR (telo-qPCR), Terminal Restriction Fragment (TRF) analysis, slot blotting, and quantitative

fluorescence *in situ* hybridization (qFISH)), and more recently Luminex-based assay (Kibriya et al., 2014), which might account for experimental differences.

Third, in many tissues, LLPMC can be renewed, even at a low rate, from resident progenitor or stem cells (see e.g.,(Spalding et al., 2013)), in particular upon injury or disease (Decary et al., 1997).

Nevertheless, if there is no definitive evidence that the TL of LLPMCs decreases over their lifetime, these various publications (summarized in Table 2) invite us to consider the existence of age-regulated telomere maintenance-mechanisms specific to LLPMCs. In agreement with this view, yeast telomeres undergo a profound reorganization when the cells enter into quiescence (Guidi et al., 2015; Maestroni et al., 2017, 2020). Moreover, as described below, our knowledge of the mechanisms of telomere maintenance suggests that some replication-independent scenarios could lead to TL shortening in LLPMCs (Figure 10).

Post-mitotic tissues/ cells	Organism	TL measurement	TL variations	References	
Skeletal muscle	Zebrafish	TRF	Shortening	(Lee and Jun, 2019; Quinn et al., 2013; Wang et al., 2016)	
	Mouse	telo-qPCR	TL shortening in isolated muscle fibers exposed to oxidative stress	(Ludlow et al., 2014)	
			TL shortening during chronological aging	(Baek et al., 2019)	
	Human	TRF	Decrease in short TL	(Decary et al., 1997)	
			TL shortening	(Daniali et al., 2013)	
			TL shortening in healthy adults	(Magi et al., 2018)	
			TL shortening in healthy adults	(Chahine et al., 2019)	
		Slot Blot	TL shortening	(Robin et al., 2020)	
	Brain	Mouse	telo-qPCR	Hippocampus TL shortening during aging	(Baek et al., 2019)
			telo-qPCR and qFISH	Neuron TL decreases in a cell cycle independent manner during aging	(Ain et al., 2018)
Human		TRF	TL shortening in cerebral white matter in Japanese adults up to 70 years old	(Nakamura et al., 2007)	
		Luminex- based method (GTEX donors)	TL shortening in the cortex and hippocampus. No TL variation in cerebellum	(Demanelis et al., 2020)	
Cardiomyocytes	Mouse	qFISH	TL decreases rapidly after birth triggering telomere dysfunction and cell cycle arrest	(Aix et al., 2016)	

			Significant TL shortening in 30 vs 4 months old mice	(Anderson et al., 2019)
Oocytes	Mouse	telo-qPCR and qFISH	TL shortening	(Yamada-Fukunaga et al., 2013) (Ge et al., 2019)
	Human	qFISH	Decrease telomere intensities in oocyte and other ovarian cells in pre and postmenopausal vs fetal-newborn samples	(Uysal et al., 2021)
Retina	Mouse	telo-qPCR	Decrease in TL during mouse aging	(Baek et al., 2019)
Adipocytes/ Fat	Human	telo-qPCR	TL is shorter in subcutaneous and visceral fat of obese and type 2 diabetes patients	(Monickaraj et al., 2012)
			TL decreases in adipocytes exposed to oxidative stress	(Monickaraj et al., 2013)
		TRF	TL shortening in subcutaneous fat	(Daniali et al., 2013)

Table 2. Telomere shortening during LLPMC aging.

Various methods are broadly used to measure TL: Terminal Restriction Fragment (TRF), Quantitative Fluorescence in situ Hybridization (qFISH), telomere quantitative Polymerase Chain Reaction (telo-qPCR) and Slot blotting. Adapted and completed from Jacome Burbano MS, Gilson E, 2020 (Jacome Burbano and Gilson, 2020)

DMD: Duchenne Muscular Dystrophy

### 2.2.1 Oxidative damage

It is established that an increase in oxidative stress can lead to TL shortening in dividing cells by interfering with telomere DNA replication and telomerase activity (Barnes et al., 2019b). The fact that isolated skeletal muscle fibers (Ludlow et al., 2014) and adipocytes (Monickaraj et al., 2013) display telomere shortening upon oxidative stress suggests the existence of a replication-independent, oxidative stress-dependent mechanism of TL shortening. Because the main DNA damage caused by oxidative stress is 8-oxoguanosine (8-oxoG), the G-richness of telomeres makes them a preferential target for oxidative lesions (Ahmed and Lingner, 2018; Fouquerel et al., 2016, 2019c; Opresko et al., 2005). Several replication-independent events caused by the presence of 8-oxoG at telomeres could lead to TL shortening. First, oxidative damage can disrupt TRF2 binding to telomeric DNA (Ahmed and Lingner, 2018; Fouquerel et al., 2019c; Opresko et al., 2005) which is expected to relax T-loop formation, rendering the telomere more sensitive to degradation (see below). Second, repair by the initiation of base excision repair (BER) by the OGG1 DNA glycosylase can introduce a nick, which could result in creation of a double-strand break (DSB) by a single-strand nuclease, thus fragmenting the distal part of telomeres. An alternative possibility is the formation of 8-oxoG on both strands in the immediate subtelomeric region as a result of the presence of degenerate telomeric DNA repeats leading to nearby nicks on both strands, resulting in DSB (Figure 10). Interestingly, it was recently shown that telomeric DSBs caused by oxidative damage are suppressed by increased formation of three-stranded structures containing a DNA:TERRA hybrid and a displaced DNA strand (telomeric R-loop) in a TRF2-dependent manner (Tan et al., 2020). It is possible that downregulation of TRF2 during LLPMC aging (Robin et al., 2020; Wagner et al., 2017) favors DSB formation upon increased oxidative damage via a decreased rate of telomeric R-loop formation.

To determine precisely how oxidative damage can shorten TL in LLPMC requires further studies (Trajano et al., 2018).

### 2.2.2 Telomere degradation

One of the main functions of telomeres is to protect chromosomal DNA termini from unwanted nuclease action while controlling the formation of the limited 3'-overhang necessary for t-loop formation and telomerase action. This is based on a complex process during the cell cycle involving the shelterin subunits POT1 and TRF2, the TRF2-associated factor Apollo, as well as various types of DNA repair enzymes, nucleases, recombinases and replication factors (Lam et al., 2010; Wu et al., 2012b; Ye et al., 2010b). In particular, the telomeres of yeast and mammalian cells are capable of preventing extensive 5' resection by the Exo1 exonuclease (Figure 4) (Garvik et al., 1995; Ngo and Lydall, 2010; Wu et al., 2012b). These mechanisms were studied in dividing cells and described in the first chapter of this manuscript. It is possible that some of the actors controlling telomere DNA integrity become dysfunctional during LLPMC aging, promoting telomeric DNA degradation (Figure 10).

### 2.2.3 Telomere recombination

The t-loops could serve as intermediates for an intrachromatid deletion event leading to marked telomere shortening (Ancelin et al., 2002; Cesare and Reddel, 2008; Pickett et al., 2009; Wang et al., 2004). Indeed, the DNA strand-invasion formed at the basis of T-loops could promote the formation of Holiday junctions, which, if processed by a resolvase, can lead to t-loop excision and TL shortening. In normally protected telomeres, the access of resolvases to t-loop is restricted by TRF2 (Poulet et al., 2009; Saint-Léger et al., 2014; Schmutz et al., 2017). Therefore, the downregulation of TRF2 reported in LLPMCs (Robin et al., 2020; Wagner et al., 2017) or its displacement upon oxidative damage (see above) could favor t-loop excision and TL shortening (Figure 10).

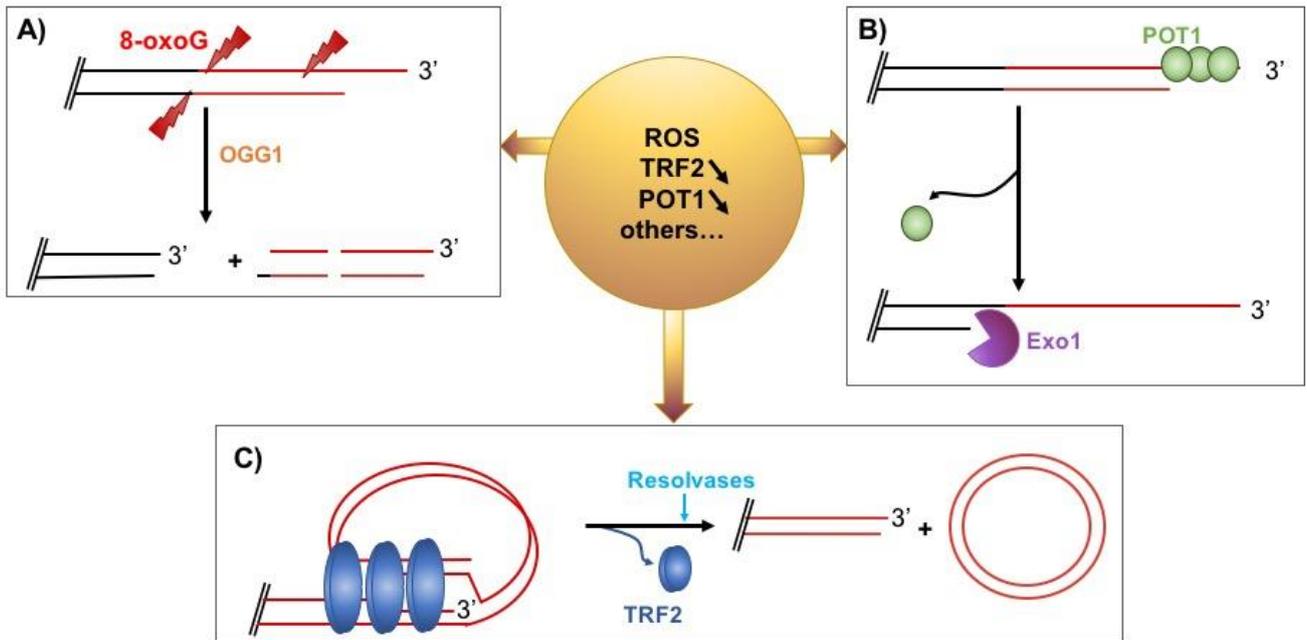


Figure 10: Possible mechanisms of TL shortening in LLPMC.

A) The OGG1 enzyme, in order to initiate the base excision repair, generates single-strand nicks at 8-oxoguanosine (8-oxoG) upon oxidative stress. These nicks can result in DSB by the action of a single-strand nuclease on the opposite strand or if they occur in proximity, in both strands. B) Telomeric factors, e.g., POT1, protect DNA extremities against nucleases. If over lifetime, the expression of these factors is downregulated, telomeric DNA can be degraded over a long distance by the action of exonucleases such as Exo1. C) TRF2 prevents resolvases to recombine the basis of T-loop leading to its excision. During aging, if TRF2 is downregulated, T-loop can be recombined leading to T-loop sized TL shortening. Taken from (Jacome Burbano and Gilson, 2020)

### 2.3 Changes in shelterin expression during LLPMC aging

Not only the TL changes during aging but also the expression of the shelterin protein that form the protective chromatin cap. Whether these telomeric chromatin changes are coordinated with TL shortening and their contribution to the aging phenotype are exciting questions for future studies.

The trend appears to be a general decrease in the expression of the shelterin subunits during aging in a tissue-specific manner. The first indication of such an age-related decrease was the finding that, during replicative senescence, the expression of TRF2 is downregulated in a p53-dependant manner via degradation by Siah1 (Fujita et al., 2010; Lototska et al., 2020). Whether this also occurs upon p53 activation in

LLPMC is unknown. In fact, few studies have investigated shelterin expression during aging in post-mitotic organs. In zebrafish, the mRNA levels of the genes encoding the six shelterin subunits decreases with age in various tissues, including brain and muscle (Wagner et al., 2017). Whether this decrease occurs in LLPMCs is unclear because, unlike humans, zebrafish have a considerable regenerative capacity in post-mitotic organs (Beffagna, 2019; Chablais and Jazwinska, 2012; Marques et al., 2019; Wan et al., 2012). In skeletal muscle, the TRF1 protein level decreases with age in mouse (Derevyanko et al., 2017). TRF1 overexpression in old mice rescued aging-related phenotypes such as cognitive and neuromuscular capacities. In human biopsies from individuals of different ages, I contributed to a work showing that, of the six shelterin subunits, only TRF2 showed a marked decrease in expression level (Robin et al., 2020).

## 2.4 Telomere deprotection and senescence during LLPMC aging

A better indicator of telomere function than TL and shelterin expression is its anti-DDR function as revealed by the recruitment at telomeres of DDR factors, such as gH2AX and 53BP1. This is usually assessed as the colocalization of a telomere marker (PNA recognizing telomeric DNA or antibodies directed against TRF1 or TRF2) by confocal microscopy and expressed as the number of telomere dysfunction-induced foci or TIF (Takai et al., 2003) (also named telomere-associated foci or TAF). There are reports of several examples of age-related TIF accumulation in LLPMCs and post-mitotic organs : cardiomyocytes during mouse and human aging (Anderson et al., 2019; von Zglinicki et al., 2021), mouse skeletal muscle myofiber (von Zglinicki et al., 2021), mouse oocytes (Ge et al., 2019), mouse osteocytes (Farr et al., 2016), human melanocytes (Vitorelli et al., 2019), mouse retinal ganglion cells (Oubaha et al., 2016) and baboon hippocampus (Fumagalli et al., 2012b).

The fact that deprotected telomeres can lead to LLPMC senescence is supported by several reports: i) the accumulation of TIFs in aging mouse cardiomyocytes triggers senescence, as attested by cellular hypertrophy, *p21* and *p16* upregulation, senescence-associated distension of satellite DNA (SADS), SASP and SA- $\beta$ -gal positivity (Anderson et al., 2019). ii) Mice deficient in telomerase, fourth-generation *terc*<sup>-/-</sup> mutants, displayed a p21-dependent senescence phenotype

compared to the wild-type in cortical and Purkinje neurons (Jurk et al., 2012). iii) Short TL in neurons activates *p53* (Ferrón et al., 2009). iv) Dysfunctional TRF2 in mature neurons activates *p21* expression (Zhang et al., 2006, 2007). v) Osteocytes from old mice have a larger number of TIFs and express *p16*, *p21*, and *p53* and secrete SASP factors (Farr et al., 2016). vi) Aging adipocytes show hypertrophy correlating to telomere dysfunction (TL shortening and TIF) (el Bouazzaoui et al., 2014; Monickaraj et al., 2013).

Noteworthy, these age-related telomere deprotection and senescence events do not always correlate with TL and shelterin changes but to age-related oxidative damage, which can directly damage telomere structure (Anderson et al., 2019).

## 2.5 Protection-independent role of telomeres during LLPMC aging

Interestingly, TL shortening and shelterin downregulation in LLPMC do not always lead to telomere deprotection and senescence as in TRF2-compromised human myofiber (Robin et al., 2020) or in skeletal muscle of old baboons (Jeyapalan et al., 2007). Whether telomere changes directly affect aging-related processes other than chromosome end protection has important implications for the hierarchical relationships among aging hallmarks.

Much data indicate that telomeres directly regulate oxidative metabolism: i) Antioxidant proteins are specifically associated with telomeres (Aeby et al., 2016); ii) There is indication that the catalytic subunit of telomerase TERT can be localized in mitochondria (Haendeler et al., 2009; Zheng et al., 2019), where it protects metabolism and DNA maintenance (Ahmed et al., 2008; Miwa et al., 2016) in neurons upon oxidative stress (Spilsbury et al., 2015); iii) Telomerase RNA can be processed within the mitochondria, giving it a role in mitochondrial function (Cheng et al., 2018a; Zheng et al., 2019); iv) The shelterin subunits (with the exception of TRF1) have mitochondrial protective functions (Kim et al., 2017); v) The shelterin subunit TIN2 can be localized in mitochondria, where it induces mitochondrial dysfunction and ROS production (Chen et al., 2012); vi) Telomere dysfunction activates *p53*, which in turn binds and represses PGC-1 $\alpha$  and  $\beta$  promoters, leading to mitochondrial dysfunction (Chang et al., 2016; Sahin et al., 2011); vii) TRF2 regulates the expression of the subtelomeric mitochondrial Sirtuin SIRT3, leading to mitochondrial dysfunction in skeletal muscle

(Robin et al., 2020). Some of the mechanisms connecting telomere structure to mitochondrial integrity in LLPMCs appear to be independent of telomere protection, e.g., through a shuttle between telomere and mitochondria (Spilsbury et al., 2015) or through telomere-dependent transcriptional regulation of the subtelomeric *SIRT3* gene (Robin et al., 2020).

The transcriptional regulatory role of telomere factors in LLPMC concerns not only *SIRT3* but also several genes involved in LLPMC differentiation (Ye et al., 2014): i) TRF2 inhibits the activity of repressor element-1-silencing transcription factor (REST), a key repressor of neuronal genes and, consequently, favors neurogenesis (Ovando-Roche et al., 2014; Zhang et al., 2008, 2011). ii) A non-telomeric isoform of TRF2 (TRF2-S) interacts directly with mRNAs to facilitate their axonal delivery (Zhang et al., 2015). iii) TRF2 binds at several telomeric interstitial sequences (ITS) throughout the genome, where it activates the expression of neuronal genes; for instance, several involved in heparan sulfate proteoglycan biosynthesis, vesicular transport, ion transport and axon growth (Biroccio et al., 2013; Cherfils-Vicini et al., 2019; Simonet et al., 2011; Ye et al., 2014). iv) In skeletal muscle cells from patients with facioscapulohumeral muscular dystrophy, the expression of the subtelomeric gene *SORBS2* is upregulated as compared to control cells with longer telomeres (Robin et al., 2015).

Overall, the capacity of telomere to modulate gene transcription in subtelomeric regions (Telomere Position Effect of TPE) and genome-wide is likely to play an important role during LLPMC aging (Ye et al., 2014). Recently, a cluster of differentially expressed genes (DEG) with age, within the 2 MB closest to telomere was noticed in human skeletal muscle data from the GTEX database (version 8) (Dong et al., 2021). This observation suggests that in the aged muscle, shorter telomeres lead to subtelomeric gene silencing withdrawal. Hence, telomere shortening and telomere changes during LLPMC aging are likely to modulate chromatin landscape and transcriptional profile, determining cellular fate.

## 2.6 A telomere aging clock for LLPMC?

The current model is that telomere dynamics, intrinsically linked to cell division, cannot act as a clock for LLPMC aging. Here, I discuss several recent findings that

challenge this view, revealing various types of telomere changes occurring during LLPMC aging. Moreover, there is evidence that these changes contribute to pathologic myocardium (Chang et al., 2016, 2021; Terai et al., 2013), skeletal muscle and fat aging (Carneiro et al., 2016; Daniali et al., 2013) and to neurodegeneration (Levstek et al., 2020; Mamdani et al., 2016; von Zglinicki, 2002).

Because the telomere changes programmed during LLPMC aging do not result from intrinsic mechanisms linked to successive cell division, what is the nature of the aging clock that orchestrates them? For instance, the increased oxidative stress during aging can have a direct impact on telomere structure (Anderson et al., 2019). A tempting hypothesis would link the telomere changes of aging LLPMC to the accumulation of senescent cells in the same or other organs, which could send extracellular signals triggering telomere changes in LLPMCs and other more short-lived post-mitotic differentiated cells. This is for instance the case of senescent melanocytes sending paracrine signals to keratinocytes (Victorelli et al., 2019) and also neutrophils triggering senescence of neighboring cells via ROS induction, directly targeting telomeres (Lagnado et al., 2021; reviewed in Jacome Burbano et al., 2021).

The consequences of telomere changes in LLPMCs are not restricted to DDR activation and can impact cell function independently of senescence by regulating cell type transcription and by directly impacting mitochondrial function. For instance, the downregulation of TRF2 during skeletal muscle aging represses the expression of the subtelomeric gene SIRT3 required for mitochondrial integrity (Robin et al., 2020). These findings suggest a new type of cellular aging, distinct from the classical senescence. It would be helpful to determine whether other cellular processes that decline during LLPMC aging, such as proteostasis or autophagy, are also directly impacted by telomere changes.

Overall, there is a need to better understand in LLPMCs the mechanisms which can shorten TL, the cellular consequences of TL shortening, the regulation in space and time of the expression of the telomere-associated factors as well as their multiple functions. Altogether, these understandings will greatly clarify the role played by telomeres in the sequence of events leading to LLPMC aging.

Another valuable source of knowledge should come from studies on the role of telomeres in the aging of organisms that are mostly composed of post-mitotic somatic cells such as the worm *Caenorhabditis elegans* or the fly *Drosophila melanogaster*. Strikingly, the molecular organization of telomeres in these organisms appears quite

different from the one in vertebrates with, notably, for the fly, an absence of TRF1 and TRF2 orthologs (Raffa et al., 2013) and an absence of telomerase as well as of simple telomeric DNA simple repeats (Casacuberta, 2017). Only a few and sometimes seemingly contradictory reports were published on the role of telomeres changes during the aging of these organisms (see for instance (Joeng et al., 2004; Raices et al., 2005) ). Interestingly, the fly telomeres are known to exert TPE (Cryderman et al., 1999), suggesting that telomere modifications during fly LLPMC aging could affect subtelomeric gene expression, as it was observed in humans and yeast (Ye et al., 2014). In *C. elegans*, an absence of double stranded telomeric DNA binding proteins suggested that telomere molecular mechanisms were distant from mammalian mechanisms. However, recently, two proteins, DTN-1 and DTN-2, have been discovered (Yamamoto et al., 2021). DTN-1 and DTN-2 bind to telomeric DNA in germline but also in post-mitotic somatic cells and are part of a telomeric complex that includes the worm POT-1 and POT-2, ssDNA binding proteins. DTN-1 and DTN-2 have redundant functions in germline maintenance and DTN-1 specifically regulates telomeric DNA length (Yamamoto et al., 2021). Overall, these results highlight the value of *C. elegans* as a model to study telomeres in a post-mitotic organism. One can anticipate that more studies will focus on *C. elegans* telomeric proteins and molecular mechanisms of telomere during development and aging or aging-related diseases. For example, as this nematode is already used as a model for neurodegeneration diseases, it would be of interest to underly telomere implication in these phenotypes.

## 3 FOXO3a, an aging related factor

### 3.1 FOXO3a and longevity

#### 3.1.1 FOXO3a generalities

FOXO3a (Forkhead box O3), also known as FKHLR1, is a member of the FOX transcription factors. The subclass O: FOXO, is conserved among metazoans (Bridge et al., 2010). Their DNA binding domain, winged helix or forkhead domain, recognizes a 5'-[AG]TAAA[TC]A-3' consensus DNA sequence and it is evolutionary highly conserved. In mammals, there are four known Forkhead family members: FOXO1, FOXO3, FOXO4 and FOXO6 (Morris et al., 2015). In humans, FOXO1, FOXO3 and FOXO4 are widely basal expressed, whereas FOXO6 is mainly expressed in the brain. However, FOXO3a appears to play a major role on skeletal muscle homeostasis (Sandri et al., 2004).

FOXO3a regulation are led by a vast range of upstream pathways. Its activity is modulated by multiple post-translational modifications (PTM): phosphorylation, acetylation, methylation, ubiquitination (Wang et al., 2016b) and by its interaction with several proteins (Brunet et al., 2004; Hopkins et al., 2018; Tsai et al., 2008; Wang et al., 2012a). The most described FOXO3a PTM is the Akt/PI3K pathway mediated phosphorylation at T32, S253 and S318. Insulin-like growth factor 1 (IGF1) stimuli induces Akt/PI3K kinase activity, leading to FOXO3a nuclear exportation and sequestration into the cytoplasm by 14-3-3 interaction (Brunet et al., 1999). Hence, FOXO3a transcriptional activity is disrupted and could be degraded via the proteasome pathway (Dobson et al., 2011).

Although this is an example of PTM inducing FOXO3a negative regulation, there are other kinases (such as AMPK, MST1 and JNK) that activates FOXO3a, triggers its translocation into the nucleus (Greer et al., 2007; Wang et al., 2016b; Yu et al., 2013) and/or leads to FOXO3a binding to proteins.

Besides, several factors are involved in the FOXO3a acetylation/deacetylation dynamics to finely regulate its activation (CBP/p300, SIRT1, SIRT2, SIRT3, SIRT6) (Bertaglia et al., 2012; Brunet et al., 2004; Khongkow et al., 2013; Tseng et al., 2013; Wang et al., 2007). For instance, the CREB binding protein (CBP)/p300 induced acetylation (K242, K259, K262, K271) deactivates FOXO3a, whereas its deacetylation by SIRT2 or SIRT3, under oxidative stress, strengthened DNA-binding (Tseng et al., 2013; Wang et al., 2007). Consequently, SIRT2 and SIRT3 are likely to enhance

FOXO3-mediated transcriptional activity. Counterintuitively, SIRT2, along with SIRT1 mediated FOXO3a deacetylation, have been described to promote FOXO3a poly-ubiquitination by Skp2, a subunit of the E3 ubiquitination ligase, and further degradation by the proteasomal pathway (Wang et al., 2012b). Many other FOXO3a PTM have been studied and certainly there are other to be discovered (reviewed in Wang et al., 2016) but the detail will not be described in this manuscript.

Apart from the Forkhead/ DNA -binding domain (157-251 aa), the FOXO3a protein is composed by other four main domains (Figure 11):

- The N-terminal part (1-156 aa) contains one of the three Forkhead family conserved regions (CR): CR1.
- At the middle (271-461) a region containing two subparts of the conserved region 2: CR2A and CR2B.
- A transactivation domain (461-482 aa) composed by CR2C that binds to CBP/p300 and SIRT1 (Brunet et al., 2004; Wang et al., 2012a).
- At the C-terminal part, there is another transactivation domain (483-673). It contains the conserved region 3 (CR3) and it is known to bind to ATM and CBP/p300 (Adamowicz et al., 2016; Chung et al., 2012; Tsai et al., 2008; Wang et al., 2012a). FOXO3a affinity to CBP/p300 is enhanced by AMPK phosphorylation at Ser626.



Figure 11 : FOXO3a protein structure.

CR: conserved region (1 to 3); FH: Forkhead domain; NLS: Nuclear localization sequence

Both CR2C and CR3 transactivation domains seem to be required for FOXO3a transcriptional activity. They could trigger a greater stabilization by CBP/p300 binding (Wang et al., 2012a). The FH domain appeared to have a low affinity with CR3 (Wang

et al., 2008). These studies suggest that FOXO3a protein conformation depends on its interaction with other proteins and PTM.

The canonic role of FOXO3a, as a transcription factor, is to activate or suppress gene transcription by binding to promoter regions, helped by co-activators like CBP/p300 (Webb et al., 2016). Its activity is inhibited by the insulin and IGF-1 signaling pathway (IIS). As a matter of fact, FOXO3a regulates gene expression involved in a numerous of cellular pathways (Figure 12) such as oxidative stress response, autophagy, apoptosis, stem cell maintenance, etc. It is considered as a stress response factor and it is associated to homeostasis (Salih and Brunet, 2008). Strikingly, FOXO3a appears as a central major regulator to ensure survival. FOXO3a role in pathways composing the hallmarks of aging will be explained below.

The fact that this protein is highly conserved across evolution metazoans, giving “insights from Hydra to humans” (Davy et al., 2018), suggests FOXO3 and more generally the FOXO family members have crucial functions in organism survival.

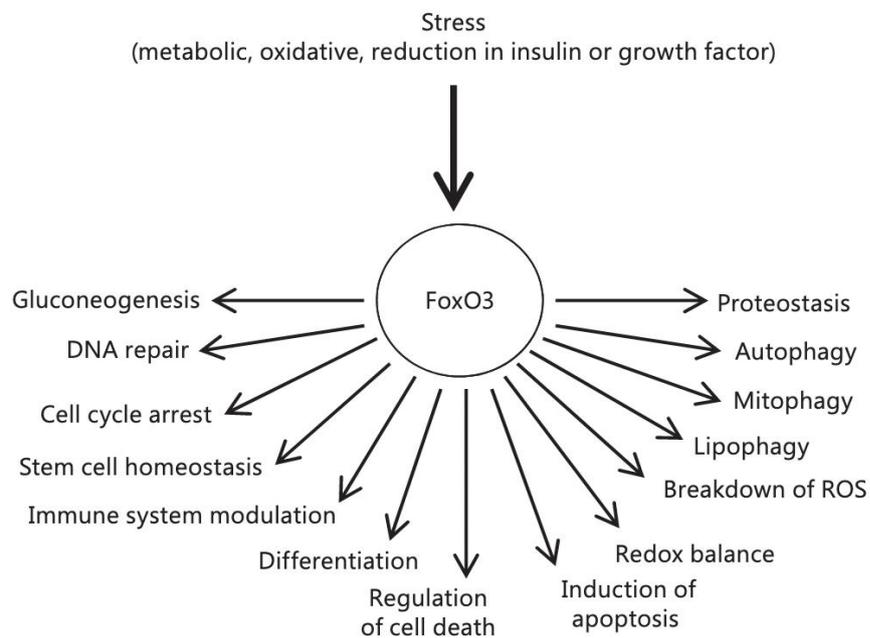


Figure 12: FOXO3a implication in cellular pathways.

Figure adapted from Morris et al., 2015, showing the range of cellular signaling pathways in which FOXO3a is involved. It confirms that FOXO3a is a major survival regulator.

It is essential to take under consideration that FOXO3a nuclear localization has been used as a shortcut to analyze this protein activation, but FOXO3a can also be translocated into the mitochondria (Celestini et al., 2018; Peserico et al., 2013). In metabolic stressed cancer cells (HCT116), FOXO3a phosphorylation at residues Ser12 and Ser30 by MEK/ERK and AMPK, respectively; and Ser30 in normal cells (NIH3T3) leads to FOXO3a mitochondrial translocation (mtFOXO3a). Importantly, the FOXO3a protein is cleaved at the N-terminal part (1-148 aa) when translocated into the mitochondria. In response to stress, mtFOXO3a binds to mitochondrial DNA (mtDNA) along with Transcription Factor A, Mitochondrial (TFAM), SIRT3 and the mitochondrial RNA polymerase, leading to mitochondrial stress response gene expression (Celestini et al., 2018). Therefore, these results suggest there is a dynamic nuclear-cytoplasmic-mitochondrial FOXO3a shuttle and crosstalk to finely regulate stress stimuli response (Fasano et al., 2019).

### 3.1.2 First description: longevity extension

Aging was generally considered as an inevitable and futile problem, not worth to get any particular attention. Nonetheless, it was noticed, in some species, that environmental conditions and reproduction could affect individual's lifespan. Some gene screenings were performed but any of them successfully conclude whether long lived mutants were due to longevity extension and not to other factors such as reproduction (Fasano et al., 2019). During the early 90's, a cutting-edge research performed by Cynthia Kenyon in *Caenorhabditis elegans* (Kenyon et al., 1993), showed that insulin/ IGF-1 pathway can affect worm longevity through genes expected to be involved in stress response (Riddle et al., 1981). It was the very first prove that lifespan extension can be genetically modulated, a major finding for the aging research field. Importantly, the longevity phenotype was independent from a progeny/ reproduction problem. In brief, the *C.elegans* orthologs of the Insulin receptor (IGF-IR) and FOXO are the abnormal dauer formation genes *daf-2* and *daf-16*, respectively. *daf-2* mutants, lived twice longer than WT worms (mean lifespan 42 vs 18 days) (Kenyon et al., 1993) (Figure 13). Importantly, the longevity extension phenotype was disrupted in double *daf-2; daf-16* mutant worms. This major result showed for the first time a link

between *daf16/FOXO3a* and longevity. *daf-16* mutant worms have a similar lifespan than WT worms (Kenyon et al., 1993).

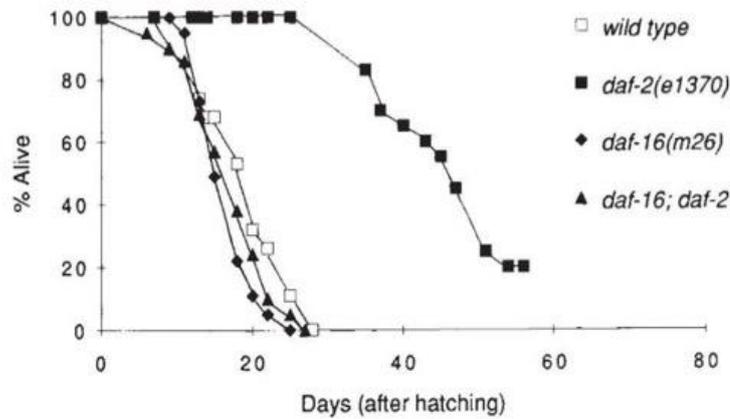


Figure 13: First prove of genetic implication in longevity.

Survival graph taken from (Kenyon et al., 1993). *daf-2/IGF1R C. elegans* mutants live twice longer. This longevity extension requires *daf-16/FOXO*.

I would like to comment that at the time of the *daf-2* mutant extension discovery it was unknown which kind of proteins were encoded by *daf-2*, *daf-16* and other proteins of the insulin/IGF-1 pathway. They were determined four years later as an ortholog of the insulin receptor and a Forkhead transcription factor (Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997). Understanding that longevity could be modulated by a transcription factor was ground-breaking and opened a plethora of new studies.

## 3.2 FOXO3a animal models

### 3.2.1 Caenorhabditis elegans: FOXO/DAF-16 origins

Apart from being the laboratory organism model in which *daf-16/FOXO3a* implication in lifespan was first described, *C. elegans* gave some insights about gene expression modulation (Figure 14). Indeed, during the beginning of the 2000's, some studies determined that genes downstream DAF-16/FOXO3a are involved in metabolism, stress response, thus, influencing lifespan (Lee et al., 2003; Murphy et al., 2003). For instance, among genes dependent on DAF-16, there are genes involved in

the antioxidant capacity (*ctl-2, sod-3, dod-10*), fatty acid metabolism (*fat-7, dod-9, pnk-1*), general mitochondria metabolism (*gei-7, dod-13, dod-1, C39F5.5, F54D5.7, Y106G6H.7*), heat shock (*hsp-16.1, hsp-12., hsp-16.49*, etc) antimicrobial genes (*spp-1*) (Lee et al., 2003; Murphy et al., 2003; Zečić and Braeckman, 2020).

In accordance to these findings, DAF-16 is required for survival and/ or enhanced homeostasis upon several cellular stress stimuli, such as heat shock resistance (Hsu et al., 2003), starvation, oxidative stress resistance (Gurkar et al., 2018; Henderson and Johnson, 2001), developmental growth under UVB induced DNA damage and defective DNA damage repair (Gurkar et al., 2018; Mueller et al., 2014).

### 3.2.2 FOXO in the evolutionary distant Cnidarians: Hydra and corals

*Hydra* and corals are part of the Cnidarians group and there are of major interest due their regeneration capacities and the cohabitation with photosynthetic symbionts, respectively, and to their extremely long lifespan (Bridge et al., 2010; Ottaviani et al., 2020).

Specially, *Hydra* does not show aging-associated or senescence-associated phenotypes. It has been hypothesized that three populations of *Hydra* multipotent stem cells: ectodermal and endodermal epitheliomuscular stem cells and interstitial stem cells in continuous self-renewal giving rise to differentiated cells: gonads, neurons, etc (Tomczyk et al., 2015). Interestingly, a single FOXO gene was discovered in *Hydra magnipapillata* and further studied in *Hydra vulgaris* (Bridge et al., 2010), confirming that the FOXO proteins are highly conserved among metazoans, even in evolutionary distant species (Figure 14). FOXO was highly expressed in the three stem cell lineages, decreased upon differentiation and its activity seems to be modulated (exogenous FOXO-GFP nuclear translocation) by the PI3K/Akt pathway and JNK kinases as in other species (Boehm et al., 2012; Bridge et al., 2010). In *Hydra*, FOXO proteins are required for stem cell maintenance, suitable growth phenotype, heat shock resistance and it is likely to be involved in apoptosis and innate immune system (Boehm et al., 2012; Bridge et al., 2010; Lasi et al., 2010; Martins et al., 2016).

A paradox of the ROS-related aging theory is the long-lived reef coral: corals shelter photosynthetic algae symbionts producing a roller-coaster of oxidative environment modulated by the circadian clock: oxidative stress exposure during the day light and hypoxia during night (Al-Horani et al., 2003). Recently, a night/day

transcriptomic analysis in *Stylophora pistillata* displayed a FOXO signature (Ottaviani et al., 2020). Two FOXO downstream genes (HEY and PDTH3 ortholog genes) were proposed as stress resistance and anti-senescence candidates. This omic profile suggest FOXO is a major regulator of the hyperoxia/hypoxia coral transition.

### 3.2.3 FOXO3a and *Drosophila melanogaster*, the dietary restriction mediated lifespan extension exception

In the fruit fly, *Drosophila melanogaster*, a *DAF16/ FOXO3a* ortholog: *dFOXO* was described in 2003 (Figure 14) (Jünger et al., 2003; Kramer et al., 2003; Puig et al., 2003). Nevertheless, two years earlier it was shown that the insulin receptor pathway was also involved in this fly longevity (Clancy et al., 2001; Tatar et al., 2001). Mutants in the drosophila insulin-like receptor (*chico* gene) leads to dwarf flies, with an up to 85% longevity extension (Clancy et al., 2001). *dFOXO* activation is also modulated by Akt/PI3K mediated phosphorylation as in *C. elegans* and mammals and it is expressed in adult fat body. *dFOXO* activation in fat body prolongs 20 to 50% flies lifespan and reduced it fecundity (Giannakou et al., 2004). Importantly, activated *dFOXO* in the adult pericerebral fat body decreases the expression of the *Drosophila* insulin-like peptide (*dilp-2*), expressed in neurons (Hwangbo et al., 2004). Although *dFOXO* is not required for dietary restriction mediated *Drosophila* lifespan extension, it is partially involved in this process (Giannakou et al., 2008). *dFOXO* is involved in autophagy, stress response, cellular growth arrest, neuromuscular junction, and neuronal homeostasis (Birnbaum et al., 2021; Demontis and Perrimon, 2010; Giannakou et al., 2008; Gruntenko et al., 2016; Jünger et al., 2003; Puig et al., 2003). Moreover, *dFOXO* transcriptional activity decreases with aging in female fruit flies (Birnbaum et al., 2019).

### 3.2.4 Mouse model: mammalian FOXOs are redundant

In the laboratory mouse (*Mus musculus*), as well as in other mammals, there are four FOXO proteins (Figure 14): Foxo1, Foxo3, Foxo4 and Foxo6. While Foxo1, Foxo3 and Foxo4 sequences are highly conserved and they are ubiquitously expressed, Foxo6 is mainly expressed in the brain, and it is differentially regulated. In order to determine whether each of these FOXO proteins has a specific role, single knock out mice were first described. Foxo1 knock-out was lethal at E11 (Hosaka et al.,

2004), embryos displayed an important vascular damage (Dharaneeswaran et al., 2014; Furuyama et al., 2004). Foxo3 KO mice are viable but females present fertility problems at young age due to damaged ovarian follicle growth (Castrillon et al., 2003; John et al., 2008). Moreover, Foxo3 KO mice displayed an impaired B cell development (Hinman et al., 2009), hyperactivation of helper T cells (Lin et al., 2004) and dermatitis was frequently noticed starting at 10-24 months (Paik et al., 2007). Foxo3 is required for dietary restriction mice lifespan extension (Shimokawa et al., 2015). Although, in the first Foxo mice description, Foxo4 KO mice did not show any abnormality (Hosaka et al., 2004), it has been observed that Foxo4 induces myocardial infarction early inflammation response by neutrophil infiltration (Zhu et al., 2015). Specific KO of Foxo4 in mice endothelial cells induced a better survival after myocardial infarction. Finally, Foxo6 null mice had abnormal dendritic spine morphology and short/ long memory consolidation (Salih et al., 2012). These different mouse strains suggest that the mammalian FOXO proteins have independent and specific roles.

FOXO proteins functions are related to cellular growth, proliferation and apoptosis, suggesting them as good tumor suppressor candidates. Even if each Foxo null mouse strain displayed a mild harmful phenotype, it has been hypothesized that this FOXO proteins could have redundant functions, explaining why some single Foxo KO phenotypes are minor. Therefore, an inducible triple mutant mouse strain was created: *Mx-Cre<sup>+</sup>;FoxO1/3/4<sup>L/L</sup>* by Ji-Hye Paik and colleagues (Paik et al., 2007). Cre widespread expression was induced at 4-5 weeks of age, preventing Foxo1 null embryonic lethality. Whereas in control mice and *Mx-Cre<sup>+</sup>;FoxO1<sup>L/L</sup>*, *Mx-Cre<sup>+</sup>;FoxO1/3<sup>L/L</sup>*, *Mx-Cre<sup>+</sup>;FoxO1/4<sup>L/L</sup>* any lymphoma was detected after 100 weeks, the triple Foxo mutant mice developed lymphomas starting at week 25 (Paik et al., 2007). Foxo3 null mice have a modest increase of tumor phenotype at very old age but the overall cancer incidence was not increased after carcinogen treatment (Paik et al., 2007). As well, triple mutant had bigger brain due to deregulated neural stem cells (NSC) proliferation leading to an extinction of the NSC pool (Paik et al., 2009).

These results determined that FOXO proteins have redundant roles in tumor suppression, but it could also be the case for other cellular processes regulated by the FOXO proteins, such as autophagy (Audesse et al., 2019).

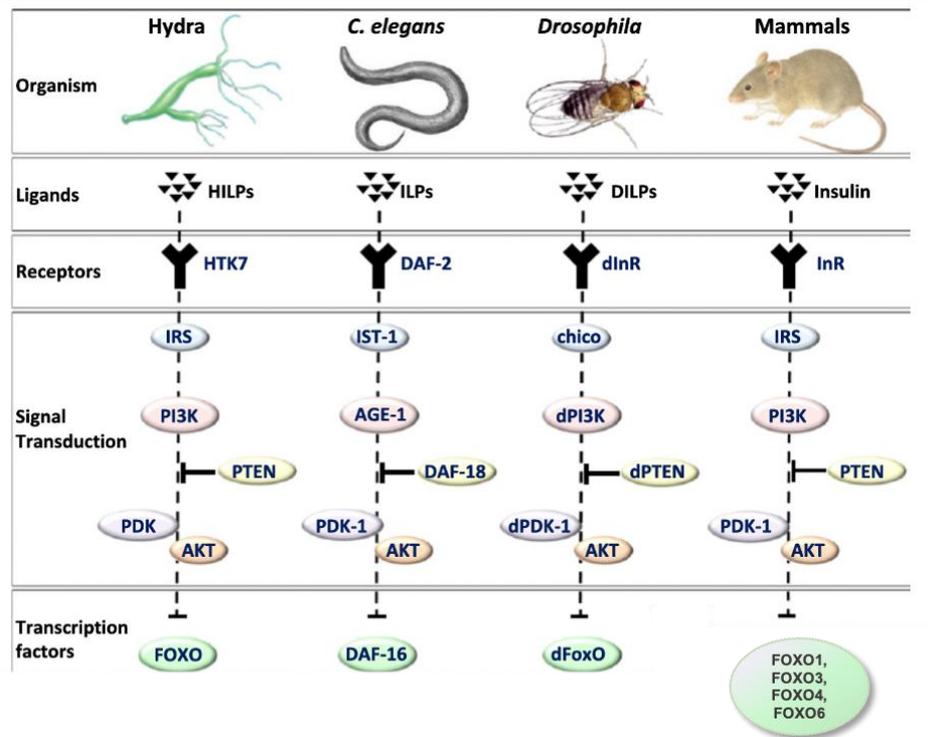


Figure 14: DAF-16/FOXO proteins regulation in several animal models

Figure adapted from (Martins et al., 2016). FOXO proteins are highly conserved across evolution, and they are all regulated by the respective AKT/PI3K pathway of these studied species.

### 3.3 FOXO3a SNPs in human population

As explained above, during the XX<sup>th</sup> century, longevity modulation was associated to environmental conditions and the genetic impact was neglected. However, just after the IIS pathway-mediated longevity extension discovery, the Apolipoprotein E: *APOE*, was associated with human longevity. In fact, the  $\epsilon$ 4 allele was less frequent in centenarians (Schächter et al., 1994). Therefore, it opened doors to determine other genes and alleles associated with human longevity.

Then, in order to study the variation in genes of the insulin/IGF-1 (IIS) signaling pathway and their correlation with human longevity, five candidate genes were characterized in American men with Japanese ancestor (more than 95 years old vs less than 81 years old). Among these genes only *FOXO3a* was associated with extended human longevity (Willcox et al., 2008). Three SNPs within *FOXO3a* intron 2 were associated with this cohort longevity: *rs2764264*, *rs13217795*, *rs2802292*. Then, the *rs2802292* G-allele (minor) was further confirmed to be associated with men longevity compared to the T-allele (major) in other old (centenarian) human cohorts:

German, French, Southern Italian, Han Chinese, Danish (Anselmi et al., 2009; Flachsbart et al., 2009; Li et al., 2009; Sanese et al., 2019; Soerensen et al., 2015) and meta-analysis (Bao et al., 2014; Broer et al., 2015; Sun et al., 2015). The *rs2802292* G-allele was associated with higher mRNA *FOXO3a* expression (Banasik et al., 2011). As there are several longevity associated SNPs within the intron 2 of *FOXO3a* it was suggested this genomic region may be part of a regulatory chromatin major structure. Therefore, the 90 bp around the *rs2802292* SNP locus was analyzed for its enhancer properties. Strikingly, the *rs2802292* G-allele creates a Heat Shock Element (HSE) binding site for the transcription factor Heat shock factor protein 1 (HSF1) (Grossi et al., 2018). Besides, HSF1 binds also to an HSE at the *FOXO3a* promoter region, thus, HSF1 is an intermediary between *FOXO3a* intron 2 and promoter, by chromatin loops, inducing an enhancer activity. HSF1 DNA-binding at *FOXO3a* intron 2 locus occurs after stress stimuli (oxidative stress, heat shock, glucose deprivation) and increases *FOXO3a* mRNA expression (Grossi et al., 2018; Sanese et al., 2019) (Figure 15).

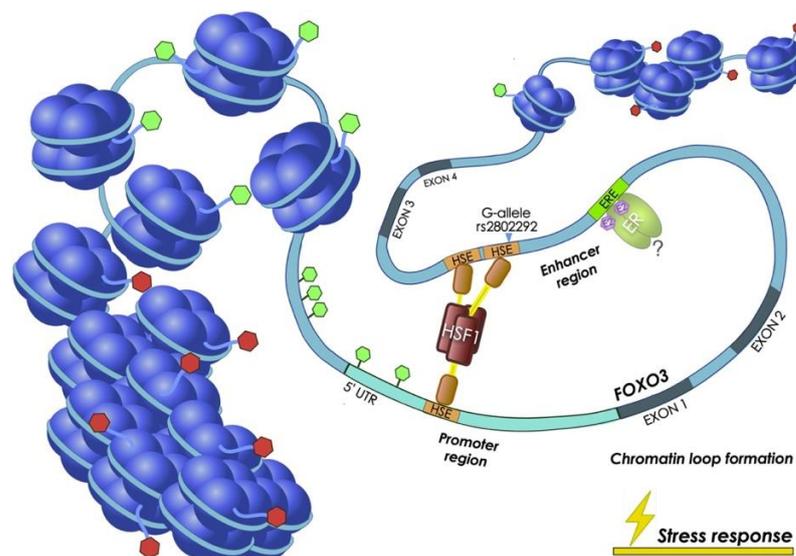


Figure 15: FOXO3a longevity associated SNPs and enhancer activity

Figure taken from (Sanese et al., 2019). G-allele of the *rs2802292* SNP creates a HSE site at the *FOXO3a* intron 2. HSF1 recognizes and binds to this HSE site and to a HSE site at *FOXO3a* promoter, triggering a chromatin loop conformation with enhancer abilities. Therefore, *FOXO3a* expression is increased. Green and red tags on chromatin represent active and inactive epigenetic marks, respectively. They regulate the accessibility to regulatory factors. HSF1: heat shock factor 1; HSE: heat shock element; ER: estrogen receptor; E2: estradiol; ERE: estrogen responsive element.

Although, I described here specially the rs2802292 SNP, other *FOXO3a* SNP have been associated with longevity (Anselmi et al., 2009; Bao et al., 2014; Flachsbart et al., 2017; Li et al., 2009; Morris et al., 2016; Sanese et al., 2019; Teumer et al., 2016). Recently, two *FOXO3a* variants: rs4946935 (A/G) and rs12206094 (C/T), showed a close association with longevity in German cohort and it was validated in French and Danish samples (Flachsbart et al., 2017). As well, the rs4946935 SNP, A-allele instead of the G-allele, creates a binding site for the SFR (Serum Response Factor) transcription factor, giving an enhancer capacity to this allele (Flachsbart et al., 2017). SFR also binds to *Igf-1* promoter (Flachsbart et al., 2017). Moreover, within human chromosome 6, *FOXO3a* gene has been observed to get in contact with 46 other neighboring genes through long range chromatin loops, via the CCCTC-Binding Factor (CTCF) factor (Donlon et al., 2017). This mechanism seems to be triggered by a longevity associated haplotype of *FOXO3a* SNPs, which is relatively common in Asian populations (Donlon et al., 2017). Consequently, *FOXO3a* locus variants might be involved in other genes regulation not only through its transcription factor role but also by creating topologically associated domains (TADs). This long-range interactions at chromosome 6, with *FOXO3a* locus at the middle, has been named “the aging hub” (Donlon et al., 2017; Grossi et al., 2018; Sanese et al., 2019).

Some of the longevity related SNPs were associated with protection against aging and aging-related diseases: reduced incidence in cardiovascular diseases, stroke and cancer, higher daily activity, reduction in all cases reduction (Banasik et al., 2011; Willcox et al., 2008, 2017; Zettergren et al., 2018).

Overall, these studies determined that *FOXO3a* is involved in human longevity through different SNPs. Importantly, even if there are some meta-analysis research, *FOXO3a* variants and frequency might be associated to a certain population cohort. Its effects on aging and aging-related diseases might be dependent on each population. Hence, further studies remain to be done taking account the global population. The current insights on *FOXO3a* SNP, suggest that some variations of this gene give an advantage by increasing stress tolerance. For instance, a higher *FOXO3a* expression in some tissues leads to a better antioxidant capacity, DNA damage repair and metabolic homeostasis, explaining why some alleles are correlated with lower risk of cardiovascular diseases, cancer and other age-related diseases. To do so, *FOXO3a* must be involved in several cellular pathways, notably in the so called hallmarks of ageing: deregulated nutrient sensing, loss of proteostasis, epigenetic alterations,

genomic instability, altered intracellular communication, telomere attrition, cellular senescence, mitochondrial dysfunction and stem cell exhaustion (López-Otín et al., 2013).

### 3.4 FOXO3a is involved in the hallmarks of aging.

Although relevant evidence has shown that FOXO3a is involved in nutrient sensing, stem cell maintenance, proteostasis, mitochondrial metabolism, epigenetics alterations, cellular intercommunication and genome stability, FOXO3a function on cellular senescence or telomere attrition remains elusive. This major role in a plethora of cellular processes associated with aging, set FOXO3a as a central regulator of aging.

#### 3.4.1 FOXO3a in the nutrient sensing

Among the hallmarks of aging in which FOXO3a, or the FOXO proteins, are involved, the nutrient sensing pathway was the first to be described. Three main pathways have been associated to nutrient sensing or dietary restriction: the IIS pathway, AMPK/mTOR pathway and SIRT1 regulation (López-Otín et al., 2013).

The insulin like-growth factor 1 (IGF-1) is mainly produced by hepatocytes but also by other cell types, in response to growth hormone response. IGF-1 has anabolic roles: cell growth and inhibited apoptosis. As its name suggests, IGF-1 is similar to insulin and both inform about glucose presence and induce signaling to control glucose homeostasis (Bartke et al., 2003). Low levels of IGF-1 have been associated with dietary restriction (Dunn et al., 1997; Greer et al., 2007; Jiang et al., 2020; Lettieri-Barbato et al., 2016; Redman et al., 2010). In mice, caloric restriction decreases IGF-1 levels, activates FOXO3a and prolongs lifespan (Dunn et al., 1997; Shimokawa et al., 2015). Upon starvation, FOXO3a is translocated into the nucleus (Figure 16). Surprisingly, IGF-1 and AKT levels decrease in mice during aging (Schumacher et al., 2008). Hence, dietary restriction could be felt as a stress activating FOXO3a to trigger a metabolic, antioxidant response and slow cellular growth.

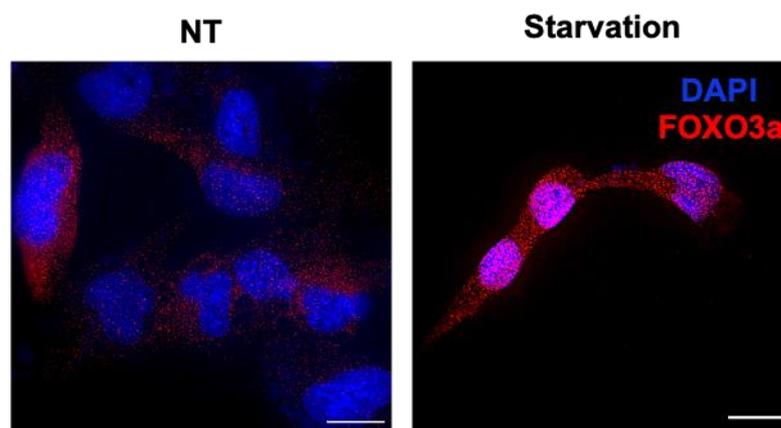


Figure 16: FOXO3a nuclear translocation under starvation.

BJ-HELT human fibroblasts after one week of serum deprivation. Under normal conditions FOXO3a is mostly cytoplasmic, but it is translocated into the nucleus after serum starvation, suggesting FOXO3a activation. Jacome Burbano, unpublished image.

Besides, as previously described AMPK activates FOXO3a, but it also inhibited TOR (Target of rapamycin or mTOR in mammals) (Greer et al., 2007; Kimura et al., 2003). From yeast to human, TOR is activated by nutrients, it activates protein translation, cellular growth and blocks autophagy (Blagosklonny, 2010). mTOR is involved in aging-related diseases such as type II diabetes, cancer or atherosclerosis (Stallone et al., 2019). FOXO3a and mTOR pathways have opposite effects. Whereas FOXO3a is activated upon nutrient deprivation, mTOR pathway is inhibited.

Finally, under dietary restriction, SIRT1 expression is increased by FOXO3a in association with p53 in rat cells (Nemoto et al., 2004). Moreover, SIRT1 deacetylates and activated PGC1 $\alpha$  that regulates mitochondrial biogenesis and glucose metabolism (Rodgers et al., 2005).

Therefore, FOXO3a is directly or indirectly involved in the three regulating pathways of nutrient response. During aging the nutrient sensing mechanisms are less effective, it might lead to an inefficient activation and FOXO3a modulation of dietary restriction and stress benefits.

### 3.4.2 FOXO3a, a stemness requirement

One of the theories of aging is known as the stem cell aging theory. It exposes that stem cells pluripotent capacity decreases during aging and stem cell pools are

reduced (Ahmed et al., 2017). It implies that at old age the regenerative capacity to guarantee tissue homeostasis and cell replacement is damaged. A considerable number of studies, in different stem cell types, have shown that FOXO proteins are required to maintain stem cells status and prevents it differentiation. In *C. elegans*, somatic FOXO3a expression appears to be required for germline stem cell maintenance (Qin and Hubbard, 2015). Some of these studies in mammals have considered three FOXO proteins: FOXO1, FOXO3 and FOXO4, as a general function to preserve stemness (García-Prat et al., 2020). However, some studies have concluded that FOXO3a is essential for progenitor/stem cells conservation (Gopinath et al., 2014; Miyamoto et al., 2007; Renault et al., 2009).

Importantly, in quiescent satellite cells (muscle stem cells), FOXO3a is required to preserve quiescent state and prevent cell cycle entry, thus, FOXO3a is needed for muscle regeneration after several rounds of muscle injury (Gopinath et al., 2014). Recently, a heterogeneity has been observed in mouse quiescent satellite cells. These cells displayed two distinct transcriptional profiles that correlates with low or high expression levels of CD34 (García-Prat et al., 2020). On the one hand, CD34 high cells were called genuine long term quiescent satellite cells, they have lower metabolic activity, a better capacity for myofiber regeneration, self-renewing and higher FOXO proteins expression (FOXO1, FOXO3a, FOXO4). On the other hand, CD34 low cells were designated as in a primed state, and they are more prone to enter in the cell cycle, start differentiation and mitochondrial OXPHOS genes are upregulated. Genuine quiescent satellite cells are well conserved in old mice (18-22 months), but a decline is observed at around 28 months old and adopt some characters of the primed quiescent cells (García-Prat et al., 2020). The transcriptional differences between genuine and primed state were blurred in geriatric mice (28-30 months old). FOXO (*FOXO1*, *FOXO3a*, *FOXO4*) triple KO specifically in satellite cells showed that FOXO proteins are required for satellite cells regeneration, self-renewal and to maintain quiescent satellite cells genuine state until extreme old age (García-Prat et al., 2020). FOXO inhibition leads to a transcriptional profile closer than primed state satellite cells, even at young age. Therefore, FOXO proteins define muscle stem cell subdivision (genuine or primed), guarantee a quiescent stem cell diversity until old ages and thus, they determine satellite cells fate, regeneration and renewal capacities. Besides, FOXO3a constitutive active form in human mesenchymal stem cells enhances cellular

stress resistance, regenerative aptitudes, prevents transformation and cellular aging (Yan et al., 2019).

Overall, these results show that FOXO are essential for stem cell maintenance and response over lifetime, being essential on tissue regeneration and aging. It has been proposed to use stem cells (for example mesenchymal stem cells) modified to have a constitutive active FOXO3a form to enhance stem cell replacement therapy and promote tissue protection (Yan et al., 2019).

### 3.4.3 FOXO3a and epigenetics alterations

During aging several epigenetic changes have been described such as redistribution of DNA methylation (general hypomethylation and CpGs islands hypermethylation), histone modifications and euchromatin/ heterochromatin status variation principally characterized as a heterochromatin loss (López-Otín et al., 2013; Zhang et al., 2020). It is important to take into consideration epigenetics alterations as a cause of aging-related transcriptional modifications. As explained above, some FOXO3a SNPs within long-lived human cohorts develop an enhancer activity in FOXO3a locus and binds to neighboring gene locus (46) creating TADs or long-range chromatin loops that might affect gene expression via chromatin higher organization alteration (Donlon et al., 2017; Flachsbarth et al., 2017; Grossi et al., 2018; Sanese et al., 2019). Therefore, longevity associated human SNPs might be essential to preserve a better transcriptional response, even at old ages.

Moreover, FOXO3a protein binds to SIRT1, SIRT2, SIRT3 and SIRT6 NAD<sup>+</sup> dependent deacetylases and p300 histone acetyltransferase (Brunet et al., 2004; Khongkorn et al., 2013; Tseng et al., 2013; Wang et al., 2007, 2012a). As a transcription factor, FOXO3a modulates gene expression and could bind to coactivators like CBP/p300 that are known to remodel chromatin (Ogiwara et al., 2011). Finally, FOXO3a induces SIRT1, SIRT6 and represses DNA methyltransferase 3B (DNMT3B) expression (Nemoto et al., 2004; Yang et al., 2014; Zhang et al., 2019). First, SIRT1 deacetylates histones H1, H3 and H4 but also other substrate such as FOXOs proteins or PGC1- $\alpha$  and it is a pro-longevity factor (Chen et al., 2020). Second, SIRT6 is a histone H3 deacetylase (H3K9ac, H3K56ac, and H3K18ac) involved in heterochromatin silencing of repetitive DNA regions (Tasselli et al., 2017). Interestingly, SIRT6 functions have been associated with several biological processes

implicated in protection against aging and aging-related diseases, stem cell maintenance and metabolism regulation (Tasselli et al., 2017). Third, DNMT3B is a *de novo* DNA methyltransferase targeting centromeres, pericentromeres, subtelomeres and active genes intragenic regions (Yang et al., 2014). Its expression decreases during lifetime (35-44 vs 55-75 years old) in European female cohort (Ciccarone et al., 2016).

Although there are only some emergent results showing FOXO3a direct or indirect implications in epigenetic regulation, further studies are required to determine other FOXO3a mechanisms or factors involved in chromatin remodeling and epigenetic alterations during aging.

#### 3.4.4 FOXO3a, role in inflammation

Altered intracellular communication is also impaired at old ages. It is observed by a dysfunctional immunosurveillance and increased inflammation, specially via the senescence associated secretory phenotype or SASP, leading to long distance cell communication (López-Otín et al., 2013). In tumor-associated dendritic cells, FOXO3a binds to NF $\kappa$ B to prevent a pro-inflammatory phenotype (Thompson et al., 2015) and NF $\kappa$ B induced secretome favors senescence and T cell hyperactivation (Chien et al., 2011; Lin et al., 2004). In the context of Rheumatoid arthritis, an auto immune and aging-related disease, FOXO3a is inhibited by TNF $\alpha$  signaling, which leads to upregulation of several pro-inflammatory cytokines such as CXCL9, CXCL10, CXCL11 and the co-stimulator Tumor necrosis factor ligand superfamily member 18 (TNFSF18) (Brandstetter et al., 2019). Recently, in mice deficient for FOXO3a, it has been determined that FOXO3a binds to *IL-21* promoter to induce its expression in Follicular helper T cells, leading to cell differentiation, thus, playing a major role in antibody production and germinal center B cell maturation (Qi et al., 2020). IL-21 is a secreted interleukin at the interphase between innate and adaptive immunity. IL-21 secreted by Follicular helper T cells, binds to IL-21 receptor at the surface of germinal center B cells to determine cellular fate and antibody production (Qi et al., 2020). In old mice (15 months old), IL-21 injection induces thymopoiesis (T cell lymphopoiesis), thus, it rejuvenates T-cell pool at old ages (Al-Chami et al., 2016).

Overall, whereas these results show that FOXO3a is involved in immune cells function, maintenance and in their secretory phenotype, it would be of interest how FOXO3a inhibition affects SAPS and its intra-organ effect. Given that FOXO3a is a tumor-suppressor, this role in cell communication could be used as a cancer therapy to avoid chronic inflammation.

### 3.4.5 FOXO3a, a major autophagy regulator

Proteostasis processes are composed by protein biogenesis, stabilization after correct folding, protein trafficking and degradation by proteasome or elimination by autophagy (Balch et al., 2008). They are critical to control protein quality and quantity at a given time. Nevertheless, proteostasis balance is damaged at old ages and in age-related degenerative diseases, leading to misfolded proteins, aggregates accumulation and erroneous protein quantity according to physiological requirements (Hipp et al., 2019). For instance,  $\alpha$ -synuclein aggregates are observed in Parkinson's disease and might contribute to its pathogenesis (Anandhan et al., 2017).

Autophagy is a process by which damaged organelles (e.g., mitophagy) or cytoplasmic proteins, aggregates that could induce proteotoxicity are removed into lysosomes for degradation. Autophagy mechanisms are conserved during evolution from plants to metazoans (Hughes and Rusten, 2007) and are associated to starvation related degradation. FOXO proteins are required for autophagy in fruit flies, *C. elegans*, mouse (Audesse et al., 2019; Demontis and Perrimon, 2010; Mammucari et al., 2007, 2008; Martins et al., 2016; van der Vos et al., 2012; Webb and Brunet, 2014; Xiong et al., 2012; Zhao et al., 2007). FOXO3a has an impact on autophagy through autophagy genes expression regulation, such as *LC3b*, *Bnip3*, *Beclin 1*, *Atg4b* and *Atg12l* (Milan et al., 2015; Webb and Brunet, 2014). Besides, proteostasis pathway genes are a conserved target of FOXO proteins from mouse, *Drosophila*, *C. elegans* and human (Webb et al., 2016). Remarkably, a ChIP-seq of 12 weeks old mice revealed that among 451 autophagy related genes, 151 are direct FOXO3a targets in neural stem and progenitor cells, including genes involved at different steps of the autophagy process: initiation (*Rbcc1*, *Ulk1*), autophagosome formation (*Gabarap*, *Atg5*, etc) and degradation (*Atg9a* and others). Additionally, in the same dataset, 43 out of 83 mitophagy genes were also FOXO3a targets, such as *Fis1* and *Optn* (Audesse et al., 2019).

FOXO3a role in autophagy was first characterized in muscle cells (Mammucari et al., 2007) but it has been confirmed in other cell types such as neurons, and adult neuronal progenitors, primary renal proximal tubular cells, cardiomyocytes, hematopoietic stem cells (Audesse et al., 2019; Sengupta et al., 2009; Warr et al., 2013; Webb and Brunet, 2014).

The second mechanism of protein degradation is the ubiquitin proteasome system or UPS, that degrades exclusively cytosolic proteins (short-lived, damaged or misfolded), one by one (Hipp et al., 2019). It is composed by four main steps: i) thioester bond between ubiquitin activating enzymes (E1) to the carboxyl ubiquitin terminus by an ATP consuming reaction; ii) the ubiquitin is transferred to ubiquitin conjugases (E2); iii) finally, E2 associates with ubiquitin ligases (E3), which recognizes substrate proteins and transfers E2 ubiquitin tag to a lysine of the substrate, and further polyubiquitinated chain; iv) substrate is send to the 26S of proteasome for degradation (Gong et al., 2016). During aging there is an UPS activity decline that could open the way for aging-related diseases such as Alzheimer and Parkinson disease (Webb and Brunet, 2014). Importantly, in skeletal muscle cells, FOXO proteins directly controls E3 ubiquitin ligases *MUSA1*, *atrogen-1*, *SMART* and *Murf-1* expression and proteasome subunits (*Psme4/PA200*, *Psm1*, *Psmc4/Rpt3* and *Psm11/Rpn6*) (Milan et al., 2015, 2015; Sandri et al., 2006; Stitt et al., 2004; Webb and Brunet, 2014). However, these ubiquitin ligases are also genes associated with muscle atrophy, also named atrogenes, because they degrade myofibrillar proteins upon catabolic stress (starvation, muscle denervation, etc) (Sandri et al., 2004; Stitt et al., 2004). Indeed, FOXO proteins activation induces muscle waist and atrophy of mature myofibers (Sandri et al., 2004, 2006). Remarkably, although FOXO proteins are redundant, FOXO1 nor FOXO4 knockout mice are protected from atrophy after muscle denervation but FOXO3a KO mice are significantly safeguarded (Milan et al., 2015). These results suggest that among the FOXO proteins, FOXO3a has a major role on muscle homeostasis.

It is noteworthy that long-lived post-mitotic cells, such as neurons or myofibers, capacities of renewal and repair driven by the proteostasis system are essential to stay young. Therefore, a finely tuned balance on FOXO proteins, specially FOXO3 expression and downstream regulation must be carried on. While it should provide a functional protein/ organelle degradation and recycling system in mature muscle fibers,

it must avoid enlarged muscle atrophy. FOXO protein's pivotal role equilibrium in this context might be finely controlled at young ages but damaged at old age.

### 3.4.6 FOXO3a and mitochondrial dysfunction

Mitochondrial alterations were rapidly associated with aging, as seen by the free radical aging theory, proposed by Denham Harman in the middle of the XX century. It proposes that reactive oxidative species (ROS) increasing accumulation during lifetime, progressively induce mitochondrial dysfunction and general damage leading to cellular aging (Harman, 1965). Although this theory has been strongly contested, oxidative damage is still considered a major DNA, RNA and protein cause of damage. Among endogenous classes of ROS there are superoxide radicals ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals ( $\cdot OH$ ).

FOXO3a protects from oxidative stress via upregulation of antioxidant gene expression, especially MnSOD2 (transforms  $O_2^-$  into  $H_2O_2$ ) and catalase: CAT (transforms  $H_2O_2$  in  $H_2O$ ) (Figure 17) (Kops et al., 2002; Nemoto and Finkel, 2002).

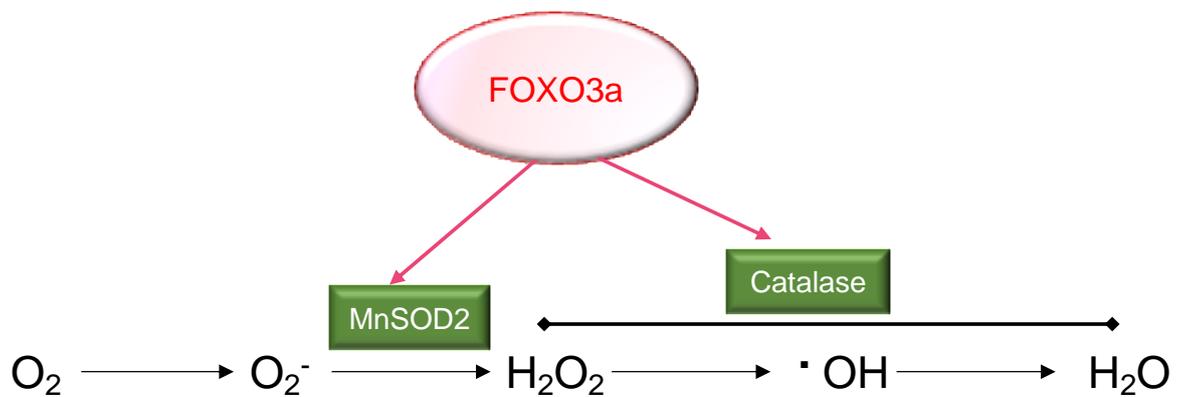


Figure 17: FOXO3a mediated ROS regulation by MnSOD2 and Catalase antioxidants.

FOXO3a upregulates antioxidants MnSOD2 and CAT expression, which will reduce superoxide radicals ( $O_2^-$ ) into hydrogen peroxide ( $H_2O_2$ ) and then into water to decrease ROS accumulation.

FOXO3a and PGC1- $\alpha$  co-immunoprecipitate together in vascular endothelial cells and bind to the same oxidative response genes promoter regions. PGC1- $\alpha$  expression is regulated by FOXO3a and it is required as co-activator for FOXO3a transcriptional activation of these genes (*SOD2*) (Olmos et al., 2009).

It has now been well established that FOXO3a can be translocated into mitochondria, by Ser30 and facultatively Ser12 phosphorylation, where it accomplishes several functions (Celestini et al., 2018).

FOXO3a binds to mitochondrial DNA (mtDNA), along together with TFAM, SIRT3 and mitochondrial RNA polymerase to control metabolism in stressed cancer cells via regulation of mitochondrial gene expression (Celestini et al., 2018; Peserico et al., 2013). This mitochondrial FOXO3a/SIRT3/mtRNAPol axis informs about nutrient deprivation and induces cellular processes for adaptation. Moreover, FOXO3a inhibits c-Myc dependent mitochondrial gene expression by upregulating c-Myc antagonist: the MAD/MXD repressor complex (Ferber et al., 2012). Besides, FOXO3a activation decreases the number of mtDNA copies, mitochondrial proteins amount and slow down respiratory chain complexes activity leading to reduced ROS accumulation (Fasano et al., 2019; Ferber et al., 2012).

Overall, FOXO3a is a major regulator of mitochondrial functions. According to its canonical role, FOXO3a regulates nuclear genes involved in oxidative response but can also modulate mitochondrial gene transcription by directly binding to mtDNA. It is a stress sensor that regulates mitophagy, mitochondrial healthy physiology and metabolism to avoid mitochondrial damage dependent premature aging (Trifunovic et al., 2004).

#### 3.4.7 FOXO3a in DNA damage and repair

Another hallmark of aging is genomic instability and DNA damage has been proposed as a central regulator of aging (López-Otín et al., 2013; Schumacher et al., 2021). Several premature aging syndromes (progeroid syndromes) are caused by mutation in DNA repair or genome maintenance genes. For instance, Werner Syndrome is caused by mutations in the *RecQ* helicases genes family and Cockayne Syndrome is caused by excision repair genes mutation *ERCC6* and *ERCC8* (Kyng and Bohr, 2005; Schumacher et al., 2021; Taghdiri et al., 2017).

Remarkably, DNA damage induces DAF-16/FOXO3a expression in *C.elegans* after UV-induced damage and in MEFs or in human primary fibroblasts exposed to mutagen agents such as bleomycin (Mueller et al., 2014; White et al., 2020). FOXO3a is critical to preserve genome stability as it is required to avoid genomic rearrangement,

$\gamma$ -H2AX foci accumulation, homologous recombination (HR) and non-homologous end joining (NHEJ) (White et al., 2020). In *C. elegans*, DAF-16/FOXO3a is required to promote development progression despite persistent DNA damage in somatic cells, via its binding with the GATA transcription factor EGL-27 to upregulate DNA damage response genes (Mueller et al., 2014). Besides, FOXO3a can delay the G2-S phase upon stress conditions and it directly targets Growth arrest and DNA damage-inducible protein GADD45 alpha (Gadd45a) to contribute to DNA repair in mammalian cells (Tran et al., 2002).

Finally, in response to DNA damage agents, FOXO3a competes with NOTCH1 for ATM-binding, and then to the ATM -Chk2-p53 complex (Adamowicz et al., 2016; Chung et al., 2012; Tsai et al., 2008). It is required to induce their phosphorylation and localization at damaged foci. Although under mild stress FOXO3a promotes ATM, under acute stimuli FOXO3a triggers DNA-damage induced apoptosis (Tsai et al., 2008). FOXO3a is a mediator for ATM association to the histone acetyltransferase KAT5/Tip60 to enhance ATM activation (Adamowicz et al., 2016).

Overall, these researches proved that FOXO3a is implicated in genome stability through induction of DNA repair gene transcription (Mueller et al., 2014; Tran et al., 2002) but also by modulating protein-protein interaction, phosphorylation and protein complex chromatin retention (Adamowicz et al., 2016; Chung et al., 2012; Tsai et al., 2008). DAF-16/FOXO3a role on genome instability have been observed upon treatments inducing mainly double stranded DNA breaks (UV, bleomycin). It would be of interest to study whether FOXO3a is involved in the repair other types of DNA damages. Due to its impressive role on mitochondrial homeostasis and ROS control, it is noteworthy to determine whether FOXO3a is also involved in oxidative stress induced DNA damage.

#### 3.4.8 FOXO3a in cellular senescence

Cellular senescence is a process in which cells undergo irreversible cell cycle arrest and a pro-inflammatory secretome (Gorgoulis et al., 2019). Senescence is triggered by cellular replication related telomere shortening (reaching the Hayflick limit), oncogenic stress (OIS), oxidative stress and genotoxic stress.

Senescent primary human fibroblasts (HDF) displayed decreased FOXO3a expression in senescent cells (Kyoung Kim et al., 2005). Young HDF inhibited for FOXO3a have an enlarged and flattened: a senescent-like morphology and one third were positive for  $\beta$ -galactosidase (Kyoung Kim et al., 2005). Several studies on Akt mediated senescence induction (or premature senescence) suggest that upon Akt activation, FOXO proteins are repressed inducing ROS accumulation (in part due to SOD2 downregulation), leading to cell cycle arrest via p53/p21 or p15/p16 pathways and senescence (El Maï et al., 2020; Imai et al., 2014; Kyoung Kim et al., 2005; Miyauchi et al., 2004; Nogueira et al., 2008). Importantly, human primary fibroblasts exposed to an antioxidant treatment decreases the percentage of  $\beta$ -galactosidase positive cells and display a young-like morphology (Kyoung Kim et al., 2005), showing that FOXO effects on senescence relays on its response to oxidative stimuli. The Akt/FOXO pathway seems to be a central position to decide cellular fate between quiescence, senescence and apoptosis (El Maï et al., 2020; Imai et al., 2014; Nogueira et al., 2008).

#### 3.4.9 FOXO3a and telomere attrition

The last hallmark of aging that will be discussed in this manuscript is telomere attrition (López-Otín et al., 2013). As discussed at the beginning of this introduction, telomeric DNA length (TL) is shortened at each cellular division but it is also affected by other endogenous or exogenous processes and stressors.

I found very few data on the literature interested to determine a link between FOXO3a (survival/stress response) and telomeres, two major pathways of aging. For now, two papers addressed this question.

In the first one, spleen cells TL was quantified in a transgenic mouse model in which FOXO3a transactivation activity is impaired (Roberts et al., 2011). There was any effect of FOXO3a inactivated form on TL compared to WT mice. Nevertheless, this study is not conclusive because it uses a point mutation model that does not reduce murine *FOXO3a* mRNA or protein amount (Youngson et al., 2011). Therefore, it suggests that the FOXO3a transcription factor canonical function is not important on TL regulation, but it does not consider non canonical FOXO3a activities. López-Otín and colleagues considered in 2013 that only TL variations could be a feature of aging. However, though balanced DNA length is critical for aging and aging-related diseases,

other factors involved in telomere homeostasis might also affect cellular aging (chromatin higher order organization controlled by long-range loops, telomere protection, relocation of telomeric factors, etc). Given that Youngson and colleagues did a punctual mutation in *FOXO3a* exon 2 and that protein expression was not affected, compared to control mice, it could be hypothesized that the other protein domains and conformation are conserved. Therefore, it is important to determine whether FOXO3a protein is involved in general telomere homeostasis.

The second work focuses on the longevity associated *FOXO3a* SNP *rs2802292*- G allele and its correlation with TL in two human cohorts (25-94 and 40-70 years old) (Allsopp et al., 2019). As expected, in homozygotes for the major T-allele (TT) or heterozygotes (GT), leucocyte TL was progressively decreased during lifetime. Strikingly, TL was conserved during aging in homozygotes for the minor G-allele (Allsopp et al., 2019), being the first demonstration of a genetic regulation of telomere attrition rate. Hence, as the *rs2802292* G-allele creates an enhancer site that upregulates FOXO3a expression (see section FOXO3a and epigenetics alterations) and it is correlated with lower TL shortening rate, one can imagine that FOXO3a longevity associated SNPs mediate their health and longevity benefits, at least in part, through TL maintenance at old ages.



Figure 18: FOXO3a a central piece of the hallmarks of aging.

Modified from (López-Otín et al., 2013). FOXO3a is an anti-aging and pro-longevity transcription factor involved in many cellular processes such as nutrient sensing, mitochondrial homeostasis, autophagy, etc. It regulates aging via its canonical function of gene expression modulation, but also by binding to some proteins or complexes and some *FOXO3a* gene SNPs have enhancer and chromatin remodeler functions.

DR: Dietary Restriction

In summary, FOXO3a and the DAF-16/FOXO proteins play a central role in the aging process, taking a part in each of its major features (Figure 18). The FOXO example discussed here unveils that the physiological decline observed at old ages is not the consequence of 9 separate and individually regulated signaling pathways. Instead, all these hallmarks are interconnected, sharing common targets or effectors. Therefore, the aging process is driven by a network of regulations, pathways and factors that must not be consider separately.



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## *Results*

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## First objective of the project

During aging, long-lived post-mitotic cells (LLPMC), such as skeletal muscle cells or neurons, display an altered proteostasis, autophagy, frequently associated with an accumulation of ROS and even some marks of senescence. It is important to comment that the “marks of senescence” taken into consideration are those that have been described in mitotic cells. The case of the human skeletal muscle aging is that a decline on regenerative capacity and mass loss is detected around at 40 years old. The loss of satellite cells does not appear to be involved in the development of sarcopenia, at least in mouse model (Fry et al., 2015). Nonetheless, over time, skeletal muscle experiences metabolic and transcriptional changes, motor unit reorganization or dismantlement, altered autophagy, fast fibers (type II) progressive loss (Etienne et al., 2020; Lexell et al., 1988; McCormick and Vasilaki, 2018; Miljkovic et al., 2015; Renault et al., 2002a). The time-dependent driver(s) of this muscle aging phenotype is (are) still elusive.

At the beginning of my PhD, little was known about telomere status during post-mitotic cells aging. Whether telomeric functions were involved in LLPMC aging, as it is in mitotic cells, was neglected given that LLPMC do not divide. A consensus is still lacking on the dynamic of TL in LLPMC during aging. Nevertheless, telomere homeostasis and subsequent regulation not only depends on TL but also on chromatin alterations, telomeric DNA integrity, telomeric protein expression and relocalization. Therefore, we hypothesized that chromatin structure and telomeric protein expression could be involved. I contributed to the project looking forward to answering this question. First, we studied shelterin expression human skeletal muscle biopsies, at different ages. Among the six sheltering human proteins only TRF2 expression decreases with age. Strikingly, TRF2 expression decline was brutal, and it starts in young adults (around 20 years old).

Hence, the first aim of our work was to determine the consequences of TRF2 downregulation in skeletal muscle.



# Mitochondrial function in skeletal myofibers is controlled by a TRF2-SIRT3 axis over lifetime

Jérôme D. Robin<sup>1,2</sup> | Maria-Sol Jacome Burbano<sup>1</sup> | Han Peng<sup>3</sup> | Olivier Croce<sup>1</sup> | Jean Luc Thomas<sup>4</sup> | Camille Laberthonniere<sup>2</sup> | Valerie Renault<sup>1</sup> | Liudmyla Lototska<sup>1</sup> | Mélanie Pousse<sup>1</sup> | Florent Tessier<sup>1</sup> | Serge Bauwens<sup>1</sup> | Waiian Leong<sup>3</sup> | Sabrina Sacconi<sup>1,5</sup> | Laurent Schaeffer<sup>4</sup> | Frédérique Magdinier<sup>2</sup> | Jing Ye<sup>3</sup> | Eric Gilson<sup>1,3,6</sup>

<sup>1</sup>Université Côte d'Azur, CNRS, Inserm, Institut for Research on Cancer and Aging, Nice (IRCAN), Medical School of Nice, Nice, France

<sup>2</sup>Marseille Medical Genetics (MMG), U1251, Aix Marseille University, Marseille, France

<sup>3</sup>International Research Laboratory in "Hematology, Cancer and Aging", Shanghai Jiao Tong University School of Medicine/Ruijin Hospital/CNRS/Inserm/Nice University, Pôle Sino-Français de Recherche en Sciences du Vivant et Génomique, Shanghai Ruijin Hospital, Shanghai, China

<sup>4</sup>Neuromuscular Differentiation Group, Institut NeuroMyoGene (INMG), UMR5310, Inserm U1217, Ecole Normale Supérieure de Lyon, Lyon, France

<sup>5</sup>Peripheral Nervous System, Muscle and ALS, Neuromuscular & ALS Center of Reference, FHU Oncoage, Pasteur 2, Nice University Hospital, Nice, France

<sup>6</sup>Department of Medical Genetics, Archet 2 Hospital, FHU Oncoage, CHU of Nice, Nice, France

## Correspondence

Eric Gilson, Université Côte d'Azur, CNRS, Inserm, Institut for Research on Cancer and Aging (IRCAN), Medical School of Nice, France.  
Email: eric.gilson@unice.fr

Jing Ye Ruijin Hospital of Schanghai, Shanghai Jiao Tong University, Shanghai, China.  
Email: yj11254@rjh.com.cn

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## Abstract

Telomere shortening follows a developmentally regulated process that leads to replicative senescence of dividing cells. However, whether telomere changes are involved in postmitotic cell function and aging remains elusive. In this study, we discovered that the level of the TRF2 protein, a key telomere-capping protein, declines in human skeletal muscle over lifetime. In cultured human myotubes, TRF2 downregulation did not trigger telomere dysfunction, but suppressed expression of the mitochondrial Sirtuin 3 gene (*SIRT3*) leading to mitochondrial respiration dysfunction and increased levels of reactive oxygen species. Importantly, restoring the Sirt3 level in TRF2-compromised myotubes fully rescued mitochondrial functions. Finally, targeted ablation of the *Terf2* gene in mouse skeletal muscle leads to mitochondrial dysfunction and sirt3 downregulation similarly to those of TRF2-compromised human myotubes. Altogether, these results reveal a TRF2-SIRT3 axis controlling muscle mitochondrial function. We propose that this axis connects developmentally regulated telomere changes to muscle redox metabolism.

## KEYWORDS

aging, mitochondria, postmitotic cells, skeletal muscle, telomeres

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## 1 | INTRODUCTION

Aging is characterized by an overall decline in the maintenance of tissue homeostasis, organ function, and stress response. In tissues, the accumulation of senescent cells (i.e., permanent cell cycle arrest in response to various types of stress or tissue remodeling) has emerged as an important contributor to aging, mainly through nonautonomous cell mechanisms driving chronic inflammation and tissue degeneration (Baker et al., 2016; van Deursen, 2014; López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013). Tissue aging is also characterized by the progressive decline in the function of long-lived postmitotic cells, such as myofibers and neurons.

Normal skeletal muscle aging is mainly driven by changes occurring in postmitotic, fully differentiated multinucleated myofibers such as progressive alterations in transcriptional and metabolic programs, dismantlement of neuromuscular junctions, and progressive loss of the most powerful fast fibers (Bua et al., 2006; Lexell, 1995; Miljkovic, Lim, Miljkovic, & Frontera, 2015; Renault et al., 2002). A decline of muscle stem cells was associated with the etiology of sarcopenia, an age-associated loss of skeletal muscle mass and strength (Snijders & Parise, 2017). However, sarcopenia cannot be only explained by the loss of stem cell over lifetime (Fry et al., 2015). Thus, the age-dependent mechanisms triggering muscle aging and their links to the cellular senescence pathways remain largely unknown (Fry et al., 2015; Lexell, 1995; Miljkovic et al., 2015; Renault et al., 2002).

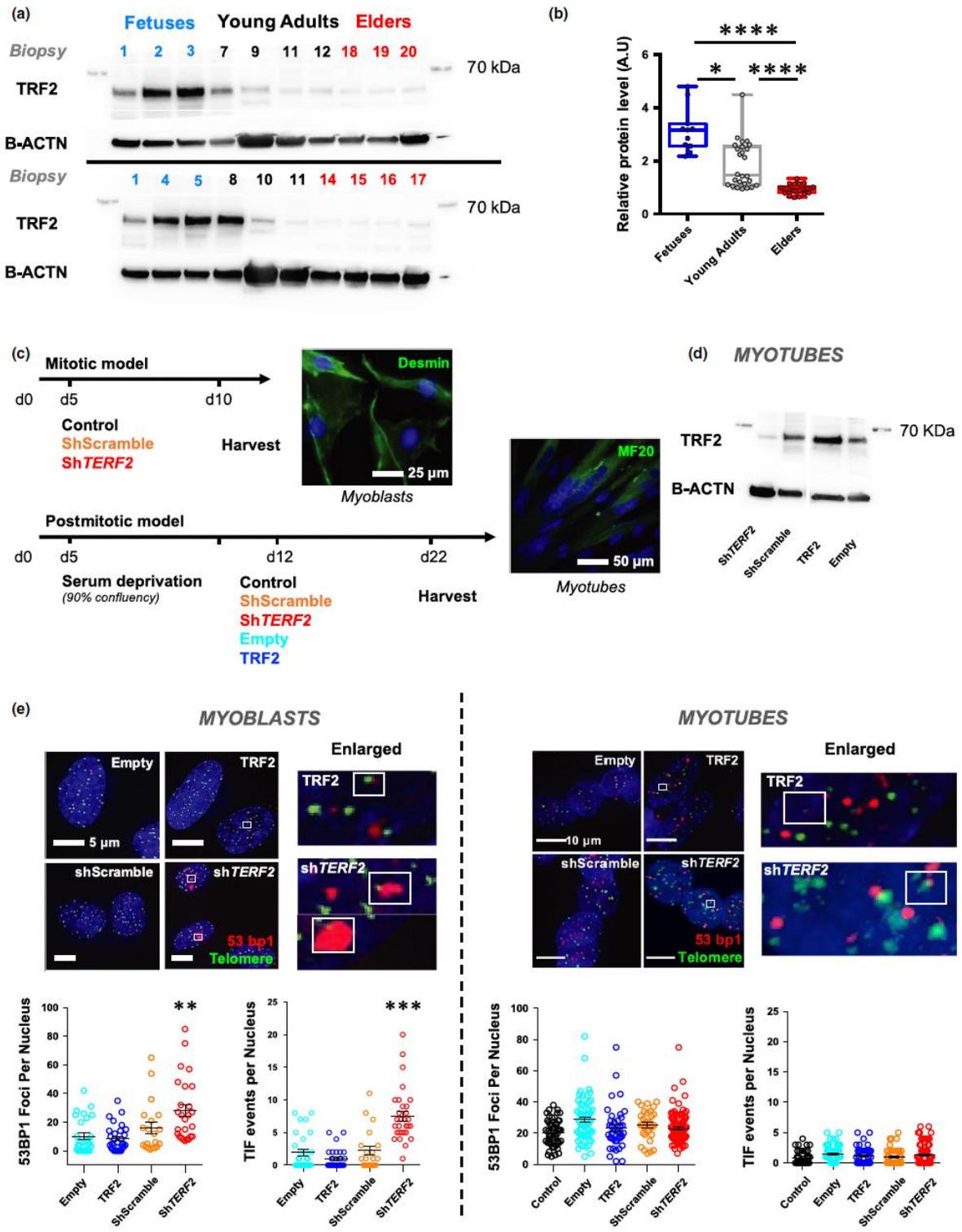
Unlike stem cells, wherein division and mobilization can be considered as a temporary rejuvenation mechanism due to the accumulation of molecular alterations such as telomere shortening, long-lived postmitotic cells mainly rely on their capacity for repair, quality control, and intracellular renewal (i.e., autophagy, mitophagy, and proteostasis). It is generally accepted that the age-dependent increase in reactive oxygen species (ROS) production by dysfunctional mitochondria and the inherent inability of autophagy and other cellular mechanisms to remove damaged molecules are responsible for progressive functional decline in long-lived postmitotic cells (Bua et al., 2006; Linnane, Marzuki, Ozawa, & Tanaka, 1989; Rubinsztein, Mariño, & Kroemer, 2011). Therefore, the current model for aging in these cells relies on the accumulation of molecular damage, mitochondrial dysfunctions, and increased ROS production, which progressively alter their transcriptional program, metabolism, and functional performance. Whether these aging events result from developmentally regulated mechanisms or a simple cumulative effect is still elusive.

Among known mechanisms of aging, the unrelenting erosion of telomeres acts as a developmentally regulated process driving replicative senescence (Gilson & Géli, 2007; López-Otín et al., 2013). Besides requiring the telomerase to be fully replicated, telomeres have several peculiarities and adopt specific chromatin conformations involving telomeric DNA looping (t-loop), the binding of specific protective factors such as the shelterin complex and non-coding RNA (i.e., TERRA; Gilson & Géli, 2007). In humans, the shelterin complex comprises six proteins: the telomeric repeat-binding factor 1 (TRF1; also known as TERF1); the telomeric repeat-binding factor 2 (TRF2; also known as TERF2); the protection of telomeres 1 (POT1); the ACD shelterin complex subunit and telomerase recruitment factor (TPP1); the repressor activator protein 1 (RAP1); and the TRF1-TRF2 interacting nuclear protein 2 (TIN2). This complex binds to telomeres, favors t-loop formation, and prevents unwanted activation of the DNA damage response (DDR).

The current view on telomere dynamics in long-lived postmitotic cell aging has been revisited in recent studies that reported telomere shortening in cell differentiation (Flores et al., 2008), muscle and fat aging (Carneiro et al., 2016; Daniali et al., 2013), stressed neurons (Zglinicki, 2002), and pathologic myocardium (Chang et al., 2016). Consistent with the existence of a telomere maintenance mechanism specific to postmitotic cells, yeast telomeres undergo a profound nuclear reorganization when cells enter into quiescence (Guidi et al., 2015), a process reminiscent of the postmitotic state in multicellular species.

Notably, a wealth of data indicates that two key aging hallmarks (López-Otín et al., 2013), telomere and oxidative metabolism, are intimately connected. Due to its high content in guanine, telomeric DNA is easily oxidized, leading to the accumulation of 8-oxo-guanine, which disrupts the binding of telomere protective factors (Opresko, Fan, Danzy, Wilson, & Bohr, 2005) and prevents extension by telomerase (Aeby, Ahmed, Redon, Simanis, & Lingner, 2016; Fouquerel et al., 2016). Concomitantly, telomeres shorten under oxidative stress, whereas antioxidant treatments are associated with their reduced erosion (Ahmed & Lingner, 2018; Passos et al., 2007; Zglinicki, 2002), and antioxidant proteins are specifically associated with telomeres (Aeby et al., 2016). The catalytic subunit of telomerase, TERT, can be localized in the mitochondria where it plays a protective role for metabolism and DNA maintenance (Ahmed et al., 2008). Telomerase RNA can be processed within the mitochondria (Cheng et al., 2018), and some shelterin subunits have been shown to have mitochondrial functions (Chen et al., 2012; Kim et al., 2017). Telomere dysfunction

**FIGURE 1** Specific TRF2 downregulation in skeletal muscle and myotubes does not trigger telomere damages. (a) Immunoblots and associated quantifications (in independent duplicates), (b) of whole protein extracts from human biopsies (Figure S1) collected at different ages with antibodies against TRF2 (upper panel) and B-ACTIN used as a loading control (lower panel). Biopsies were grouped in three categories: fetuses; young adults (17–35 yo); and elders (>60 yo). TRF2 level decreases with age, young adults versus elders,  $p < .001$ ; fetuses versus elders,  $p < .001$  (Kruskal–Wallis multiple comparisons test;  $\alpha = 0.05$ ). (c) Schematic representation of the strategy used. Myoblasts stained with anti-desmin antibody and myotubes with MF20 antibody are shown as illustrations; control refers as the untransduced condition. (d) TRF2 immunoblots of transduced human myotubes using B-ACTIN as a loading control.  $\epsilon$  53BP1 staining and TIFs in transduced myoblasts and myotubes performed using a telomeric probe (PNA, green) and 53BP1 (red) antibody indicating dsDNA damage. Means  $\pm$  SEM are shown. Only foci within multinucleated cells, that is, corresponding to postmitotic myotubes were counted ( $n > 40$  nuclei per condition). No statistical difference in myotubes is seen between the different conditions (TRF2 overexpression or knockdown and respective controls; ANOVA, Kruskal–Wallis multiple comparisons test,  $\alpha = 0.05$ ). \* $p < .05$ ; \*\* $p < .01$ ; \*\*\*\* $p < .001$



activates p53, which in turn binds and represses PGC1- $\alpha$  and PGC1- $\beta$  promoters leading to mitochondrial dysfunction (Chang et al., 2016; Sahin et al., 2011). Overall, it appears that telomere dysfunction triggers mitochondrial alteration and *vice versa*. It is a tempting hypothesis that such an "explosive" regulatory loop might drive long-lived postmitotic cell aging. In further support for a role of telomeres in postmitotic cell aging are the differential expression of the TRF2 shelterin subunit in cardiomyocytes upon physical exercise or heart failure (Oh et al., 2003) and the extratelomeric roles of this protein in neuronal gene expression (Robin et al., 2014; Yang et al., 2011; Ye, Renault, Jamet, & Gilson, 2014).

In this study, we explored changes in the telomere state *in vivo* during muscle development and aging and their functional significance *in vitro* using differentiated skeletal muscle fibers (myotubes). We found that TRF2 expression and telomeric DNA length start decreasing in human skeletal muscles of young adults, indicating important developmentally regulated changes in telomere composition at the end of muscle development, which are accentuated in elders. Remarkably, *TERF2* downregulation in human myotubes led not to telomere shortening and deprotection but to changes in mitochondrial respiration and elevated ROS levels through a direct mechanism involving transcriptional regulation of the *SIRT3* subtelomeric gene encoding a mitochondrial Sirtuin. Besides, using a transgenic mouse model abolishing *Terf2* expression in skeletal muscle, we did not observe telomeric deprotection but changes in mitochondrial respiration, increased oxidative environment, and Sirt3 downregulation.

## 2 | RESULTS

### 2.1 | TRF2 level negatively correlates with age in human skeletal muscle biopsies

Rapid muscle cell proliferation occurs during embryogenesis, decreases progressively during the fetal stage, and becomes negligible

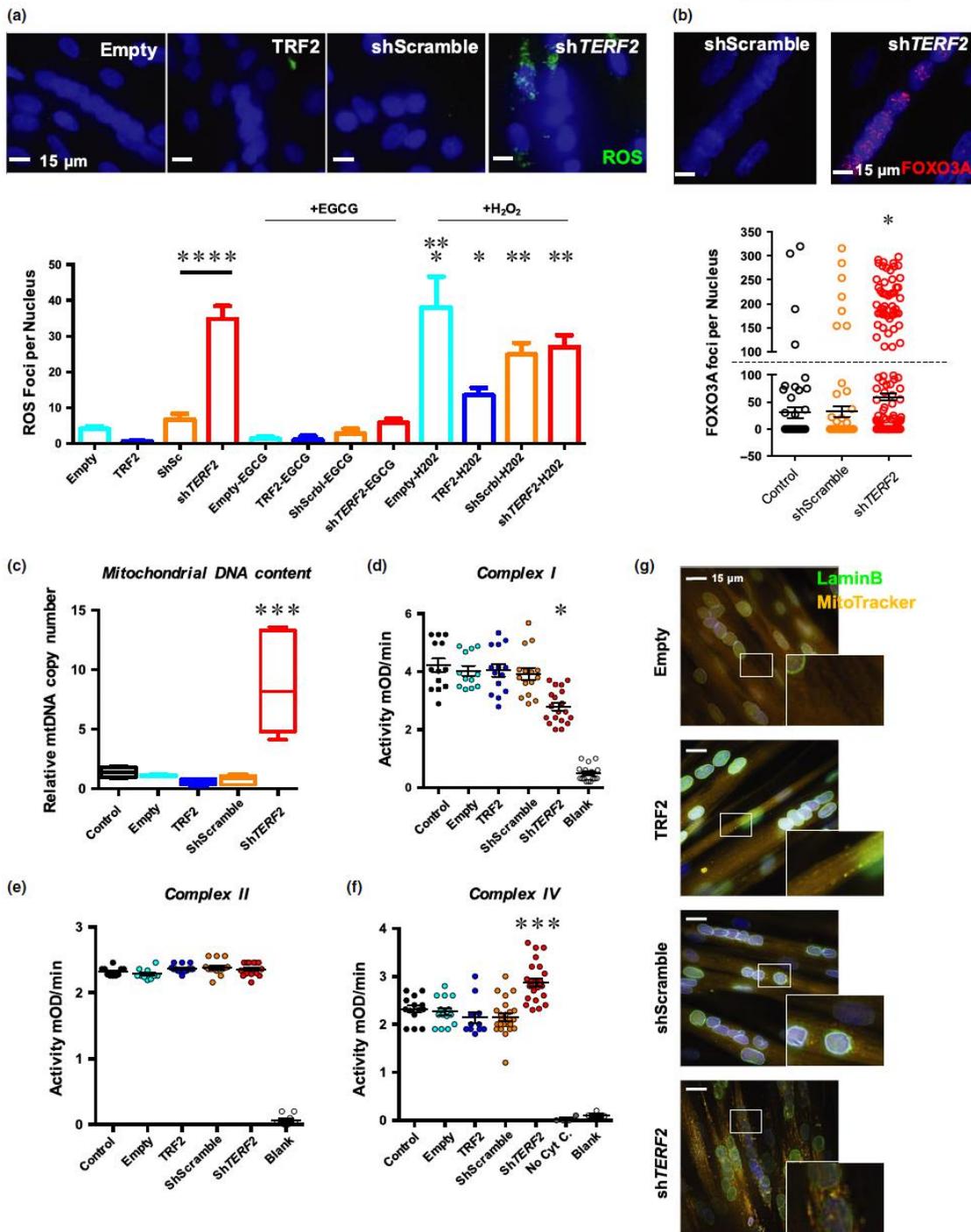
in early adolescence (Lepper, Conway, & Fan, 2009; Miljkovic et al., 2015; Murphy, Lawson, Mathew, Hutcheson, & Kardon, 2011; Relaix & Zammit, 2012). As a consequence, the proportion of satellite cells decreases from ~30% to 5%–6% between the postnatal period and early teens (Dayanidhi & Lieber, 2014; Romero, Mezmezian, & Fidziańska, 2013; Schultz, 1974). Adult skeletal muscle is mainly composed of postmitotic differentiated multinucleated myofibers and 5%–6% of quiescent satellite cells (Moss & Leblond, 1971; Schultz, Gibson, & Champion, 1978) suggesting a minimum influence of the pool of satellite cells in the composition of skeletal muscle tissue when addressing biological parameters such as telomeres (e.g., length, shelterin complex).

Using human skeletal muscle biopsies isolated from individuals at different ages ( $n = 20$ , fetuses to 72-year-old adults; Figure S1a), we first confirmed telomere shortening with age as reported previously (Figure S1b; Daniali et al., 2013). In a striking parallel with telomere shortening, we found that TRF2 protein levels decreased markedly during the third decade of life and were negatively correlated with age (Figures 1a–b and S1c;  $R^2 = .6967$ ). Importantly, among the six known shelterin proteins (e.g., TRF1, TRF2, POT1, RAP1, TPP1, and TIN2), only TRF2 levels were significantly associated with this age-associated pattern (Figure S1d), suggesting a specific role of TRF2 in muscle cell function.

### 2.2 | TRF2 downregulation in human myotubes did not trigger telomere damage

To investigate the consequences of the TRF2 downregulation observed in skeletal muscle in early adulthood (Figure 1a–b), we knocked down *TERF2* expression using shRNAs in cultured myotubes. At confluence, primary myoblasts obtained from a human biopsy (Robin, Wright, et al., 2015) were switched to differentiation medium (day 5; 2% horse serum). They formed myotubes at day 10, were transduced with lentiviral particles at day 12, and

**FIGURE 2** TRF2 knockdown is associated with increased ROS and modifies mitochondrial activity in postmitotic tissues. (a) Detection of ROS foci in transduced myotubes. We report the total number of ROS foci normalized to the number of nuclei. On average, the number of nuclei per cell and DAPI intensities was identical between conditions (Figure S3).  $N > 300$  nuclei per condition, means  $\pm$  SEM are shown. Hydrogen peroxide treatment increases ROS foci number (Empty vs. Empty + H<sub>2</sub>O<sub>2</sub>,  $p = .0274$ ; Empty vs. TRF2 + H<sub>2</sub>O<sub>2</sub>,  $p = .0291$ ; Holm–Sidak's multiple comparisons test;  $\alpha = 0.05$ ). Downregulation of *TERF2* significantly increases ROS (shScramble vs. sh*TERF2*, TRCN0000004812,  $p < .0001$ ; Holm–Sidak's multiple comparisons test;  $\alpha = 0.05$ ). (b) FOXO3A staining and quantification of foci per nucleus; single nucleus cells were excluded from the analysis. Positive cells correspond to cells exhibiting  $>100$  FOXO3A foci per nucleus (cutoff symbolized by dashed line).  $N = 300$  nuclei per condition, means  $\pm$  SEM are shown. *TERF2* knockdown is associated with an increase of positive FOXO3A cells ( $n = 7$  vs.  $n = 80$  positive nuclei, shScramble vs. sh*TERF2*, respectively,  $p < .0001$ ; chi-square test;  $\alpha = 0.05$ ). (c) Relative quantification of mitochondrial DNA content in transduced myotubes. Sh*TERF2*-transduced myotubes show an increase in mitochondrial DNA content (shScramble vs. sh*TERF2*,  $p = .0003$ ; Kruskal–Wallis multiple comparisons test;  $\alpha = 0.05$ ).  $N = 6$  per condition (biological triplicate in technical duplicate), means  $\pm$  SEM are shown. (d–f) Mitochondrial complex I (d), II (e), and IV (f) activity in transduced myotubes. Sh*TERF2*-transduced myotubes exhibit specific mitochondrial defects. We report a decreased complex I activity ( $p < .001$ ) and an increased complex IV activity ( $p < .001$ , Kruskal–Wallis multiple comparison test;  $\alpha = 0.05$ ). (g) Mitochondrial network in transduced myotubes was analyzed using MitoTracker<sup>®</sup>. An average of 100 z-stacks was taken for each condition (DeltaVision Elite<sup>®</sup>, GE). Pictures from 10 to 15 independent and randomly chosen microscope fields were taken, treated postacquisition, and deconvoluted with IMARIS. Single nucleus cells were excluded from the analysis. Nuclei were stained using an anti-lamin B antibody (green) and counterstained with DAPI. A punctate mitochondrial staining is observed in myotubes transduced with sh*TERF2* suggesting mitochondrial fission. Similarly, H<sub>2</sub>O<sub>2</sub> treatments induce a punctuated mitochondrial staining (Figure S3). \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .005$ , \*\*\*\* $p < .001$



collected at day 22 (Figure 1c). Concomitantly, transduced sub-confluent myoblasts were collected 5 days after transduction. Decreased *TERF2* expression in myotubes to levels similar to those observed in early adult muscles (Figure 1a,d) revealed no major differences in cell myogenesis, fiber morphology, or number of nuclei per fiber, indicating the absence of apoptosis triggered by this downregulation (Figures 1e and S2a–b). Moreover, *TERF2* downregulation is not associated with a significant modulation of other shelterin proteins (Figure S2c). Staining of telomeric induced foci (TIFs; Figure 1e) showed an increased rate of recruitment of DDR factors to telomeres, indicating that *TERF2* downregulation triggered telomere uncapping in proliferative myoblasts, as observed in mitotic cells. Intriguingly, under all conditions tested (i.e., four different shRNAs), *TERF2* depletion did not trigger TIF formation in differentiated myotubes (Figure 1e; *TERF2* shRNA TRCN000004812; Figure S3a). Consistent with the absence of DDR activation in these differentiated cells, we did not detect phosphorylation of ATM and p53 (Figure S3b), changes in telomere length, or signs of telomere fusion (Figure S3c). Noteworthy, if myotubes do not exhibit an increased rate of TIFs upon *TERF2* downregulation, they have a higher level of basal DNA damages compared to myoblasts (Figure 1e), which likely reflects defects in the DNA repair machinery as previously reported (Vahidi Ferdousi et al., 2014), associated with their post-mitotic nature.

### 2.3 | TRF2 downregulation in human myotubes induced metabolic changes

We then investigated whether *TERF2* inhibition affects other hallmarks of aging, particularly oxidative metabolism (López-Otin et al., 2013). Upon *TERF2* depletion, we observed a significant increase in

ROS levels in myotubes (shScramble vs. sh*TERF2*,  $p < .0001$ ), similar to those induced by  $H_2O_2$  treatments (Figures 2a and S4a), as well as increased expression and activity of the *FOXO3A* key oxidative stress response and longevity regulator (Sandri et al., 2004; Figures 2b and S4b–c).

We next examined mitochondria, an important source of ROS. We observed higher levels of mitochondrial DNA (eightfold increase;  $p = .0003$ ; Figure 2c), decreased complex I, and increased complex IV mitochondrial activity (assessing NADH oxidation and cytochrome C reduction, respectively; Figures 2d–f and S4d) associated with a punctuated appearance of the mitochondrial network, suggesting mitochondrial fission (Figure 2g). Importantly, antioxidant treatment decreased ROS production and restored the mitochondrial network (epigallocatechin gallate; Figures 2a and S5a). Overall, these results indicate that TRF2 depletion triggers important mitochondrial changes. The increase in DNA content and the increased activity of complex IV could reflect compensatory mechanisms to complex I deficiency to preserve mitochondrial ATP production, as suggested by others (Kotiadis, Duchon, & Osellame, 2014; Van Bergen et al., 2011). In this context, the interplay between mitochondrial complexes seems independent of complex II, as no statistical differences were found between conditions (Figure 2e).

Given the observed mitochondrial DNA enrichment and modulated activity of the different respiratory complexes (I and IV), we further examined the global oxidative capacity of transduced myotubes by NADH-tetrazolium reductase (NADH-TR), a staining used in muscle histology for simple fiber-type characterization. We found stronger NADH-TR staining in downregulated *TERF2* myotubes (Figure S5b), suggesting a switch from glycolytic (fast, type II) toward oxidative (slow, type I) fibers, the latter being associated with development, aging, and stronger resistance to oxidative stress (Lexell, 1995; Miljkovic et al., 2015).

**FIGURE 3** TRF2 binds and modifies expression of the subtelomeric *SIRT3* gene. (a) Venn diagrams generated from a TRF2 ChIP-Seq performed in myotubes, and all data are uploaded into the GEO database under the accession number GSE88983. Significant peaks ( $p < .05$ ) were identified and annotated. A differential analysis identified the significant peak that was "lost" or "gained" by TRF2 modulations. A final Venn diagram was then produced using the genes associated with the peaks found only in the shScramble condition (e.g., lost gene); the genes related to the peaks found only in the TRF2 condition (e.g., gained gene) and genes related to ITS by an in silico analysis (see Section 4 for details). The list of 53 genes localized within 100 kb of modulated ChIP peaks and ITS can be found in Table S1. This analysis led to identification of *SIRT3* as a candidate gene. (b) Gene expression quantified by RT-qPCR in transduced myotubes normalized to three housekeeping genes (HKG: *HPRT*, *PPIA*, and *GAPDH*;  $\Delta\Delta Ct$  method).  $N = 6$  per condition (technical duplicates of biological triplicates), means  $\pm$  SEM with associated statistical significance are reported (Kruskal–Wallis multiple comparisons test;  $\alpha = 0.05$ ). *TERF2* depletion reduces transcription of *SIRT3* without modulating other main Sirtuins, correlates with *PGC1 $\alpha$*  upregulation, and is associated with reduced *SIRT3* activity as reported in (c) *SIRT3* activity in enriched mitochondria extracts from transduced myotubes. A purified *SIRT3* recombinant protein was used as positive control (Kruskal–Wallis multiple comparisons test;  $\alpha = 0.05$ ). (d–g) *SIRT3* rescue experiments and immunoblots of enriched mitochondria extracts from transduced human myotubes using HSP60 as a loading control (d). *TERF2*-modulated expression results in decreased *SIRT3* level (shScramble vs. sh*TERF2*,  $p = .0425$ ). (e–f) Mitochondrial complex I (e) and *SIRT3* activity (f) in mitochondrial extract from transduced myotubes. *SIRT3* overexpression in TRF2-depleted myotubes restores the mitochondrial-associated activity (shScramble vs. sh*TERF2*-*SIRT3*,  $p > .9$ ; in both assays, Holm–Sidak's multiple comparisons test;  $\alpha = 0.05$ , Figure S5). (g) ROS foci in transduced myotubes. We report the total number of ROS foci normalized to the number of nuclei (Figure S6).  $N > 400$  nuclei per condition, means  $\pm$  SEM are shown. *SIRT3* overexpression decreases ROS foci number upon *TERF2* downregulation (shScramble vs. sh*TERF2*-*SIRT3*,  $p = .496$ ; shScramble vs. sh*TERF2*,  $< .0001$ ; Holm–Sidak's multiple comparisons test;  $\alpha = 0.05$ ) and protects myotubes under  $H_2O_2$  treatment (Empty +  $H_2O_2$  vs. *SIRT3* +  $H_2O_2$ ,  $p = .0034$ ; ShScramble +  $H_2O_2$  vs. *SIRT3*-sh*TERF2*,  $p = .039$ ; Holm–Sidak's multiple comparisons test;  $\alpha = 0.05$ ). \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ ; \*\*\*\* $p < .0001$ ; # $p < .05$ ; ## $p < .01$  ( $H_2O_2$  conditions)



## 2.4 | TRF2 controls mitochondrial function by activating SIRT3 expression

Considering that the role of TRF2 in mitochondrial function appears to be independent of telomere protection and that TRF2 occupies extra telomeric sites where it regulates the expression of neighboring genes (Biroccio et al., 2013; Simonet et al., 2011; Yang et al., 2011; Ye et al., 2014), we searched genome-wide for TRF2 binding sites in transduced myotubes (TRF2 up- or down-regulation) using chromatin immunoprecipitation (ChIP) followed by deep sequencing (ChIP-Seq). Using peak calling tools, we identified putative TRF2 binding sites along with their genomic distribution and compared peaks between myotubes with up- or downregulation of TRF2 (Figure S6). Interestingly, some peaks correspond to TRF2-bound ITSs (interstitial telomeric sequences) previously characterized in cancer cell lines, such as a binding site at the intron of the *HS3ST4* gene (Simonet et al., 2011; Yang et al., 2011), whose expression is regulated by TRF2 (Biroccio et al., 2013). We first validated ChIP-Seq data by droplet digital PCR (ChIP-ddPCR) (Robin et al., 2014) at the TRF2-enriched peak localized within the *HS3ST4* gene (Figure S7a). Next, we generated a list of genes localized within a distance of 100kb from putative TRF2 binding sites and inversely modulated by TRF2 levels (gain and loss; Figure 3a). By crossing position of these genes with ITSs, we produced a list of 53 genes associated with TRF2 and with at least one ITS (Table S1). Among them, the *SIRT3* gene encoding for the mitochondrial Sirtuin-3 NAD-dependent deacetylase (SIRT3), which is involved in complex I activity (Ahn et al., 2008; Karamanlidis et al., 2013), mitochondrial homeostasis (Sack, 2012), and senescence (Wiley et al., 2016), represents an attractive and coherent TRF2 target regarding metabolic changes induced by *TERF2* downregulation. Using ChIP-ddPCR, we confirmed changes in TRF2 binding to the ITSs of the *SIRT3* locus as well as to the *SIRT3* promoter (Figure S7b–c). Upon *TERF2* downregulation, these binding sites were depleted in TRF2, whereas the *CICp23* subtelomeric pseudogene promoter, which is located

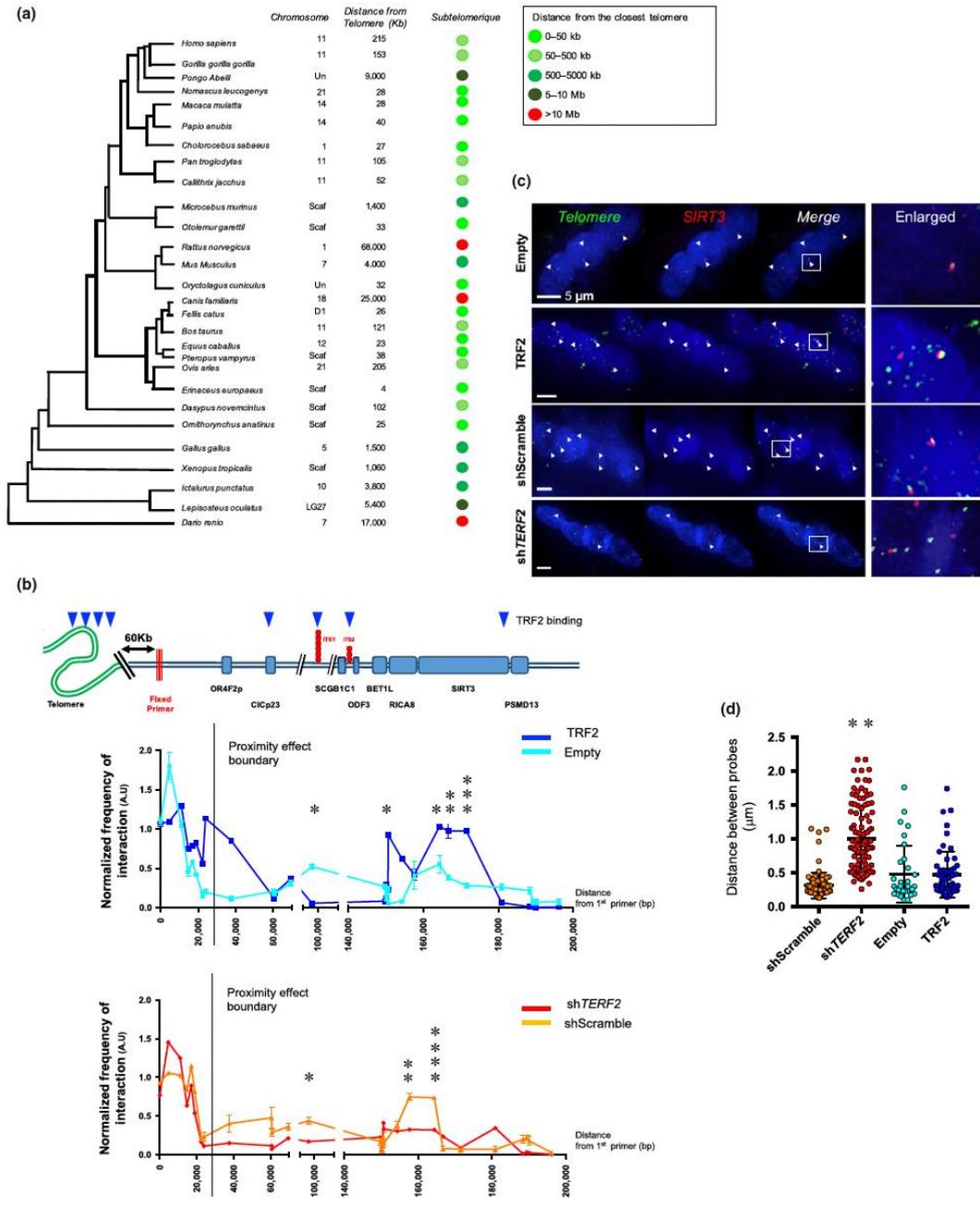
closer to the telomere, was enriched. We speculated that TRF2 binding loss triggers large-scale subtelomeric chromatin changes that expose the *CICp23* gene, which is localized between the 11p telomere and the *SIRT3* locus, to the spreading and relocalization of the shelterin protein complex, an explanation consistent with the dynamic chromatin boundaries described for other telomeres (Koering et al., 2002; Robin et al., 2014).

Next, we examined the expression levels of *SIRT3* and other SIRT3s. In postmitotic myotubes but not in dividing myoblasts, *TERF2* depletion led to specific and restricted downregulation of *SIRT3* (Figures 3b and S7), consistent with the presence of TRF2 binding sites in the vicinity of the *SIRT3* gene (Figure S7b–c). Using enriched mitochondrial extracts from transduced human myotubes, we found that SIRT3 deacetylase activity was decreased upon *TERF2* knockdown (Figure 3c). Together, these results show that TRF2 is involved in the direct regulation of *SIRT3* expression and enzymatic activity.

Notably, the expression of *PGC1 $\alpha$* , a mitochondrial regulator gene known to be positively regulated by the RAP1 shelterin subunit (Martinez et al., 2013) and repressed by p53 activation (Sahin et al., 2011), is inversely proportional to *TERF2* expression (myotubes; Figure 3b). In human myotubes, RAP1 levels were not modulated by *TERF2* downregulation (Figure S2c). These results indicate that the mitochondrial dysfunction triggered by TRF2 depletion does not result from a RAP1-mediated downregulation of *PGC1 $\alpha$*  and is consistent with our observation that TRF2 depletion does not activate p53 (Figure S3a–b). Thus, *PGC1 $\alpha$*  upregulation is likely a compensatory mechanism of the mitochondrial dysfunction and increased ROS production triggered by TRF2 inhibition rather than directly regulated by shelterin proteins.

Importantly, we asked whether the restoration of a physiological level of SIRT3 in TRF2-compromised myotubes would rescue the oxidative stress and mitochondrial dysfunction triggered by TRF2 depletion. Impressively enough, upon *TERF2* knockdown, ectopic *SIRT3* expression (Figures 3d and S7e) restored SIRT3 activity (Figure 3f), mitochondrial activity (Figure 3e, sh*TERF2* vs. Rescue,

**FIGURE 4** TRF2 depletion modifies higher-order conformation of the subtelomeric *SIRT3* gene. (a) Phylogenetic tree and *SIRT3* localization through evolution. For each species, we report the chromosomal position when available (Scaf: scaffold genome; Un: unplaced) and deduced distance from the closest telomere. Remarkably, the subtelomeric position of *SIRT3* is well conserved, more particularly among mammals and primates. (b) Chromatin conformation capture (3C) assay. Schematic representation of the 11p locus with TRF2 detected binding regions (top); genes and primer localization are aligned to the 3C performed on the 11p locus (first 2Mb), reported below. Myotubes were collected 10 days after transduction. Each measure represents the amplification of interactions involving a fixed primer (red bars) and a second primer along the 2Mb of the locus, both located in proximity of a *HindIII* restriction site. *TERF2* overexpression enhances subtelomeric looping and interaction between the distal part of the 11p subtelomere and the *SIRT3* locus (Empty vs. TRF2 position: 164,360,  $p = .0195$ ; 166,712,  $p = .0039$ ; 171,528,  $p = .0008$ ; unpaired  $t$  test;  $\alpha = 0.05$ ), whereas *TERF2* downregulation decreases these interactions (shScramble vs. sh*TERF2* position: 157,640,  $p = .0017$ ; 164,360,  $p < .0001$ ; 166,712,  $p = .074$ ; 171,528,  $p = .4785$ ; unpaired  $t$  test;  $\alpha = 0.05$ ).  $N = 6$  per data point (technical duplicates of biological triplicates), means  $\pm$  SD are shown. No difference was detected between control conditions (Figure S7). (c–d) DNA FISH in 3D-preserved transduced human myotubes and associated quantifications (c). Telomeres were labeled in green and the *SIRT3* locus in red. Distances between gravity centers of the *SIRT3* locus signal and the closest telomeric signal are reported;  $N > 40$  nuclei per condition, means  $\pm$  SEM are shown. Single nucleus cells were excluded from the analysis (e.g., less than 5% of total values). We observe a significant increase in separated signals corresponding to an increased distance between the telomere and *SIRT3* locus in myotubes transduced with sh*TERF2* (Kruskal–Wallis multiple comparisons test;  $\alpha = 0.05$ ) compared to the other conditions (shScramble, Empty, TRF2). \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$



$p = .0337$ ; Figure 3f, shTERF2 vs. Rescue,  $p < .0001$ ; Figure S7f), decreased ROS production (Figures 3g and S8a), and mitochondrial DNA content (Figure S8b). These results demonstrate that SIRT3

repression in myotubes is responsible for the changes in mitochondrial respiration and elevated ROS levels triggered by TRF2 downregulation.

## 2.5 | TRF2 created a long-distance subtelomeric loop involving the SIRT3 locus

Given the subtelomeric conserved localization of the *SIRT3* gene at a distance of approximately 200 kb from the 11p telomere in human cells (Figure 4a), and the fact that its neighboring genes are not affected by *TERF2* downregulation (Figure S9a), we hypothesized that *SIRT3* could be regulated by a discontinuous telomeric position effect (Koering et al., 2002; Robin et al., 2014) involving a subtelomeric chromatin loop (Lebrun, Fourel, Defossez, & Gilson, 2003; Wood et al., 2014). We applied chromosome conformation capture (3C) and three-dimensional (3D) DNA fluorescent in situ hybridization (FISH) in our transduced myotube model. Using 3C, we identified a TRF2-dependent chromatin loop encompassing the 11p telomere and the *SIRT3* locus (Figure 4b). This loop was observed less frequently in TRF2-compromised myotubes (position 164,360,  $p < .0001$ ) and more frequently in TRF2-overexpressing myotubes (position 164,360,  $p = .0195$ ), indicating that the formation of this loop is dependent on TRF2 level. No difference was observed between control conditions (shScramble vs. Empty;  $p > .1$ ; Figure S9b). Using specific probes against telomeres and the *SIRT3* locus, we further confirmed by 3D DNA FISH that this chromatin loop is less frequent in the nuclei of myotubes transduced with shTERF2 constructs, but conserved under upregulation conditions (Figure 4c–d).

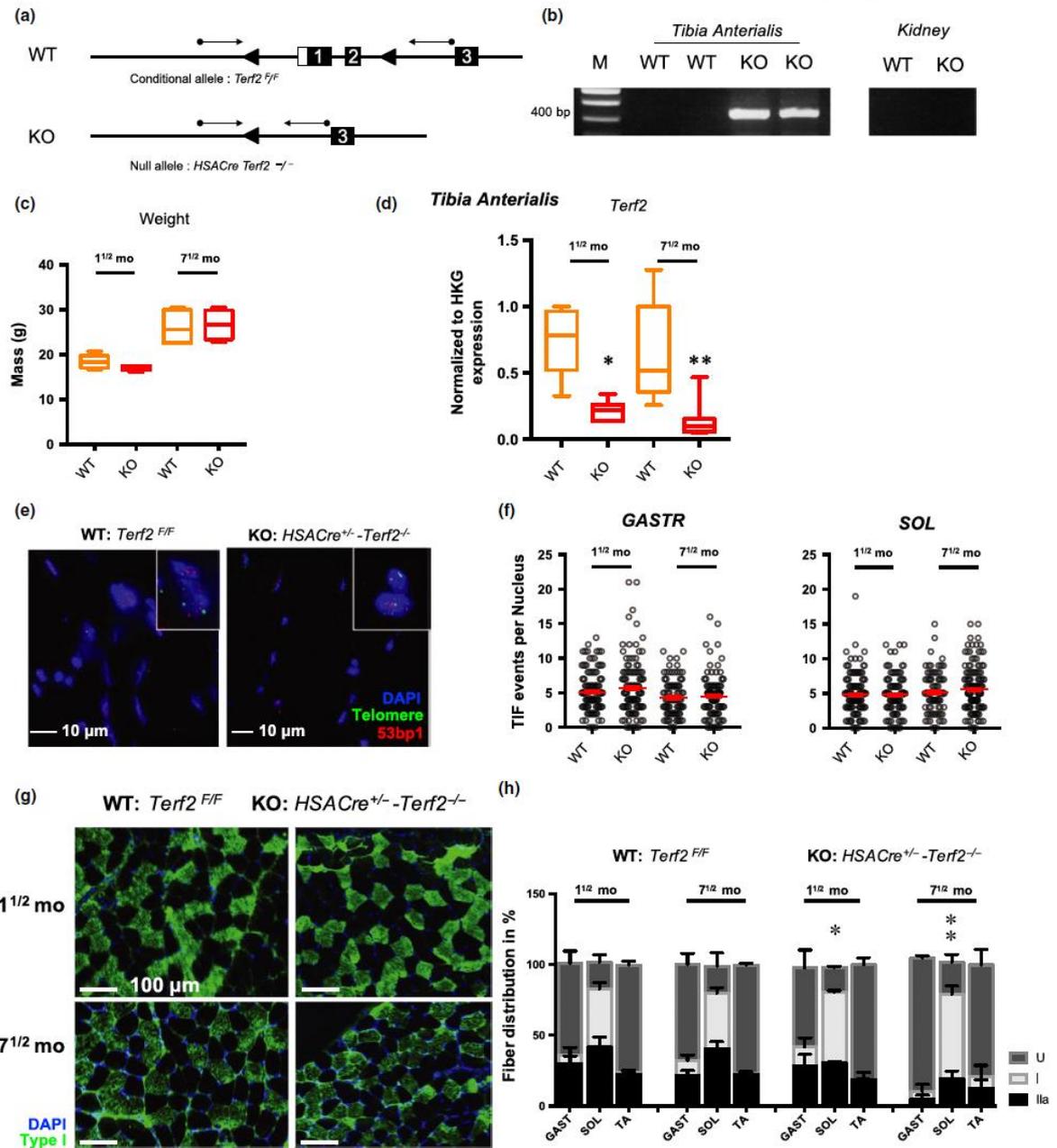
Given the striking conservation of subtelomeric localization of the *SIRT3* locus across evolution (Figure 4a), we next explored whether the ability of TRF2 to mediate a large *SIRT3* subtelomeric loop was conserved in other species. We monitored the formation of the loop and Trf2 dependence in mitotically arrested mouse embryonic fibroblasts (MEFs treated with mitomycin). In mice, *Sirt3* is localized 4 Mb from the 7q chromosome, allowing for the use of a unique 7q telomeric probe. Using 3D-FISH, we observed an interaction between the *Sirt3* locus and the 7q telomeric probe. As in human myofibers, the loop was less frequent upon *Terf2* knockdown (Figure S9c–d) confirming the role of TRF2 in the transcriptional regulation of the *SIRT3* locus.

## 2.6 | Terf2 gene ablation in skeletal muscle increases the proportion of oxidative fibers and leads to mitochondrial changes

To generalize the results obtained with human myotubes, we explored the role of Trf2 in mouse skeletal muscle mitochondria by generating mice where *Terf2* is specifically abrogated in mature fibers (*HSACre-Terf2*, Figure 5a5b). We confirmed Trf2 depletion in skeletal muscle nuclei (Figure S10) and *Terf2* downregulation in whole muscle biopsies (Figure 5d). Up to the age of 10 months, these mice exhibit apparent normal development, weight (Figure 5c), and behavior. Akin to human myotube results, transgenic mouse skeletal muscles (e.g., *gastrocnemius*, *soleus*) did not exhibit any increase in TIFs (Figure 5f–g) but showed an increased proportion of oxidative fibers in the *Soleus* as compared to controls (Figures 5h and S10b,  $p = .037$ ,  $p = .0173$  at 1<sup>st</sup> and 7<sup>th</sup> months, respectively). Then, we set out to address whether this metabolic shift was associated with mitochondrial changes. By qPCR, we found a higher level of mitochondrial DNA in transgenic mice (Figure 6a) along with a disturbed mitochondrial network represented by less grid-like structures (Figure 6b). To strengthen these observations, we tested the mitochondrial complex activities in skeletal muscles (*soleus*, *tibia anterioralis*, *gastrocnemius*), and in control tissues (Heart, Kidney). We found a modified complex I and IV activity, decreased and increased, respectively (Figures 6c and S11), restricted to skeletal muscle tissues (e.g., *soleus* and *tibia anterioralis*) and without modification of complex II. As proposed for human myotubes, the increased activity of complex IV could constitute a compensatory mechanism of complex I dysfunction leading to oxidative stress (Kotiadis et al., 2014). Once again consistent with the results obtained with human myotubes, we detected a decrease of *Sirt3* expression in enriched mitochondrial extracts and a potent activation of Foxo3a in skeletal muscle of transgenic mice (Figure 6d6f).

Altogether, these mouse in vivo results confirm the role of the TRF2-SIRT3 axis in skeletal muscle mitochondrial functions revealed in vitro in human myotubes.

**FIGURE 5** Muscle-specific *Terf2*-deficient mice do not trigger telomere damages but display increased oxidative fibers. (a) Scheme depicting the *Terf2* locus within the targeted conditional allele (*Terf2<sup>fl/fl</sup>*) and the null allele (*Terf2<sup>-/-</sup>*). PCR primers used for genotyping are indicated by arrows. (b) PCR analysis of transgenic mice. Muscle-specific *Terf2* KO allows the amplification of a 385 bp PCR product in muscle fibers. M: molecular weight markers; WT: *Terf2<sup>fl/fl</sup>* mice, KO: *HSACre-Terf2<sup>-/-</sup>*. (c) No statistical differences of weight were seen among mice of the same age. (d) Knockdown of *Terf2* in mature muscle fibers was further validated in RT-qPCR and IFs (Figure S10). *Terf2* expression quantified by RT-qPCR in RNA extracted from skeletal muscle (TA) from WT and KO mice. Each measure represents the average fold-change expression of eight independent repetitions (four mice in technical duplicate) normalized to three housekeeping genes (*Hprt*, *Ppib*, and *Gapdh*;  $\Delta\Delta Ct$  method). Means  $\pm$  SEM with associated statistical significance are reported (Kruskal–Wallis multiple comparisons test;  $\alpha = 0.05$ ). (e) Telomeric induced focus (TIF) analysis in transgenic WT and KO mice and associated quantification reported in (f) in the *soleus* (SOL) and *gastrocnemius* (GASTR). For each group, we report the number of TIFs counted in 30 nuclei per mice for a minimum of four mice (minimum of 120 nuclei per group). Means  $\pm$  SEM are shown. No statistical differences were observed between groups (Kruskal–Wallis multiple comparisons test;  $\alpha = 0.05$ ). (g–h) Fiber-type characterization in *HSACre<sup>+/+</sup>-Terf2<sup>-/-</sup>* mice using myosin heavy chain antibodies against type I and IIa fibers for the *gastrocnemius* (GAST), *soleus* (SOL), and *tibia anterioralis* (TA).  $n = 5$  mice per group. Undetermined fibers (U) represent fibers that remained unstained after using both antibodies (e.g., against type I and type IIa fibers, Figure S10). Means  $\pm$  SEM are shown. \*  $<.05$ ; \*\*  $<.001$ ; \*\*\*  $<.0001$



### 3 | DISCUSSION

Our results revealed a downregulation of TRF2 in a representative cohort of human skeletal muscle biopsies ( $n = 20$ ) over lifetime (Figure 1). The same muscle samples exhibit progressive telomere shortening with age, validating the significance of our cohort regarding the previously observed telomere shortening during muscle aging (Daniali et al., 2013). Mimicking this in vivo regulation,

TRF2 downregulation in a fully controlled in vitro human myotube model did not trigger telomere deprotection but instead triggered mitochondrial changes reminiscent of those observed during development and aging, that is, increased ROS and oxidative metabolism (Figure 2). Moreover, we report a close overlap of our in vitro observations in vivo, using a transgenic mouse model of skeletal muscle-specific *Terf2* ablation. In both models, downregulation of *TERF2* triggered an increase of FOXO3A to the nucleus (Figures 2b

and 6e). A finding that is in agreement with previous work describing FOXO3A as a stress sensor (Milan et al., 2015; Sandri et al., 2004) and mitochondrial regulator (Ferber et al., 2012). Hence, knowing that FOXO3A function declines with age (Mueller et al., 2014) and is linked to longevity (Albani et al., 2014; Flachsbarth et al., 2009), one can hypothesize that upregulation of FOXO3A detected in our models (e.g., myotubes, transgenic mice) illustrates an age-dependent protective mechanism.

Based on these results, we propose that the TRF2 downregulation, starting in early adulthood as a developmentally regulated process, contributes to the physiological adaptation and oxidative metabolic changes occurring in muscles over lifetime (Lexell, 1995; Miljkovic et al., 2015; Powers, Radak, & Ji, 2016). An interesting mechanism would be a direct regulation of *TERF2* by muscle-specific factors involved in fiber-type switching (Murgia et al., 2017) or through changes in the Wnt and beta-catenin pathways that regulate muscle differentiation but also involved in *TERF2* regulation (Diala et al., 2013).

Reminiscent of the antagonistic pleiotropic models of telomeric DNA shortening (Harley, 1997), TRF2 downregulation might be beneficial at young ages and detrimental for muscle function at older ages. Consistent with this hypothesis, a depletion of TRF2 in mouse skeletal muscle triggers a modification of fiber-type proportion toward an increased oxidative metabolism allude for a premature aging phenotype (Alnaqeb & Goldspink, 1987; Lexell, 1995; Miljkovic et al., 2015; Verdijk et al., 2007). Beyond the scope of this study, further characterization of this transgenic model will allow one to appreciate the contribution of TRF2 in the changes and adaptation of skeletal muscle metabolism and physiology during development and aging.

At the molecular level, we elucidated a mechanism by which TRF2 alters muscle cell mitochondrial oxidative respiration. We showed that TRF2 positively regulates the expression of *SIRT3*, a key mitochondrial gene associated with human longevity (Albani et al., 2014; Flachsbarth et al., 2009) and *SIRT3*-dependent deacetylase activity (Figure 3). As described by others, *SIRT3* is a crucial regulator of basal physiology (Ansari et al., 2017) and affects human life span (Kincaid & Bossy-Wetzel, 2013). Importantly, restoring physiological *SIRT3* levels in TRF2-compromised myotubes rescued the

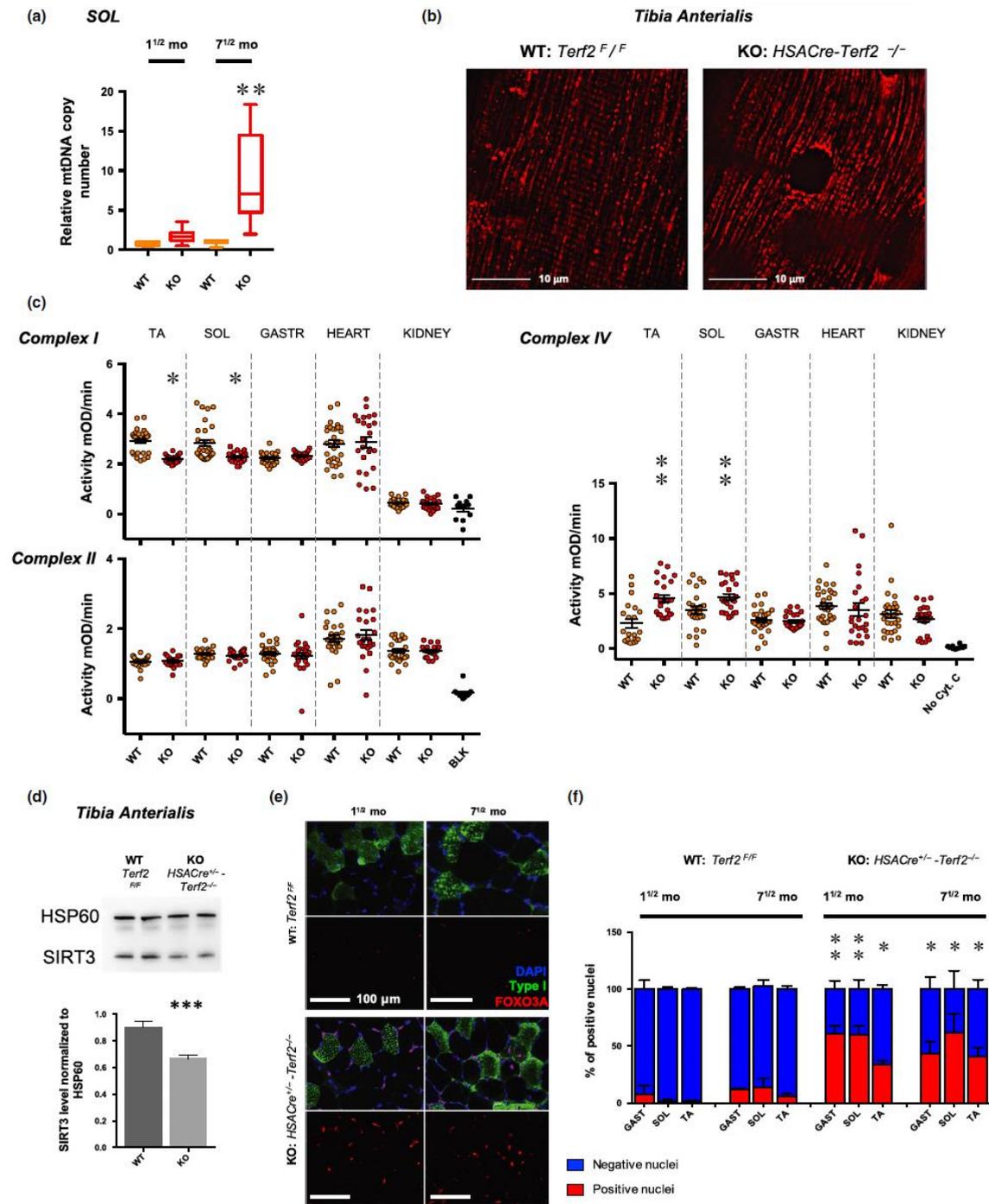
mitochondrial phenotype and decreased ROS levels to those of control cells, establishing that a TRF2-*SIRT3* axis modulates the oxidative metabolism in skeletal muscle fibers.

TRF2 appears to be directly involved in *SIRT3* transcriptional regulation by binding to several sites around the subtelomeric *SIRT3* locus and mediating the formation of a long-distance subtelomeric chromatin loop between the *SIRT3* locus and its proximal telomere (Figure 4). The subtelomeric location of the *SIRT3* locus is highly conserved throughout evolution. Remarkably, a similar Trf2-dependant long-range loop between the *Sirt3* locus and the telomere is also observed in mouse mitotically arrested cells (Figure S9) and Trf2 ablation in mouse skeletal muscle leads to *Sirt3* downregulation, highlighting the functional importance of the TRF2-mediated chromatin loop in regulating *SIRT3* gene expression and, subsequently, oxidative metabolism.

In contrast to the subtelomeric loops encompassing the *TERT* gene at chromosome 5p (Kim et al., 2016) and the *SORBS2* gene at the 4q35 locus (Robin, Ludlow, et al., 2015), the telomere-*SIRT3* loop discovered in this study is required for gene activation as previously observed (*TEAD4*; Robin et al., 2014). The underlying mechanism linking subtelomeric loops to gene regulation and the peculiar telomere protection at play in long-lived postmitotic cells are now key questions for future studies. Whether telomere shortening concomitant to TRF2 downregulation in skeletal muscle destabilizes the telomere-*SIRT3* loop, as observed for other loci (Kim et al., 2016; Robin, Ludlow, et al., 2015; Robin et al., 2014), is an interesting hypothesis. Further, if telomere shortens in human tissues, including skeletal muscle (Daniali et al., 2013), diminution of TRF2, as already reported through lifetime in animal models (e.g., mice, zebrafish; Wagner et al., 2017), remains to be established in other human tissues. Noteworthy, recent studies focusing on another postmitotic tissue (e.g., heart) also link telomere length and TRF2 level to metabolic changes (Chang et al., 2018, 2016; Oh et al., 2003). Last, using a proliferative model (e.g., transduced myoblasts), we observed an increase of the DDR pathway (Figure 1) but no changes in *SIRT3* gene expression (Figure S7d), further arguing in favor of a telomere-dependent mechanism restricted to postmitotic tissues.

Overall, our work uncovers a TRF2-*SIRT3* axis that connects telomere changes to muscle adaptative metabolism, development,

**FIGURE 6** Muscle-specific *Terf2*-deficient mice exhibit increased mitochondrial DNA content, mitochondrial dysfunction, and nuclear Foxo3a accumulation. (a) Relative quantification of mitochondrial DNA content in the *soleus* of transgenic mice (WT and KO). *Terf2* KO mice exhibit a significantly higher mtDNA content (Kruskal-Wallis multiple comparisons test;  $\alpha = 0.05$ ).  $n = 12$  per group, means  $\pm$  SEM are shown. (b) Electroporated *tibia anterioralis* (TA), using Mito-DsRed in control and transgenic mice ( $n = 4$  per group). Electroporation of the construct allows one to stain the mitochondrial network in tissues. The mitochondrial network of KO mice (right) appears punctuated and less structured as in controls (left). (c) Mitochondrial complex I, II, and IV activity in tissues (e.g., kidney; heart; *soleus*; *tibia anterioralis*) from 40-week-old transgenic WT and KO mouse ( $n = 10$  per data point). *Terf2* KO mice exhibit skeletal muscle-specific mitochondrial defects. We report a decreased complex I activity ( $p < .05$ , paired, two-tailed Student's *t* test;  $\alpha = 0.05$ ) and an increased complex IV activity ( $p < .001$ , paired, two-tailed Student's *t* test;  $\alpha = 0.05$ ), potentially part of a compensating phenomenon. No statistical differences were observed between WT and KO kidney and heart extracts. (d) *SIRT3* immunoblots of enriched mitochondria extracts from the *tibia anterioralis* (TA) of transgenic mice using HSP60 as loading control.  $N = 5$  per group. *Terf2* abolished expression results in decreased *SIRT3* level (WT vs. KO,  $p = .0007$ ). (e–f) Foxo3a immunofluorescence and associated quantifications in muscles along with type I staining (e.g., GAST, SOL, and TA). No statistical association was found among positive nuclei and fiber types. *Terf2* KO mice exhibit a higher percentage of Foxo3a-positive nuclei (aged and muscle-matched KO vs. WT,  $p < .05$ ; Kruskal-Wallis multiple comparisons test;  $\alpha = 0.05$ ). \*  $< .05$ ; \*\*  $< .01$ ; \*\*\*  $< .001$



and aging. This finding holds the potential to drive future research on physiological and pathological changes occurring during the lifetime in muscles and other postmitotic tissues (e.g., brain, bones, or fat among others).

#### 4 | METHODS

Detailed methods and Supplemental Material can be found with this article online.

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## CONFLICT OF INTEREST

None declared.

## AUTHOR CONTRIBUTIONS

J.D.R. executed the experiments, helped design, and wrote the manuscript. L.S. and V.R. helped design. M.S.J.B., L.L., W.L., and C.L. provided technical help; H.P., F.T., E.P., and O.C. assisted and performed the bioinformatics analysis of the ChIP-Seq results. F.M. and S.S. obtained muscle biopsies. J.Y. helped project coordination. E.G. designed the project and wrote the manuscript. All authors edited the manuscript.

## DATA AVAILABILITY STATEMENT

All original unprocessed data used for this study are available through a Mendeley database link provided below. The depository includes all raw data for the building of all figures provides and raw files. We also have included a report summary file. This details the software (Prism/IMARIS) and script (ChIP-Seq) used for this study as well as specific reagents (human samples, mice, and antibodies) used to generate our results. All information is accessible at: <http://dx.doi.org/10.17632/mst92jfr.1>. ChIP-Seq raw data and analysis have been deposited to GEO database under the accessing number GSE88983. Queries regarding reagents and materials can be addressed to the corresponding authors.

## ORCID

Jérôme D. Robin  <https://orcid.org/0000-0001-5865-8372>

Frédérique Magdiniér  <https://orcid.org/0000-0002-0159-9559>

Eric Gilson  <https://orcid.org/0000-0001-5738-6723>

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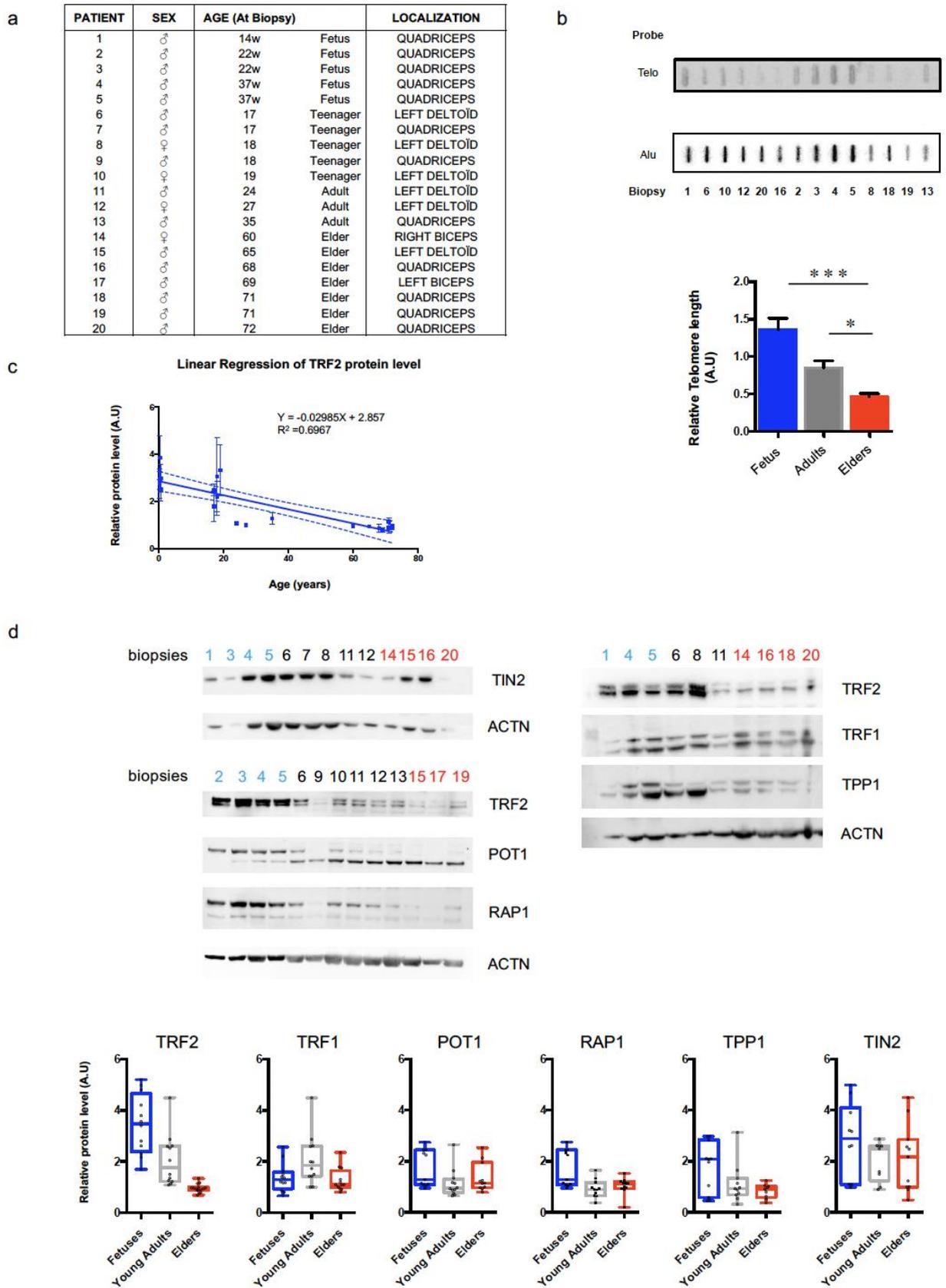
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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Robin JD, Jacome Burbano M-S, Peng H, et al. Mitochondrial function in skeletal myofibers is controlled by a TRF2-SIRT3 axis over lifetime. *Aging Cell*. 2020;19:e13097. <https://doi.org/10.1111/ace1.13097>

Figure S1

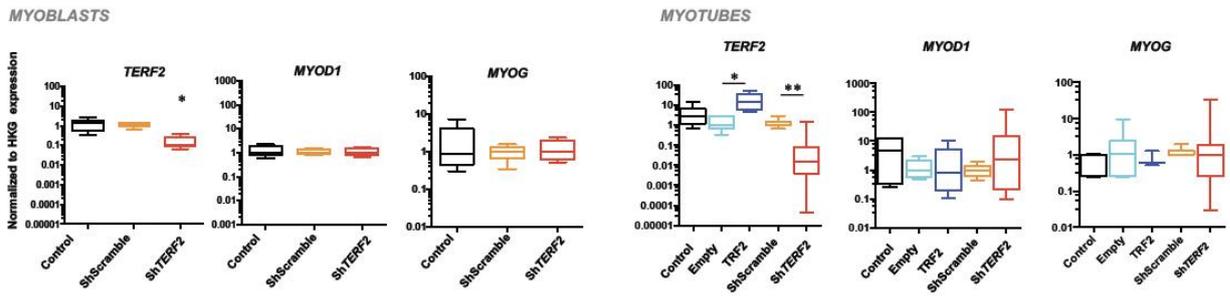


## Figure S1.

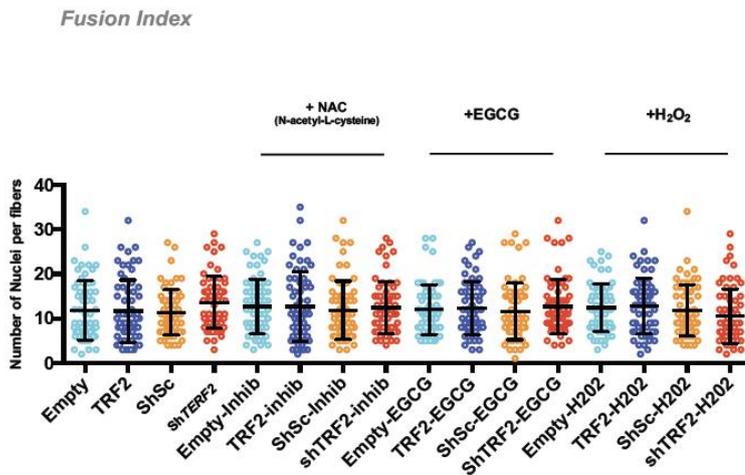
**a.** Recapitulative table of biopsies used in the study. Patient's sex, age at biopsy and localization of puncture are reported. All patients were healthy and free from muscle-related diseases. **b.** Slot blot and associated quantifications of relative telomere signal from human skeletal biopsies normalized to Alu repeats. Telomere length decreases in human skeletal biopsies with age (Fetuses vs. Adults (17/35),  $p=0.27$ ; Adults vs. Elders (60/72),  $p=0.046$ ; Fetuses vs. Elders,  $p=0.002$ ; Kruskal-Wallis multiple comparison test;  $\alpha=0.05$ ). **c.** TRF2 protein level correlates with age in skeletal muscle biopsies. The 95% intervals of confidence are indicated by discontinuous blue lines as well as the equation and associated goodness of fit ( $R^2$ ). **d.** Immunoblots and associated quantifications (bottom) of whole protein extracts from human biopsies collected at different ages with antibodies against TRF2, TRF1, TPP1, TIN2, RAP1, POT1 and B-ACTIN used as loading control (lower panel). Biopsies were grouped in 3 categories: Fetuses; Young Adults (17-35 yo) and Elders (>60 yo).

Figure S2

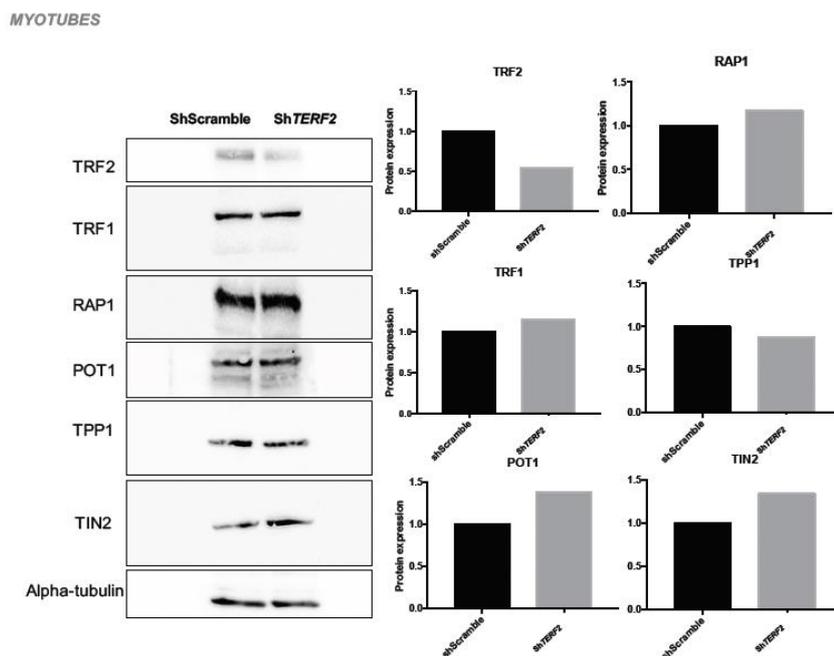
a



b



c



## Figure S2.

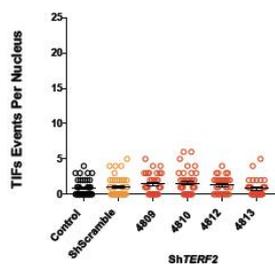
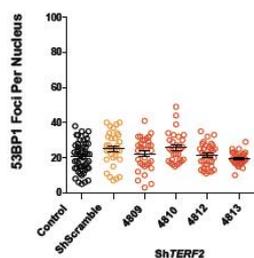
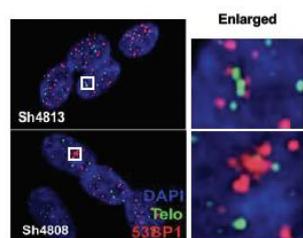
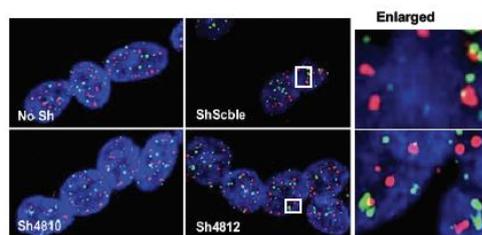
**a.** Gene expression quantified by RT-qPCR in transduced myoblasts and myotubes. Cells were collected 5 and 10 days after transduction for myoblasts and myotubes respectively. Each measure represents the average fold-change expression of six independent repetitions (technical RT duplicates of biological triplicates) normalized to three housekeeping genes (HKG: *HPRT*, *PPIA* and *GAPDH*);  $\Delta\Delta\text{Ct}$  method). Means  $\pm$  SEM with associated statistical significance are reported for myoblasts and myotubes (Mann-Whitney multiple comparison test and Kruskal-Wallis multiple comparison test, respectively;  $\alpha=0.05$ ).

**b.** Fusion index characterization of myotubes transduced and/or treated by N-acetyl-L-Cysteine (NAC), Epigallocatechin gallate (EGCG), two antioxidant or hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Fusion index was calculated as the average number of nuclei counted per myotube at day 17 of differentiation in 10 independent and randomly chosen microscope fields (DeltaVision; GE). Single nucleus cells represented <5% of cells in all conditions. Means  $\pm$  SEM are shown.

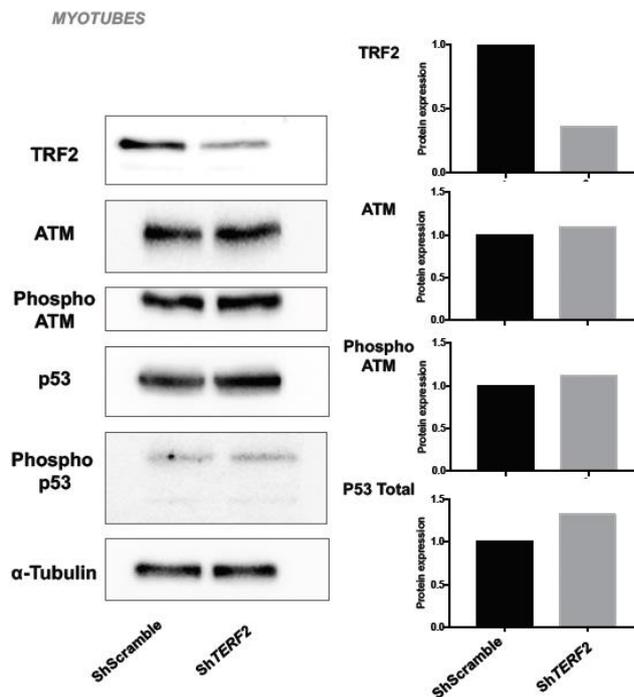
**c.** Immunoblots and associated quantifications of whole protein extracts from human myotubes transduced with sh*TERF2* and associated control (shScramble), collected at day25 with antibodies against TRF2, TRF1, TPP1, TIN2, RAP1, POT1 and alpha-Tubulin used as loading control.

Figure S3

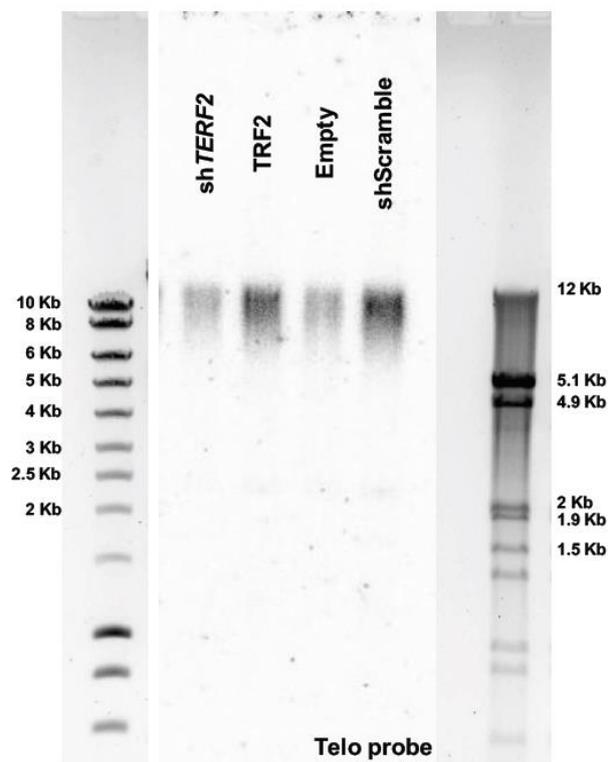
a



b



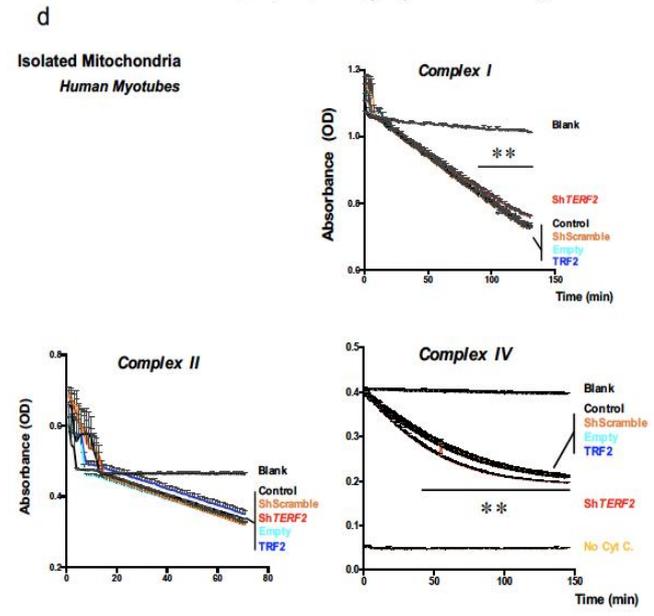
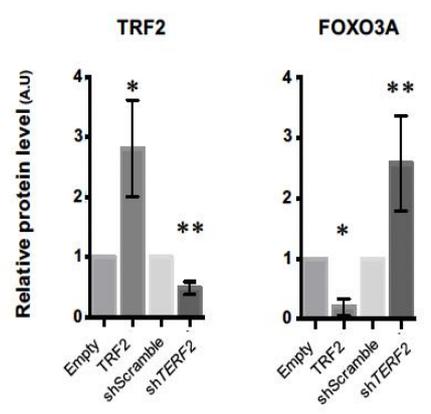
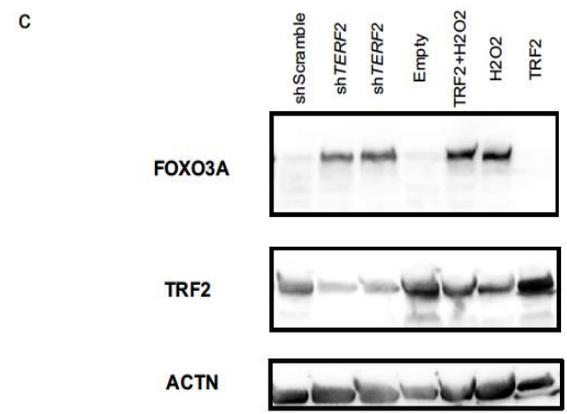
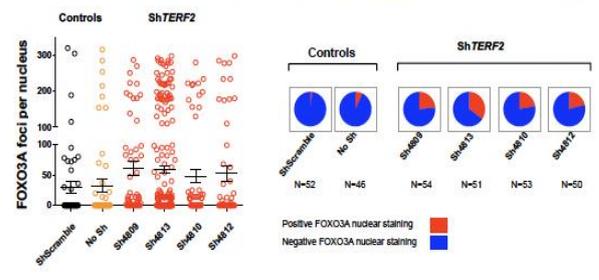
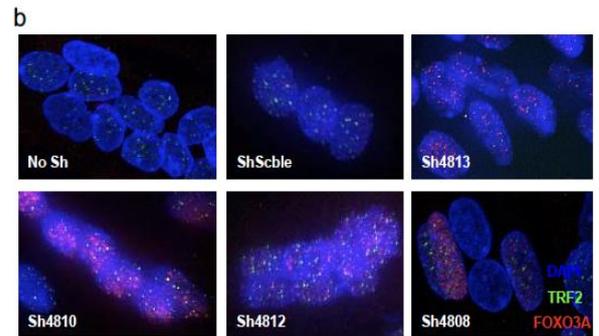
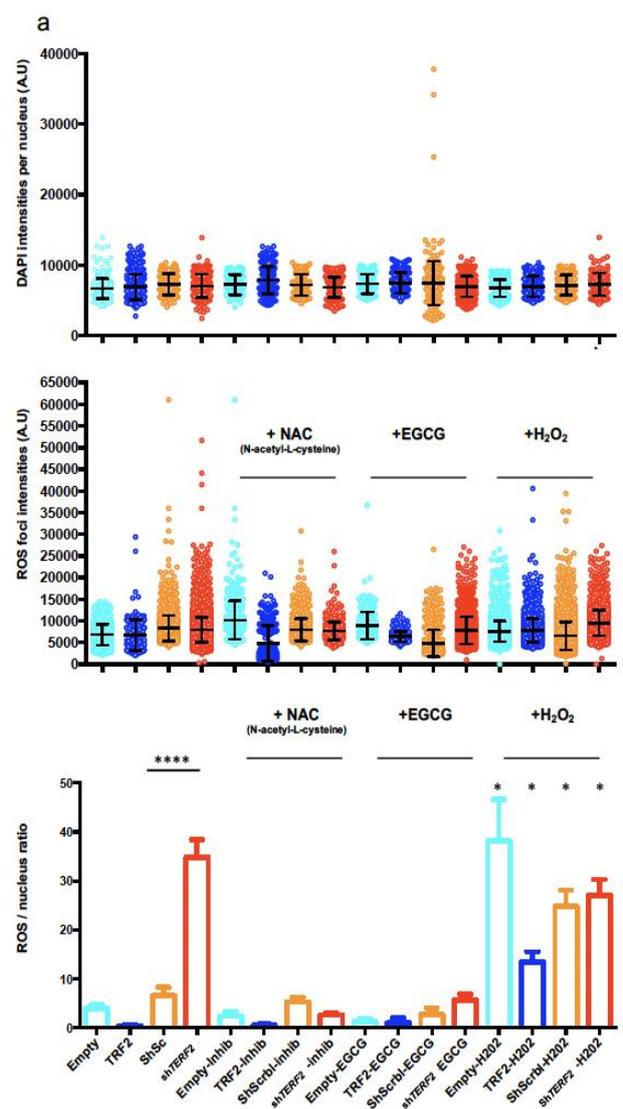
c



### Figure S3.

**a.** 53BP1 staining and TIFs analysis in transduced myotubes using four different ShRNAs against *TERF2*. No statistical difference was seen between the different conditions (ANOVA, Kruskal-Wallis multiple comparison test;  $\alpha=0.05$ ). Single nucleus cells were excluded from the analysis (e.g., less than 5% of total values). **b.** Immunoblots and associated quantifications of transduced human myotubes (shScramble and *shTERF2*; respectively) using alpha-Tubulin as loading control. We report the protein levels of DNA Damage Response (DDR) associated proteins (i.e., p53, ATM and phosphorylated counterparts). No difference was observed between conditions, suggesting that TRF2 depletion in myotubes does not trigger the DDR pathway. **c.** Telomere length was measured by Telomere Restriction Fragment analysis (TRF). TRF from myotubes transduced with the different constructs used in the study. No differences in telomere length were observed upon transduction.

Figure S4

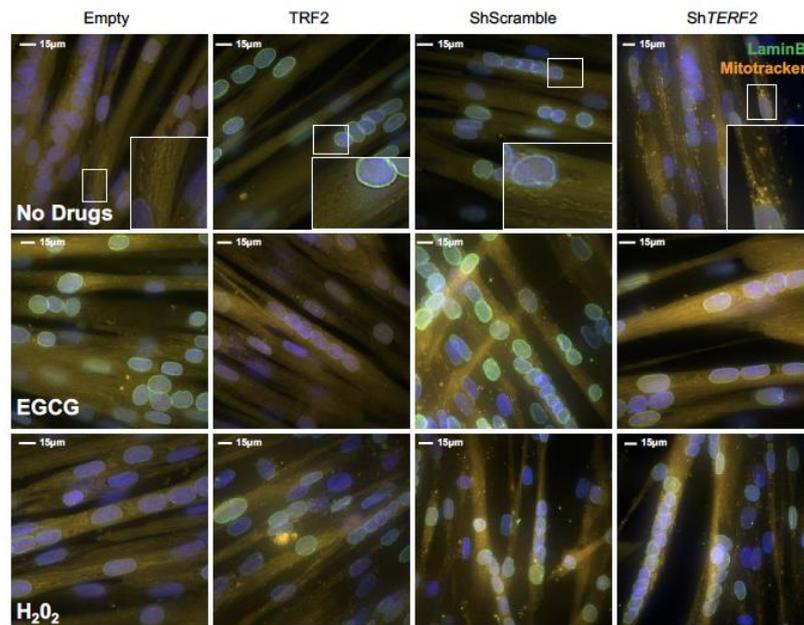


#### Figure S4.

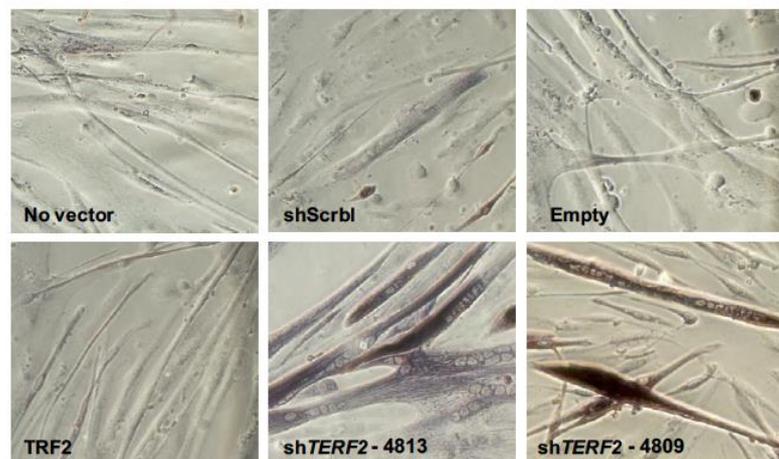
**a.** ROS-related foci intensities in transduced myotubes using the ROS-ID® detection kit (GE). An average of 100 z-stacks were taken for each condition (Deltavision Elite®, GE). Pictures from 10 to 15 independent and randomly chosen microscope fields were taken, then treated post-acquisition and deconvoluted with IMARIS. Single nucleus-cells were excluded from the analysis. DAPI intensities (top) and ROS related foci intensities (middle) are reported. All intensities fall into the same range. Outliers represented less than < 5% of the total intensities as shown by overlapping Means  $\pm$  SEM. Normalized quantification of ROS in transduced myotubes (bottom) shown as total number of ROS foci normalized to the number of nuclei (from 10-15 independent microscope fields). Hydrogen peroxide treatment increases ROS production as reported by the increased ROS foci number (Empty vs. Empty+H2O2,  $p= 0.0274$  ; Empty vs. TRF2+H2O2,  $p= 0.0291$  ; Holm-Sidak's multiple comparisons test;  $\alpha=0.05$ ). Down regulation of TRF2 significantly increases ROS (shScramble vs. sh*TERF2*,  $p< 0,0001$ ; Holm-Sidak's multiple comparisons test;  $\alpha=0.05$ ) and antioxidant treatments (e.g., NAC, EGCG) reduces ROS to control condition's levels. **b.** IF staining of FOXO3A (red), in transduced myotubes using 4 shRNAs against *TERF2*. Associated quantification of positive FOXO3A nuclei observed per conditions is reported. Single nucleus cells were excluded from the analysis (e.g., less than 5% of total values). Means  $\pm$  SEM are shown. **c.** FOXO3A representative western blots, staining and associated quantifications in transduced myotubes. Relative protein level quantifications normalized to B-ACTN and control (Empty and shScramble as reference for TRF2 overexpression and sh*TERF2* conditions; respectively; A.U,

arbitrary units). All assays were run in biological triplicates. Means  $\pm$  SEM are shown. In post-mitotic cells, increase or decrease in TRF2 level induces a stress response with FOXO3A modulation (shScramble vs. sh *TERF2*,  $p=0.0098$ ; Empty vs. TRF2,  $p=0.03$ ; Man-Whitney test;  $\alpha=0.05$ ). **d.** Kinetics of mitochondrial complex I, II and IV activity in transduced myotubes as reported by the apparatus before activity calculations. Sh *TERF2* transduced myotubes exhibit specific mitochondrial defects. We report a decreased complex I activity ( $p<0.001$ ) and an increased complex IV activity ( $p<0.001$ , Kolmogorov–Smirnov’s test;  $\alpha=0.05$ ). Activity was deduced and reported in Figure 2 by calculating the slope of the curve as suggested by the manufacturer ( $\Delta OD/$  Time), using only the time period where a linear activity was observed.

Figure S5



**NAD<sup>+</sup> / NADH enzymatic staining of transduced myotubes (NADH-TR)**



**Figure S5.**

a. Mitochondrial network in transduced and chemically-treated myotubes was analyzed using Mitotracker<sup>®</sup>. An average of 100 z-stacks were taken for each condition (Deltavision Elite<sup>®</sup>, GE). Pictures from 10 to 15 independent and randomly chosen microscope fields were taken, treated post-acquisition and deconvoluted with IMARIS.

Single nucleus cells were excluded from the analysis. Nuclei were stained using an Anti-Lamin B antibody (Green) and counterstained with DAPI. A punctate mitochondrial staining (observed as fragmented orange dots) is observed in myotubes transduced with sh *TERF2* suggesting mitochondrial fission. Similarly, H<sub>2</sub>O<sub>2</sub> treatments induce a punctuated mitochondrial staining (*i.e.*, a discontinuous orange staining, lacking global homogeneity along the myotubes structures). **b.** NAD<sup>+</sup>/NADH enzymatic staining of transduced myotubes. Nicotinamide adenine dinucleotide-nitro-blue tetrazolium reductase (NADH-TR) staining performed in transduced myotubes. Dark coloration corresponds to the deposition of purple formazan precipitate at sites of mitochondrial activity. Typically, mitochondria-rich tissues (*i.e.*, Oxidative fibers, Type I fibers) will display a darker staining than those relying on glycolysis (*i.e.*, Glycolytic fibers, Type II). TRF2 depleted myotubes exhibit a positive strong coloration, corresponding to higher oxidation of NADH, thus advocating for an oxidative phenotype (as classically seen for Type I fibers).

Figure S6

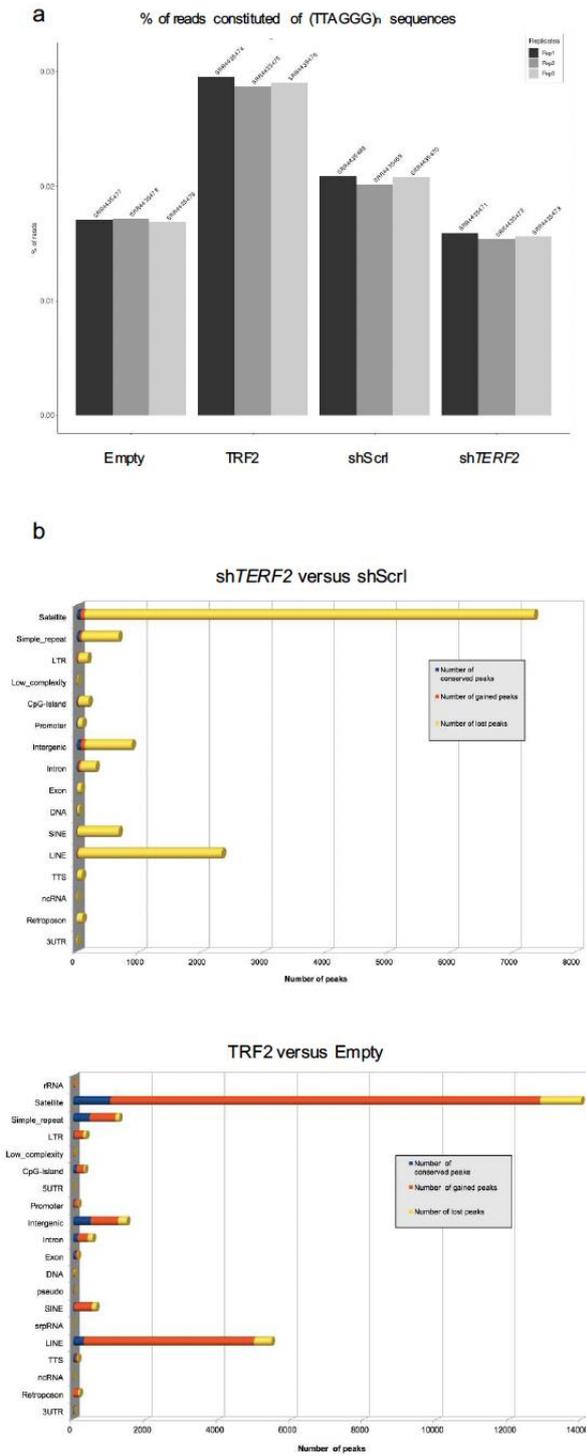


Figure S6.

a. Percentage of reads containing TTAGGG repeat motifs. Motifs were searched in raw fastq files, without considering mismatches and including reverse complement motifs. All files from the Illumina sequencing were evaluated (i.e., 3

C

Genes loss

Top Canonical Pathways		
Name	p-value	Overlap
G-Protein Coupled Receptor Signaling	1,57E-04	13,5 % 38/281
CREB Signaling in Neurons	5,09E-04	13,8 % 30/217
GPCR Signaling Pathway	5,45E-04	15,9 % 21/132
Netrin Signaling	6,98E-04	20,0 % 13/65
cAMP-mediated signaling	8,63E-04	13,4 % 39/224

GO term	Description	P-value
GO:0007186	G protein-coupled receptor signaling pathway	5,69E-9
GO:0007187	G protein-coupled recep sign path, coupled to cyclic nt second messenger	2,23E-6
GO:0046777	protein autophosphorylation	5,92E-5
GO:0007165	signal transduction	8,88E-5
GO:0007193	adenylate cyclase-inhibiting G protein-coupled recep signaling pathway	2,14E-4
GO:0007188	adenylate cyclase-modulating G protein-coupled recep signaling pathway	2,32E-4
GO:0065007	biological regulation	2,91E-4
GO:0065008	regulation of biological quality	5,24E-4
GO:0006796	phosphate-containing compound metabolic process	7,14E-4
GO:0006793	phosphorus metabolic process	7,14E-4
GO:1901564	organonitrogen compound metabolic process	9,26E-4
GO:0044248	cellular catabolic process	9,84E-4
GO:0004930	G protein-coupled receptor activity	1,43E-6
GO:0095928	G protein-coupled neurotransmitter receptor activity	5,04E-5
GO:0003824	catalytic activity	1,13E-4
GO:0044425	membrane part	1,66E-4
GO:0005887	integral component of plasma membrane	6,18E-4

Genes gain

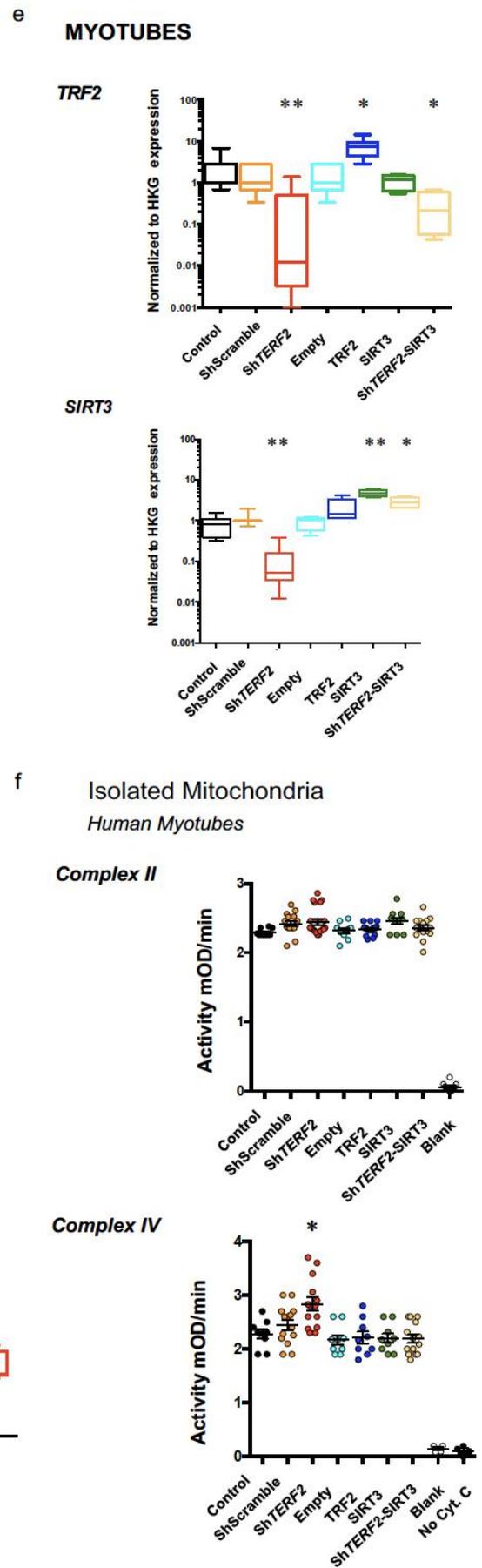
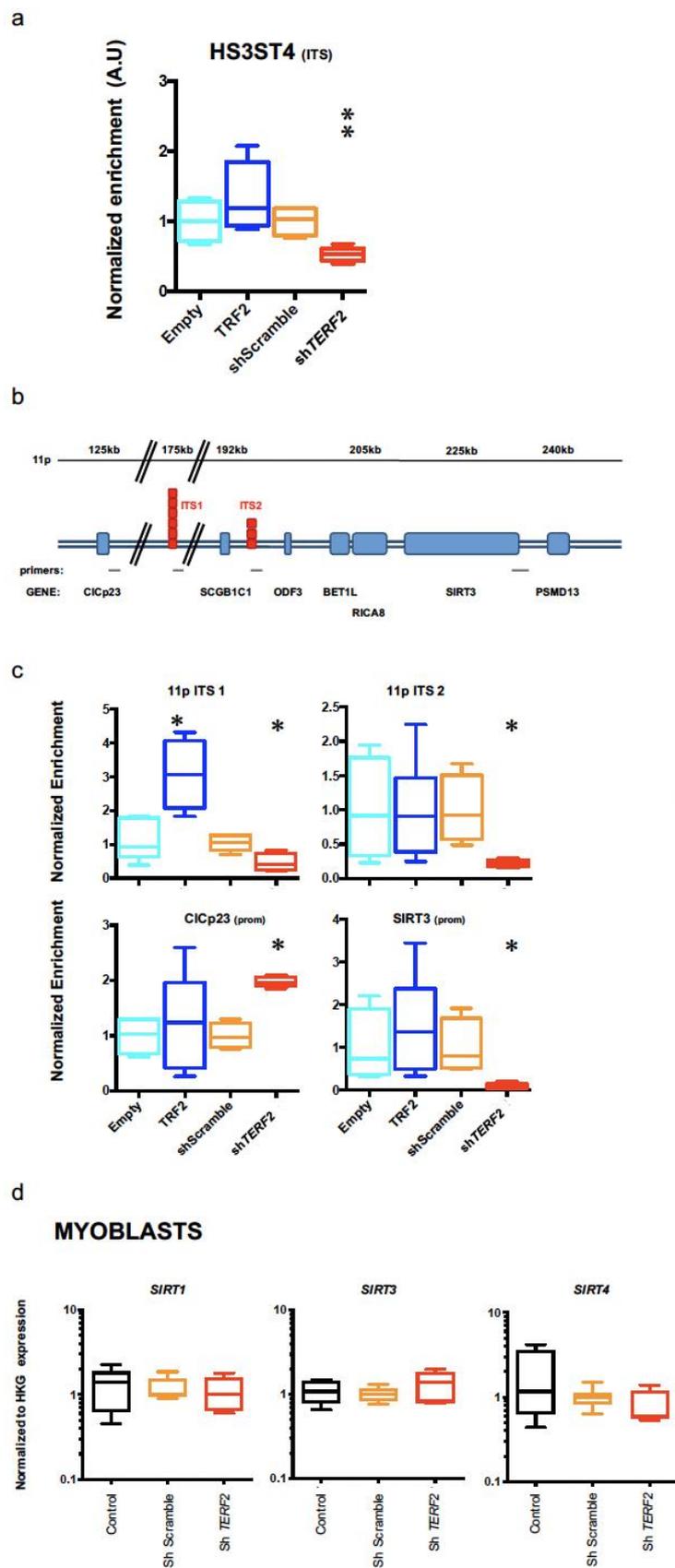
Top Canonical Pathways		
Name	p-value	Overlap
Leukocyte Extravasation Signaling	3,84E-03	11,1 % 23/208
Netrin Signaling	5,27E-03	15,4 % 10/65
Choline Biosynthesis III	6,04E-03	30,8 % 4/13
Protein Kinase A Signaling	9,42E-03	9,1 % 35/383
Iron homeostasis signaling pathway	1,24E-02	11,5 % 15/131

GO term	Description	P-value
GO:0022610	biological adhesion	4,41E-5
GO:0007155	cell adhesion	4,41E-5
GO:0098609	cell-cell adhesion	9,05E-5
GO:0032879	regulation of localization	1,16E-4
GO:0030198	extracellular matrix organization	1,41E-4
GO:0043062	extracellular structure organization	1,41E-4
GO:0071840	cellular component organization or biogenesis	1,43E-4
GO:0016043	cellular component organization	1,43E-4
GO:0033554	cellular response to stress	4,42E-4
GO:0040012	regulation of locomotion	6,51E-4
GO:2000145	regulation of cell motility	6,51E-4
GO:0051894	positive regulation of focal adhesion assembly	7,02E-4
GO:0010811	positive regulation of cell-substrate adhesion	7,02E-4
GO:0001954	positive regulation of cell-matrix adhesion	7,02E-4
GO:1901890	positive regulation of cell junction assembly	7,02E-4
GO:1903393	positive regulation of adherens junction organization	7,02E-4
GO:0002376	immune system process	7,67E-4
GO:0034613	cellular protein localization	7,85E-4
GO:0070727	cellular macromolecule localization	7,85E-4
GO:0051270	regulation of cellular component movement	7,85E-4
GO:0048523	negative regulation of cellular process	8,88E-4
GO:0050789	regulation of biological process	9,42E-4
GO:0031267	small GTPase binding	3,69E-4
GO:0017016	Ras GTPase binding	3,69E-4
GO:0051020	GTPase binding	3,69E-4
GO:0017048	Rho GTPase binding	3,69E-4
GO:0030054	cell junction	1,94E-5
GO:0005911	cell-cell junction	9,19E-5
GO:0043229	intracellular organelle	8,44E-4
GO:0070161	anchoring junction	8,78E-4
GO:0005886	plasma membrane	9,49E-4

biological replicates for each of the 4 conditions). Corresponding SRA accessions identifiers are displayed on top of the bars. The % of telomeric reads varies in parallel with TRF2 expression modulation, confirming the TRF2 specificity of the ChIP-seq experiments. **b.** Peak distributions. Annotations are provided from RefSeq database and assigned using Homer software. Graph 1 shows the peaks founded in shTERF2 samples replicates versus shScramble Peaks; graph2 peaks from surexpressed TRF2 experiment vs Empty samples. Peaks are represented in 3 groups: (i) in blue those founded in both conditions (i.e., same overlapping positions) labeled as “conserved peaks”; (ii) in red the peaks founded in only one condition (shTERF2 and TRF2) labeled as gained peaks; in yellow the peaks only presented in the control condition (ShScramble and Empty) labeled as “lost peaks”. **c.** Top canonical pathways generated with IPA (IPA Q Ingenuity®Systems, Redwood City, USA). The IPA program analyzes input list of genes provided against the Ingenuity Pathway Knowledge Base (IPKB), derived from known functions and interactions of genes published in the literature, allowing one to identify global biological networks, functions and pathways altered from a given dataset. We used genes-lists encompassing genes located within <5000 nt of identified peak(s). List 1 represents the top canonical pathways given by IPA using the genes/peaks loss upon TRF2 downregulation (shTERF2 vs. shScramble); list 2 the top canonical pathways using the genes/peaks gained in TRF2 overexpression (TRF2 vs. Empty). We have also use genes related to theses 2 lists of top pathways to generated the over represented GO terms from Gene Ontologie (GO) database, using GOrilla software (<http://cbl-gorilla.cs.technion.ac.il/>). GO terms, descriptions and corresponding P-values are represented in 2 tables. Interestingly, in both gain and loss conditions, the top pathways are related to

GPCR/cAMP/PKA/CREB signaling, suggesting a role of TRF2 in modulating this pathway in skeletal muscle cells.

Figure S7



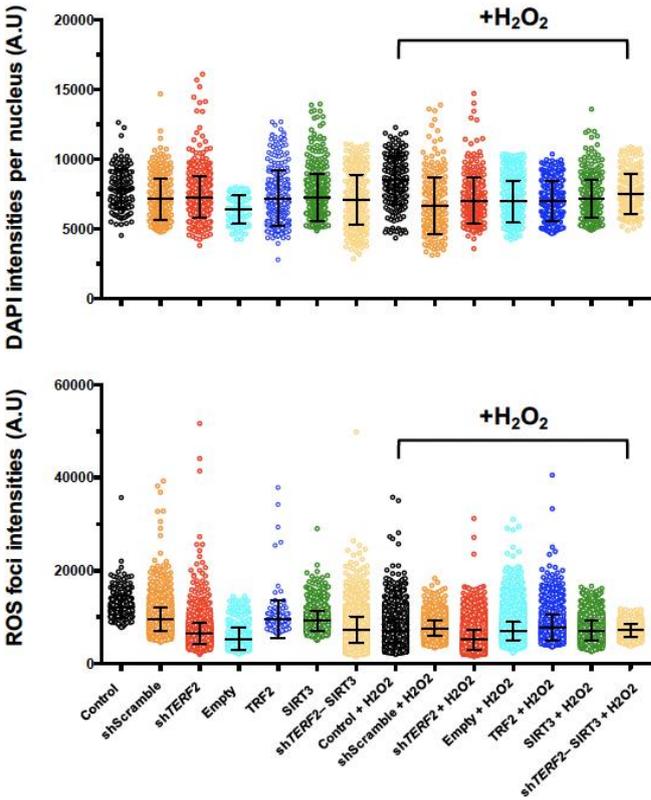
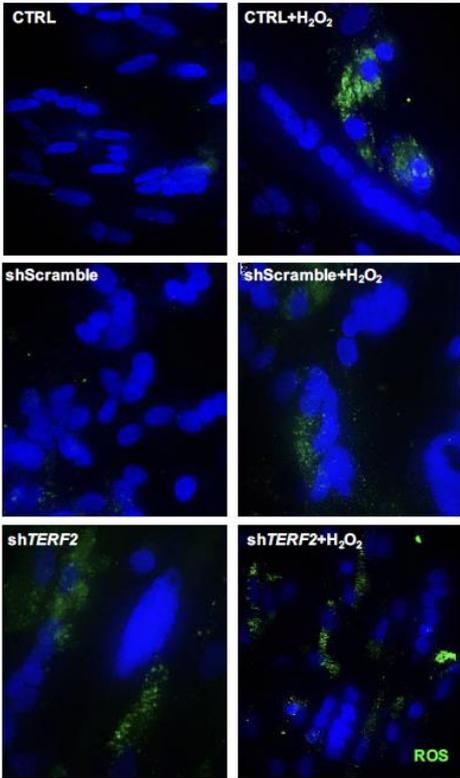
## Figure S7.

**a.** Validation of the TRF2 Chip-Seq. We used primers encompassing the *HS3ST4* TRF2 binding site (shScramble vs. sh*TERF2*,  $p= 0.0095$ ; Mann-Whitney comparison test,  $\alpha=0.05$ ). **b.** Schematic representation of the localization of the Internal Telomeric Sequences (ITS) at the 11p locus with surrounding genes and primers used for CHIP-ddPCR<sup>®</sup> reported in **c.** Quantification of TRF2 enrichment by CHIP-ddPCR<sup>®</sup> at the subtelomeric *SIRT3* region. Results were normalized to Alu repeats. At distal sites, TRF2 enrichment is decreased upon *TERF2* knock-down (shScramble vs. sh*TERF2*: 11pITS1,  $p=0.041$ ; *SIRT3* (prom),  $p= 0.0386$ ; Mann-Whitney comparison test,  $\alpha=0.05$ ), whereas the most subtelomeric site (*CICp23*) is enriched (shScramble vs. sh*TERF2*,  $p= 0.032$ ). TRF2 enrichment is increased upon *TERF2* overexpression (Empty vs. TRF2,  $p= 0.024$ ). **d.** Gene expression quantified by RT-qPCR in transduced myoblasts (as presented in Figure 1) normalized to three housekeeping genes (HKG: *HPRT*, *PPIA* and *GAPDH*;  $\Delta\Delta$ Ct method). N=6 per condition (technical duplicates of biological triplicates), means  $\pm$  SEM with associated statistical significance are reported (Kruskal-Wallis multiple comparison test;  $\alpha=0.05$ ). *TERF2* depletion reduces transcription of *SIRT6* without modulating other main Sirtuins and correlates with increased DNA damage as reported by TIFs assay (Figure 1). **e.** Gene expression quantified by RT-qPCR in transduced myotubes normalized to housekeeping genes (HKG: *HPRT*, *PPIA* and *GAPDH*;  $\Delta\Delta$ Ct method). N=6 per condition (technical duplicates of biological triplicates), means  $\pm$  SEM with associated statistical significance are reported (Kruskal-Wallis multiple comparison test;  $\alpha=0.05$ ). **f.** *SIRT3* rescue experiments using enriched mitochondria extracts from transduced human myotubes. Mitochondrial complex II and IV activity. *SIRT3* overexpression in TRF2-depleted myotubes restores the mitochondrial-associated activity (Complex IV:

shScramble vs. shTERF2-SIRT3,  $p > 0.9$ ; Holm-Sidak's multiple comparisons test;  $\alpha = 0.05$ ). \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

Figure S8

a



b

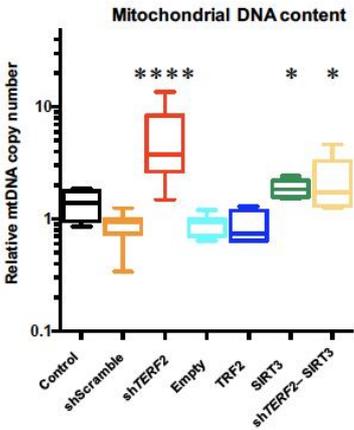
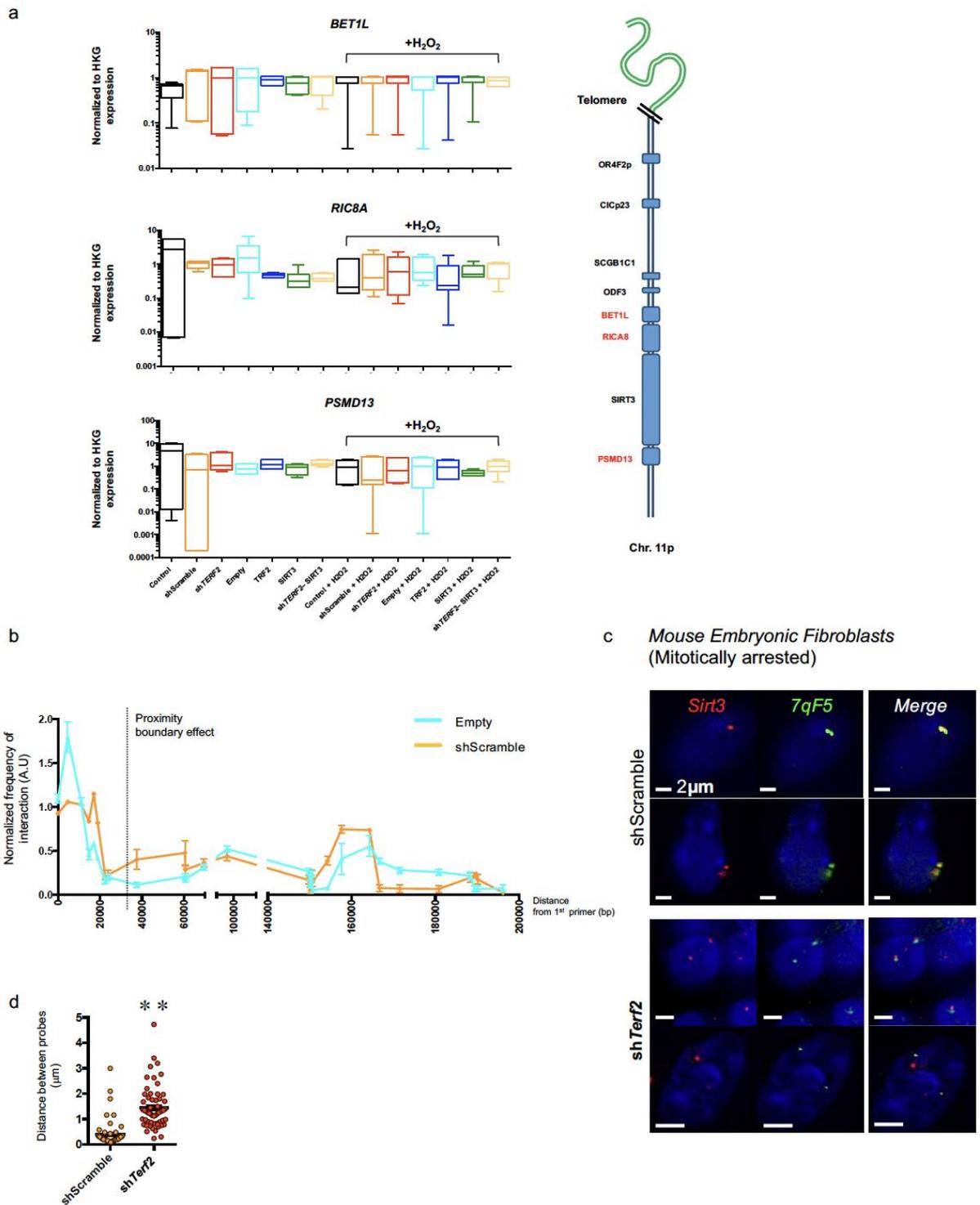


Figure S8.

**a.** ROS-related foci intensities in transduced myotubes using the ROS-iD® detection kit (GE). An average of 100 z-stacks were taken for each condition (Deltavision Elite®, GE). Pictures from 10 to 15 independent and randomly chosen microscope fields were taken, treated post-acquisition and deconvoluted with IMARIS. Single nucleus cells were excluded from the analysis. DAPI intensities (top) and ROS related foci intensities (bottom) are reported. All intensities fall into the same range. Outliers represented less than < 5% of the total intensities as shown by overlapping Means  $\pm$  SEM. **b.** Relative quantification of mitochondrial DNA content in transduced myotubes. Mitochondrial DNA (mtDNA) was quantified by qPCR and normalized to genomic DNA from three independent experiments ( $\Delta\Delta$ Ct method; tRNA-Leu and B2-microglobulin for mt and nuclear DNA; respectively). Sh*TERF2* and *SIRT3* transduced myotubes show an increase in mitochondrial DNA content (shScramble vs. sh*TERF2*,  $p < 0.0001$ ; shScramble vs. *SIRT3*,  $p = 0.03$ ; shScramble vs. sh*TERF2*-*SIRT3*,  $p = 0.025$ ; Kruskal-Wallis multiple comparisons test;  $\alpha = 0.05$ ). Importantly, the results suggest a partial rescue of *TERF2* downregulation by *SIRT3* overexpression (ROS foci, average mitochondrial DNA content). Means  $\pm$  SEM with associated statistical significance are reported. \*  $p < 0.05$ ; \* \* \* \*  $p < 0.001$ .

Figure S9

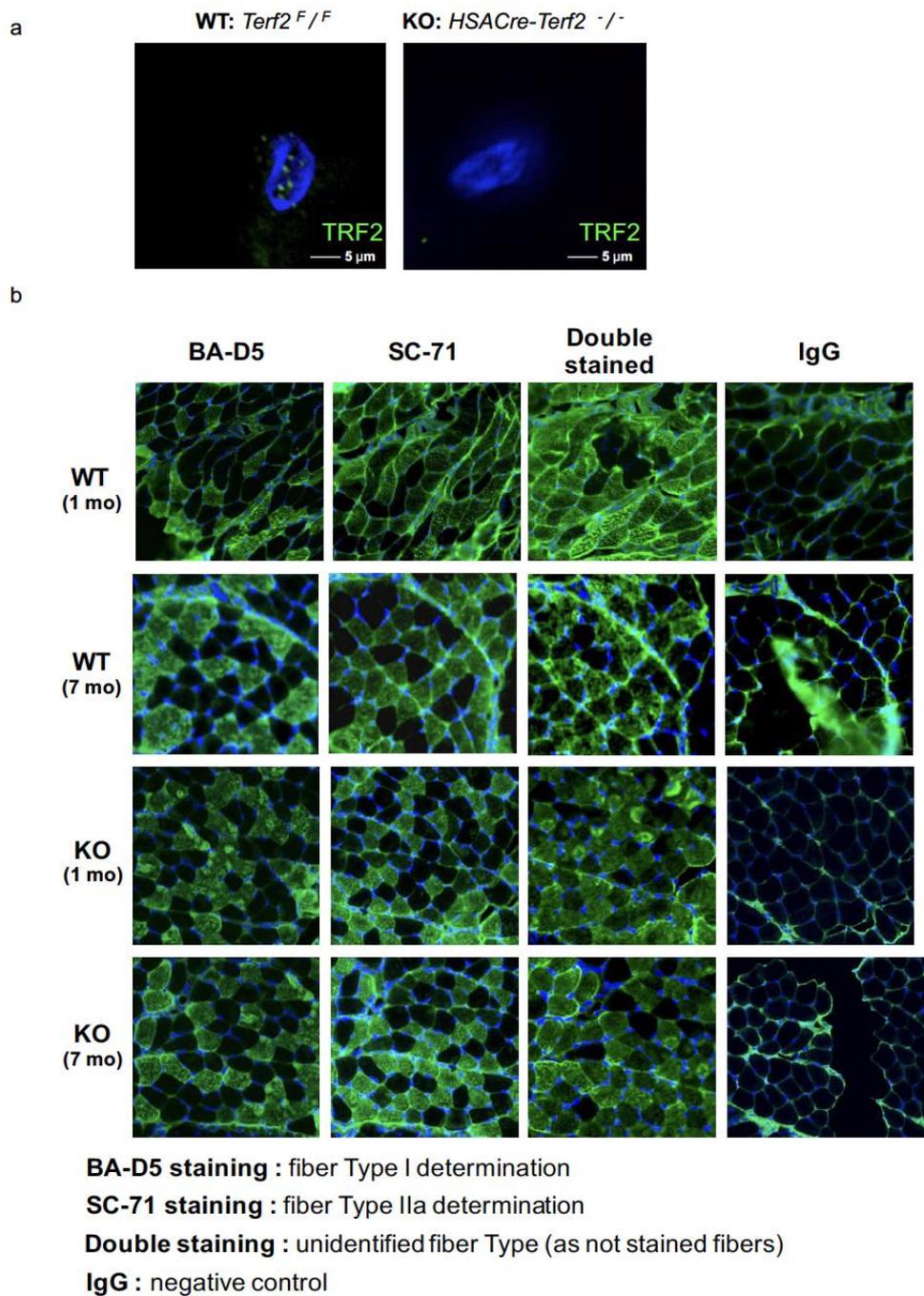


**Figure S9.**

**a.** Gene expression of genes located at the 11p locus as represented (left), in transduced myotubes. Genes surrounding *SIRT3* (in red) were quantified by RT-qPCR.

Each measure represents the average fold-change expression of six independent repetitions (technical RT duplicates of biological triplicates) normalized to three housekeeping genes (HKG: *HPRT*, *PPIA* and *GAPDH*;  $\Delta\Delta\text{Ct}$  method). No statistical difference was found amongst conditions (Kruskal-Wallis multiple comparison test;  $\alpha=0.05$ ). **b.** Chromatin Conformation Capture (3C) performed on the 11p locus (first 2Mb) in myotubes as reported in Figure 4. Cells were collected 10 days after transduction. Each measure represents the amplification of interactions involving a fixed primer and a second primer along the 2Mb of the locus, both located in proximity of a *HindIII* restriction site. No statistical difference was detected between control conditions (Empty vs. shScramble; Unpaired t Test;  $\alpha=0.05$ ). **c.** 3D-DNA FISH in transduced mouse embryonic fibroblasts (MEFs) mitotically arrested using mitomycin and associated quantification **d.** MEFs were transduced with a sh *Terf2* construct and corresponding control vector then labeled with a *Sirt3* (red) and a unique distal sub-telomeric 7q (green) probe (5Mb apart); N>40 nuclei, means  $\pm$  SEM are shown. As in human myotubes, we observe a significant increase in separated signals (corresponding to an increased distance between the 7q telomere and the mouse *Sirt3* locus) in MEFs transduced with sh *Terf2* (Two-tailed Student's t-test;  $\alpha=0.05$ ).

Figure S10



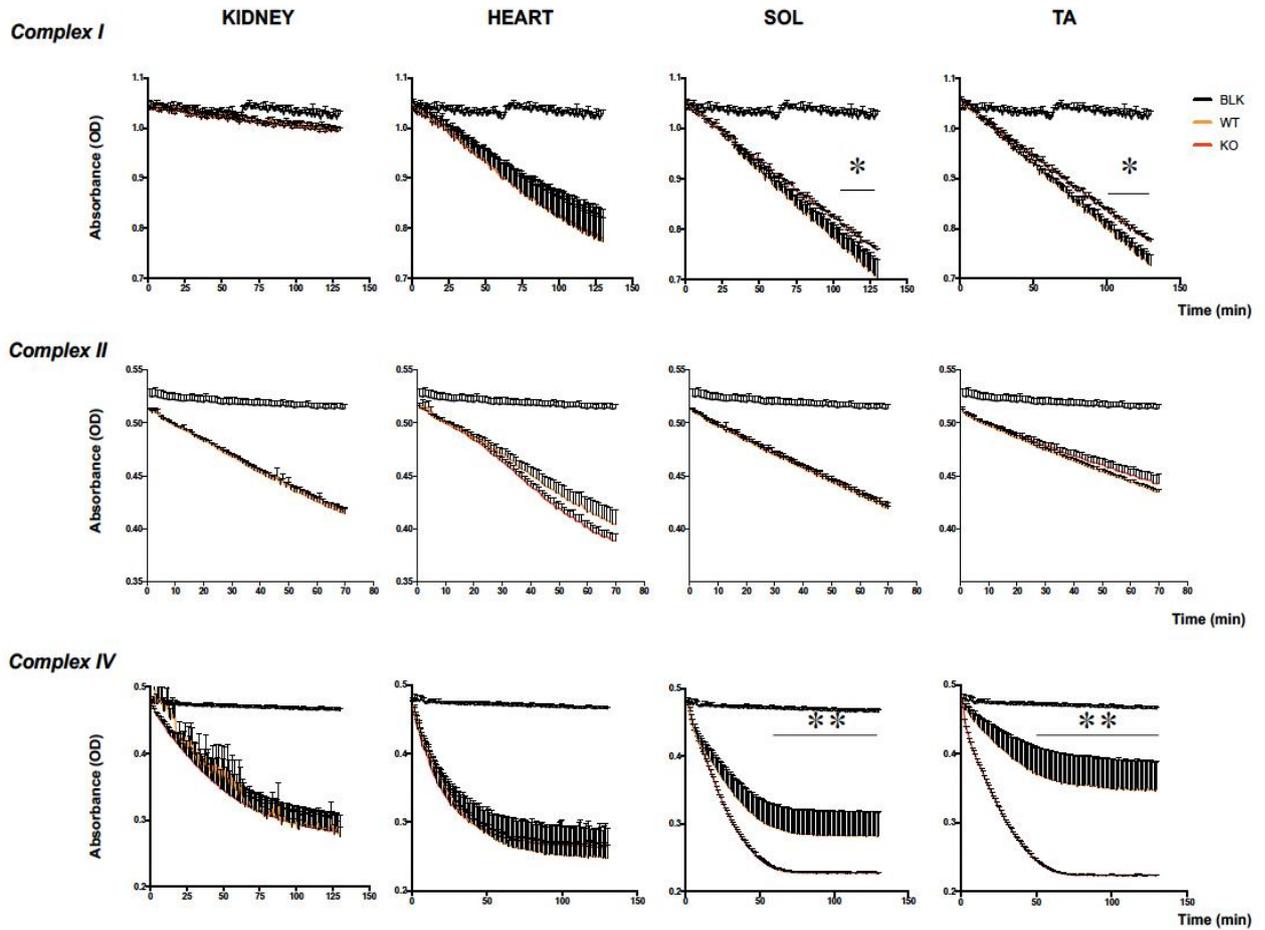
**Figure S10.**

**a.** *Terf2* immunostaining in transversal section of a WT and KO mice. *Terf2* staining was detectable in all of the WT mice nuclei but only in a portion of the KO, an expected result as the Cre activation is restricted to mature muscle fibers. **b.** representative fiber type characterization in *HSACre*<sup>+/-</sup> *-Terf2*<sup>-/-</sup> mice using myosin

heavy chain antibodies against type I (BA-D5) and IIa (SC-71) fibers. We report here the serial staining (Type I, Type IIa, Type I+ Type IIa and IgG control, from left to right; respectively) performed to discriminate the fiber type composition of the *soleus* (SOL) in 4 different mice. Undetermined fibers (reported U Figure 5h) represent fibers that remained unstained after a double stained procedure.

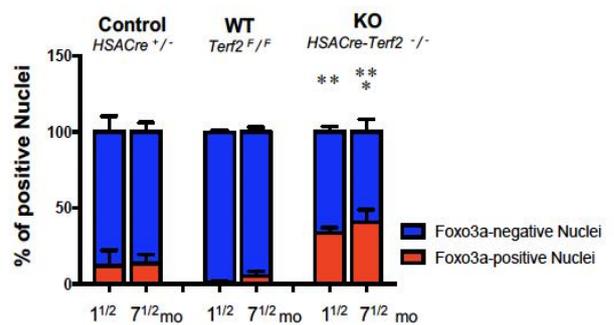
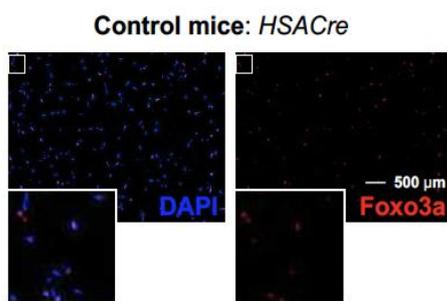
Figure S11

a



b

**Tibia Anterioris**



**Figure S11.**

a. Mitochondrial complex II, I and IV activity in 40 weeks-old transgenic WT and KO; freshly harvested tissues (e.g., Heart; Kidney; *gastrocnemius*; *soleus* and *tibia anterioris*). 5 mice from each group were processed in duplicates using the Enzyme

Activity Microplate Assay Kit (abcam) and kinetics recorded by a SPECTROstar Nano™ apparatus (BMGLabtech). No statistical differences were observed between WT and KO mice in mitochondrial complex II activity as well as activity of complex I and IV from heart and kidney tissues. **b.** *Foxo3a* staining and associated quantification in the TA by immunofluorescence. KO mice exhibit a higher percentage of positive *Foxo3a* nuclei, no statistical differences were seen between the *HSACre* and WT (*Terf2*<sup>F/F</sup>) mice (Kruskal-Wallis multiple comparisons test;  $\alpha=0.05$ ). \* <0,05; \*\* < 0,001; \*\*\* < 0,0001

## FULL METHODS

### Human biopsies and Ethic statement

The collection of fetal muscle biopsies was approved by the “Agence Française de la Biomedecine” of the Ministry of Health for legal access to the biological material in full accordance with the law (research protocol number PFS13-006). Samples were obtained after therapeutic abortion. Parents have provided written informed consent for the use of biopsies for medical research in accordance with the Declaration of Helsinki. Muscle biopsies were processed by fetopathologists from fetuses not affected by a muscular pathology. Skeletal muscle biopsies from teens and adults were obtained from the Nice Hospital (CHU l’Archet registered as protocol number DC-2015 2374) and from the Tumorothèque, Assistance Publique des Hôpitaux de Marseille, agreement n°AC-2013-1786, from voluntary healthy donors using standardized muscle biopsy protocol. All biopsies were tested for musculoskeletal pathologies with negative outcome.

**Mice – Transgenic model:** In order to generate a skeletal muscle specific TRF2 KO mice (*HSACre*<sup>+/+</sup> - *Terf2*<sup>-/-</sup>) two characterized strains were crossed. First, we obtained a TRF2 floxed mice from the Jackson laboratory (strain B6; 129P2-

*Terf2*<sup>tm1Tdl/J</sup>) that have been previously characterized<sup>1</sup>. TRF2 floxed mice (noted *Terf2*<sup>F/F</sup>) were first backcrossed with C57BL/6 strain for 6 generations to homogenate the genetic background. The mice were then crossed with the *HSACre*<sup>+/-</sup> strain expressing the Cre-recombinase under the control of the Human Skeletal Actin (*HSA*) gene promoter specifically expressed in striated muscle fibers<sup>4</sup>. The study design and protocols were controlled and approved by the Comité Institutionnel d'Éthique Pour l'Animal de Laboratoire (CIEPAL) and the Ministry of Education and Scientific Research under the number APAFIS#10444-2017052414468355v2. All experiments were performed at the ANIPHY core (ENS, Lyon), agreement n°C693880803.

**Mitochondrial DNA quantification:** DNA was extracted using a high salt precipitation and resuspended in 50µl of TE (10mM Tris HCl pH8.0; 1mM EDTA). After DNA quantification, all samples were diluted to a concentration of 3ng/µl in TE. A qPCR assay was then performed using 1µl of the prepared diluted samples. All samples were run in triplicates and biological triplicate for human myotubes (n=9 per condition); 4 mice per group (n=12 per condition) using FastStart universal SYBR Green master Mix (Roche) and a 7900HT Fast Real-time PCR system with an 384 well block module (Applied Biosystem). Melting curves were analyzed to exclude nonspecific amplification products. The program used is as followed: denaturation 95°C – 5min, followed by 40 cycles of 95°C – 30 sec, 60°C – 30s annealing, 72°C – 30s extension. PCR was stopped with a final 98°C – 10 min step. We used a set of primers specific to human as described elsewhere<sup>2</sup>, to measure the relative mitochondrial content (dubbed mtDNA and nucDNA, respectively; See Extended primer list). To determine the mitochondrial DNA content, we used the following equations:  $\Delta CT = (\text{nucDNA } C_T - \text{mtDNA } C_T)$ ; Relative mitochondrial DNA content =  $2^{2\Delta CT}$ .

**Mitochondrial Complex Enzyme Activity Assay:** following manufacturers' instructions (Abcam) mitochondrial extracts were prepared either from snap freeze tissues in isopentane/Liquid nitrogen double bath as commonly used for biochemistry assays (i.e., TA, SOL, GASTR, Heart and Kidney) or directly from freshly harvested transduced human myotubes and processed within 4 hours. Briefly, tissues were reduced into powder using a liquid nitrogen cooled mortar and pestle apparatus (Bel-Art Scienceware) and homogenize subsequently with a dounce homogenizer in ice

cold PBS. After supernatant collection, a small portion was extracted and used to determine protein concentration. The remaining solution was then adjusted to a 5.5mg/ml final concentration in PBS. Proteins were then extracted from sample by adding 1:10 of detergent solution as supplied by the manufacturer. Solution was left on ice for 30min to allow solubilization of mitochondria and thereafter centrifuged for 20min at 12000g at 4°C. Finally supernatant was aliquoted, snap freeze and stored at -80°C for storage. Aliquots were then used only once after thawing. 3 mitochondrial complexes were assessed: Complex I (MitoTox complex I OXPHOS Activity, abcam); Complex II (Complex II Enzyme Activity Microplate Assay Kit, abcam) and Complex IV (Complex IV Rodent Enzyme Activity Microplate Assay). For each assay, appropriate concentrations were determined for each tissue. We used 70ug of mitochondrial tissue extract for skeletal muscle, 100ug for Kidney and 50ug for Heart samples. Last, plates were prepared according manufacturers protocol, using technical duplicates, 2 wells for positive controls and 4 wells for background measures.

Kinetics were followed by ODs measures using a SPECTROstar Nano apparatus (BMG labtech). For each assay, a total of 10 mice (5 WT; 5 KO) were tested in duplicates, and two control tissue used (e.g., heart, kidney). Each data point represents the average of 10 measures. For human myotubes, assays were done in duplicate using biological triplicate for a total of 6 measures per data point (minimum). Activity was then deduced and reported by calculating the slope of the curve as suggested by the manufacturer ( $\Delta OD / \text{Time}$ ), using only the region where a linear activity was observed.

**Mitochondrial SIRT3 Activity Assay:** following manufacturers' instructions (Abcam ab156067) mitochondrial extracts were prepared from freshly harvested transduced human myotubes and processed within 4 hours. Briefly, cells were pelleted and lysed in mitochondrial extraction buffer, a small portion was then used to determine protein concentration. The remaining solution was adjusted to a 5.5mg/ml final concentration in PBS. Proteins were then extracted from sample by adding 1:10 of detergent solution as supplied by the manufacturer. Solution was left on ice for 30min to allow solubilization of mitochondria and thereafter centrifuged for 20min at 12000g at 4°C. Finally, supernatant was aliquoted, snap freeze and stored at -80°C for storage. Aliquots were then used only once after thawing. For each plate run, we used dilution series (10-30 $\mu$ g) of our enriched mitochondrial extracts along with: a provided positive

control (pure Recombinant SIRT3); a buffer only control, a no enzyme control and a no NAD control (no substrate) well.

Kinetics were followed by Fluorescent measures (detector at 450nm; 1min intervals) using a GloMax Discover Microplate reader (Promega). For each assay, myotubes were done in duplicate using biological triplicate for a total of 6 measures per data point (minimum). Activity was then deduced and reported by calculating the slope of the curve as suggested by the manufacturer ( $\Delta$ Fluorescence/ Time), using only the region where a linear activity was observed.

**Western Blot:** Cells were scraped from culture dish in 1X PBS complemented with protease inhibitors, mixture was spin down (800g, 10min at 4°C) and pellet stored at -80°C for further use. Whole cell lysates were prepared from cells by adding cell lysis buffer (i.e., Mueller Buffer: 50mM Hepes pH7.4, 0.1% Triton X-100, 4mM EGTA pH8.0, 10mM EDTA pH8.0, 15mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 25mM NaF, 5mM NaVO<sub>4</sub>, 100mM  $\beta$ -glycerophosphate) complemented with phosphatase inhibitors (Roche). For human biopsies, samples were first reduced into powder using a liquid nitrogen cooled mortar and pestle apparatus (Bel-Art Scienceware) and homogenize subsequently using a Precellys®24 apparatus (Cayman chemical) with ceramic beads before being briefly sonicated. Total protein concentration was determined using a Bradford Protein Assay reagent (BioRad), by measuring absorption at 750nm on a spectrophotometer. For each sample, 30 $\mu$ g of protein was resolved in a 4-15% gradient mini-protean precast polyacrylamide gels (BioRad) and transferred to PVDF low fluorescence membranes (Millipore) for 5 hours at 4°C (Wet transfer). After blocking for 1 hour with 10% skim milk in PBST (0.2% Tween-20 in PBS), the membranes were incubated overnight at 4°C with primary antibodies diluted in 5% BSA in PBST. The following primary antibodies were used: TRF2, mouse monoclonal, (clone IMG-124A, 1:1000, Imgenex); TRF2, rabbit monoclonal, (clone EPR3517(2), 1:2000, Abcam); TRF1, mouse monoclonal (ab1423, 1:2000, Abcam); RAP1, rabbit monoclonal (A300-306A, 1:1000; Bethyl); POT1, rabbit monoclonal (NB500-176, 1:1000; NovusBiological); TIN2, rabbit monoclonal (ab136997, 1:1000; Abcam); TPP1, rabbit monoclonal (A303-069A, 1:1000; Bethyl); Actin, rabbit polyclonal, (1:3000, Santa Cruz); ATM, mouse monoclonal, (clone 2C1, 1:1500, Abcam); Ser 1981 phospho-ATM, mouse monoclonal, (clone 10H11.E12, 1:1500, Cell Signaling Technology); FoxO3a, rabbit monoclonal (clone 75D8, 1:1500, Cell Signaling Technology); HSP60, goat polyclonal

(1:5000, Abcam); SIRT3, rabbit monoclonal (clone D22A3, 1:1500, Cell Signaling Technology); SIRT3, rabbit monoclonal (clone C73E3, 1:1500, Cell Signaling Technology); LC3A/B, rabbit polyclonal (1:2000, Cell Signaling Technology). The membranes were then rinsed three times in PBST for 10min and incubated 1 hour at room temperature with appropriate secondary antibodies diluted (1:15000) in Li-COR blocking reagent or 10% skim milk (e.g., IRDye® 800CW/680 Goat anti-mouse; Goat anti-rabbit; Li-COR; HRP Goat anti-rabbit IgG; HRP Goat anti-mouse IgG; HRP Donkey anti-goat IgG; Vector Laboratories). Following three rinses in PBST for 15min, membranes were imaged by IR fluorescence with the Odyssey® imaging system (Li-COR) or Fusion Solo imaging system (Vilber). For comparison between membranes, quantifications were normalized to the intensity of the ladder (2.5µl, PageRuler™ plus prestained protein ladder; ThermoScientific).

**Cell culture:** Cells used for this study were isolated<sup>3</sup> from patient 12 (Extended Data. 1) and produced as previously described<sup>4</sup>. For day-to-day maintenance, human myoblasts were seeded in dishes coated with 0.1% pigskin gelatin in 4:1 Dulbecco modified Eagle medium/Medium 199 supplemented with 15% FBS, 0.02M HEPES, 1.4mg/l vitamin B12, 0.03mg/l ZnSO<sub>4</sub>, 0.055mg/l dexamethasone, 2.5µg/l hepatocyte growth factor and 10µg/l basic fibroblast growth factor. Cultures were maintained in a 5% oxygen environment and passaged at ~60% confluency. Population doublings (PDs) were calculated as  $PD = \ln[(\text{final number of cells})/(\text{initial number of cells})]/\ln(2)$ .

Myogenicity of the cells was verified by myotubes formation following a change to differentiation medium (2% horse serum in 4:1 Dulbecco modified Eagle medium: Medium 199) when 70-90% confluent.

For infection of myotubes (Figure 1D), cells (myoblasts) were seeded in 10 cm dishes, switch to differentiation media (2% Horse Serum) upon confluence (90%) and transduced at least week after. Cells were transduced at a MOI 2 using the different shRNAs and vectors. For ectopic upregulation, we used previously validated constructs and associated control (annotated TRF2 and Empty)<sup>5</sup>. For SIRT3, we used the same backbone and cloned the *SIRT3* cDNA sequence in lieu of *TERF2* cDNA. For downregulation experiments, we used MISSION shRNAs (pLKO.1; Sigma) with references as followed: *TERF2* (TRNC0000004809; TRNC0000004810; TRNC0000004812 and TRNC0000004813), *FOXO3A* (TRNC0000010335 and TRNC00000235491) and control (SHC002).

**RT-qPCR:** Cells were lysed (RNeasy plus kit (Qiagen)) after washing with PBS, scraped (BD Biosciences) and sheared by centrifugation through Qiasredder columns (Qiagen). Total RNA purified according to the manufacturer's instructions was quantified on a Nanodrop 1000 spectrophotometer (Thermo Scientific). For Reverse Transcription (RT) 2x 500ng RNA was reverse transcribed in technical duplicates using two separate kits (SuperScriptIII, invitrogen; High Capacity cDNA RT Kit, Applied Biosystem). The cDNA was diluted 1:4 in water for quantitative RT-PCR (qRT-PCR) in triplicates using FastStart universal SYBR Green master Mix (Roche) and a 7900HT Fast Real-time PCR system with 384 well block module (Applied Biosystem). Melting curves were analyzed (SYBR green) to exclude non-specific amplification products. We confirmed amplicon size at least once on agarose gels. Crossing-threshold (Ct) values were normalized by subtracting the geometric mean of three housekeeping genes (GAPDH, PPIA and HPRT1). All Ct values were corrected by their PCR efficiency, determined by 1:2 or 1:4 cDNA dilution series.

**Immunofluorescence Assays:** Traditional immunofluorescence assays were performed as followed: cells were grown on cover slides and fixed for 10min on ice with 4% paraformaldehyde in PBS. After PBS washes, cells were incubated for 1h at room temperature in blocking solution (1%Triton X-100, 1%BSA, 5% donkey serum in PBS). Cells were then stained overnight at 4°C in blocking solution containing the respective primary antibodies (TRF2, 1:100; Foxo3A, 1:250; LaminB, 1:200). After three washes with PBS/ 0.1% Triton X-100, slides were incubated for 1h30min at room temperature with secondary antibodies (AlexaFluor 1:500) in PBS containing 0.5% Triton X-100, 5% BSA. Slides were mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, USA). Images were taken using a Delta vision elite system (GE) with a 60X oil-immersed lens (60X/TRIF – Plan Aplanachromat; Olympus).

**Telomeric Induced Foci (TIFs):** Cells were grown on cover slides and fixed for 10min on ice with 4% paraformaldehyde in PBS. After PBS washes, cells were incubated for 1h at room temperature in blocking solution (1%Triton X-100, 1%BSA, 5% donkey serum in PBS). To perform the PNA-FISH staining, cells were washed twice with SSC2X for 5min at RT and subsequently treated with RNaseA for 45min at 37°C. After additional SSC2X washes (5min, 4°C), coverslides were dried and

incubated upside-down with a hybridization solution containing the PNA probe (20µl H<sub>2</sub>O; 70µl formamide; 7µl 10% blocking B (Roche); 1µl 1M Tris pH7.2; 1µl probe) and sealed on coverslips using rubber cement. Slides were then heated at 85°C for 4min, and incubated in the dark at 37°C in a humidification chamber for 2 hours. After removal of the rubber cement, cells were serially washed in three different solutions: twice for 15min at RT with washing solution I (10mM Tris pH 7.2; 70% formamide); twice for 15min at RT with washing solution II (150mM NaCl; 50mM Tris pH7.2; 0.05% Tween 20) and twice with PBS for 5min at RT. Cells were then blocked for 1 hour with the blocking solution and immuno-stained overnight at 4°C in the blocking solution containing the primary rabbit polyclonal anti-53BP1 antibody (Novus Biologicals, 1:500). After three washes with PBS/ 0.1% Triton X-100, slides were incubated for 1h30min at room temperature with Alexa 555 Donkey anti-rabbit secondary antibody in PBS containing 0.5% Triton X-100, 1% BSA, 2.5% donkey serum. Slides were mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, USA). Images were taken using a DeltaVision elite system (GE). Co-localization events, representing telomeric DNA damages (TIFs), were counted in at least 30 nuclei per condition from three independent experiments using the IMARIS software.

**Reactive Oxygen Species (ROS):** Cells were grown on cover slides and treated as indicated in the ROS kit instructions (Enzo). Briefly, transduced differentiated cells were washed twice and 1ml of fresh differentiation media was added, with or without drugs and incubated for 30min at 37°C (N-Acetyl-L-cystein: 5mM; EGCG: 10mM; H<sub>2</sub>O<sub>2</sub>: 100µM). After additional wash and media renewal (1ml), cells were incubated for 1 hour at 37°C with a solution composed 2X ROS detection and 4µl of Oxidative stress reagent per 10ml (5mM; dilution 1:2500). The solution was added to the 1ml of fresh media to adjust to the appropriate concentration. Cells were then washed three times with 1X PBS and directly mounted using 15µl of vectashield+DAPI (no fixation). Pictures were taken using a DeltaVision Elite system (GE). An average of 100 stacks and 50 nuclei were taken per conditions. Images were then treated using IMARIS. Intensities of ROS foci and DAPI staining were used for analyses, excluding single-nuclei cells for myotubes analysis.

**Mitochondrial network:** Cells were grown on cover slides and treated as indicated in the MitoTracker® Red CMXRos manual. Briefly, transduced differentiated

cells were washed twice and incubated with MitoTracker (25nM) diluted in 1ml of fresh differentiation media, with or without drugs and incubated for 30min at 37°C (EGCG: 10mM; H<sub>2</sub>O<sub>2</sub>: 100µM). After additional washes (3 x 5min in PBS), cells were counter stained with anti-LaminB antibody. Cells, were blocked for 1 hour with blocking solution and immuno-stained overnight at 4°C in a solution containing the primary rabbit polyclonal anti-LaminB antibody (Abcam, 1:500). Next, cells were washed with PBS/ 0.1% Triton X-100, slides were incubated for 1 hour at room temperature with Alexa 488 Donkey anti-rabbit secondary antibody in PBS containing 0.5% Triton X-100, 5% BSA. Slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, USA). Images were taken using a DeltaVision elite system (GE).

**Fiber type characterization:** Mice were sacrificed, fresh muscle were dissected (e.g., *gastrocnemius-soleus* and *tibia anterioralis*) and then flash frozen (dry-ice and liquid nitrogen mixture) in an OCT block. Transverse frozen sections (10µm) of each muscle mice were prepared and keep at -80°C for storage. In order to characterize fiber types, we used two mouse antibodies directed against myosin MyHC-I (Isoform BA-D5, DSHB, University of Iowa) and MyHC-IIa (Isoform SC-71, DSHB, University of Iowa) specific to type I and type IIa fibers, respectively.

Staining procedure went as follow: muscle slides were melted at room temperature for 30min and fixed for 10min with 4% paraformaldehyde in PBS. After three PBS 1X 3 min washes, muscle slides were incubated for permeabilization in 0.5% Triton X-100 in PBS for one hour and washed again three time in PBS 1X. Slides were Blocked using a solution of M.O.M IgG diluent (Vector Laboratories, Burlingame, USA) for one hour at room temperature. Slides were then briefly washed twice in PBS 1X for 5 min and incubated with M.O.M protein diluent solution for 5min. Next, slides were incubated with primary antibodies in M.O.M protein diluent (1:20) over night at 4°C. Each slides were treated simultaneously with 4 conditions separated using a Dako pen (Agilent); MyHC-I, MyHCIIa, combined MyHC-I + MyHC-IIa and no primary; to allow characterization of fibers in 3 categories; I, IIa and Unidentified, respectively. After three washes of 5 min each in PBS 1X, slides were incubated for 40min in M.O.M protein diluent containing the secondary antibody (Alexa 488 Donkey anti-mouse; 1:200) and washed three times 5min in PBS 1X. Last, slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, USA). Images were taken using an Axio Imager 2 and a 20X lens (Zeiss)

**3D DNA FISH:** Three dimensional DNA Fluorescent in situ Hybridization was performed as previously described elsewhere <sup>6</sup>. For human myotubes we used a PNA C-Rich probe along with a probe generated by Nick translation using the BAC clone RP11-656018 (CHORI) as template. For MEFs, BACs targeting the *SIRT3* and subtelomeric 7q locus were used as template: B6Ng01-185F08 and B6Ng01-137117, respectively.

**ChIP and ChIP-Seq:** Samples for chromatin immunoprecipitation (IP) were prepared as followed. IP using TRF2 antibody (TRF2- Imgenex124A) were crosslinked for 10 min at RT and 20 min at 4°C with 0.8% formaldehyde (methanol free, ultrapure EM grade, Polysciences, Inc; Warrington PA). Reaction was stop at RT for 10 min with the addition of Glycine to a final concentration of 0.125 M. Cells were rinsed twice with ice-cold 1X PBS, scraped from the dish and pelleted after centrifugation (800g, 5min at 4°C). Next, cells were treated according to the manufacturer's guidance (Pierce Classic Protein G IP Kit, Thermo Scientific). For sonication, we used a total processing time of 15min per sample in a Bioruptor (Diagenode) using the following settings: 14 cycles; 30 Sec ON/30 Sec OFF on High power. Sonicated DNA was controlled on a 2% agarose gel, adequate sonication is achieved when a smear ranging from 200-700bp is obtained. IPs were processed using a 4°C O/N incubation (concentration of TRF2 antibody at 1.5µg); 1µl of each preparation: IP, IgG, Rabbit non-immune Serum, No crosslink control, no Antibody control and 1% input were used as controls for ddPCR analysis. Primers were designed for the promoter region of each gene, results are normalized to inputs and Alu repeats and normalized to adequate controls (e.g., Empty for TRF2, shScramble for sh *TERF2*). Each PCR primer pairs were tested on genomic DNA to verify specificity and efficiency (see primer list file).

**ChIP-Seq analysis:** DNA was sequenced on an Illumina HiSeq in single-end mode with a read length of 49nt, producing an average amount of 2.5 Million of reads per replicate and 3.8 Million for the 1% input replicates. Raw data were filtered and trimmed using Trimmomatic<sup>7</sup> reducing the reads set to -3.8% per file. Reads from each file were aligned to the human reference genome hg38 using Bowtie2<sup>8</sup> with default parameters. The aligned files from identical samples (replicates) were then

merged together and all subsequent analysis were performed using MACS<sup>9</sup> and a suite of tools including BEDTools<sup>10</sup> and BEDOPS<sup>11</sup>. Significant peaks ( $p < 0.05$ ) were identified and annotated using the UCSC database (hg38). Data, including raw files and annotated peaks have been deposited on NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>), accession GSE88983 and the list of genes associated peaks is reported in Supplemental Items 1.

**Chromatin Conformation Capture 3C:** Cell cross-linking and lysis: 4 x 10cm dishes were plated with cells and grown in muscle media until 60% confluence (average of 25 million cells). Cells were then washed once at 37°C in Solution A prior to fixation with a fresh mixture of muscle media with 0.8% formaldehyde (methanol free, ultrapure EM grade, Polysciences, Inc; Warrington PA) at RT on a shaking platform for 10min. The reaction was stopped with 1.25M Glycine (Sigma # G88980) with agitation 10min at RT and 15min on ice. Supernatant was discarded and dishes were washed twice with 10ml ice-cold Solution A. 550µl of cell lysis buffer (10mM Tris-HCl pH 8.0, 10mM NaCl, 0.2% Ige Cal CA 630 (NP-40) + 50µl protease inhibitor Sigma #P8340) was added to each dish on ice. Cells were scraped and collected in a 35ml falcon tube and spun at 300g for 10min, 4°C. Supernatant was discarded and fresh 1.1ml of 3C Lysis Buffer was added. Cells were resuspended, transferred into a 1.5ml tube and incubated on ice for 20min (chemical lysis), then mechanically lysed using a Dounce homogenizer (two 10 up and down strokes separated by 1min incubation on ice). Finally cells were spun 5min at 300g, supernatant discarded and cell pellet washed twice with 300µl of NEB2 buffer (50mM NaCl, 10mM Tris-HCl pH 7.9, 10mM MgCl<sub>2</sub>, 1mM DTT). The pellets were stored at -80°C.

Chromatin digestion: Cells suspended in a 300µl of NEB2 buffer were split in 3x 100µl tubes and an additional 262µl NEB2 buffer 1X was added to each tube. To remove un-cross-linked proteins, 38µl of 1% SDS was added to the tubes and incubated at 65°C for 10min in a thermo-shaker. SDS was quenched by addition of 44µl 10% Triton X-100 on ice (final volume of 444µl per tube). 5µl aliquots were taken per samples as undigested controls. Chromatin was then digested overnight at 37°C in a thermo-shaker by adding 400 Units of HindIII (NEB) per samples. The next day, tubes were put on ice, and 5µl aliquot per samples were taken and labeled 'digestion control'. To determine the digestion efficiency, control aliquots (digested and undigested) were de-crosslinked by adding 10µl proteinase K (NEB) in 90µl 10mM Tris-

HCl pH 7.5 and incubated at 65°C for 2 hours. The digestion efficiency was then analyzed on 0.6% agarose gels. Successful digestion is visualized as a smear with the majority of the fragments between 5 to 10Kb.

Ligation: Ligation mixes were made in 15ml falcon tubes. Each ligation tube contained an 8ml mixture composed of 800µl 10% Triton X-100, 800µl 10x ligation buffer (500mM Tris-HCl pH 7.5, 100mM MgCl<sub>2</sub>, 100mM DTT), 80µl BSA (10mg/ml NEB), 80µl ATP (100mM, Invitrogen) completed with ddH<sub>2</sub>O. Digested chromatin was transferred to each appropriate tube and 1U of T4 DNA ligase was added to each tube (cohesive ligation) and left overnight at 16°C.

DNA extraction: 25µl of 20mg/ml proteinase K was added to each tube and incubated at 65°C during the day. Next, an additional 25µl was added, and the reaction continued overnight. The next day, the 8ml reaction was cooled to RT and transferred into 50ml VWR ultra high-performance tubes. 800µl of 3M Na Acetate pH 5.5 (Ambion) and 18ml of 100% ethanol was added to each tube. DNA was precipitated 2 hours at -20°C. DNA was pelleted by spinning the tubes for 1 hour at 4°C at 12,000g. Pellets were washed ten times with 70% ethanol, and finally resuspended in 500µl TE (10mM Tris pH 8.0, 0.1mM EDTA). RNA was removed by adding 1µl of 1mg/ml RNaseA for 2 hours at 37°C. DNA was purified a second time by phenol:chloroform extraction. Briefly, 500µl phenol (pH 8) was added to the 500µl sample and vortexed for 30s. Samples were spun down in a tabletop centrifuge at max speed for 5 min. The upper layer was then transferred to a new tube and mixed with a fresh 500µl of phenol: chloroform mixture (1:1). Tubes were vortexed another 30s, spun down and supernatant transferred to a new tube where 500µl chloroform was added. Tubes were vortexed for 30s, spun down and the supernatant was transferred to a new 1.5ml tube. The phenol:chloroform procedure was repeated twice. 1/10 volume of 3M Na acetate was added to the 400-500µl supernatant retrieved (40 to 50µl) and completed with 1ml of 100% ethanol. Tubes were inverted several times to properly mix the content and left at 4°C overnight. DNA was pelleted by spinning down the tubes at max speed for 30 min at 4°C. DNA pellets were washed with 70% ethanol 3 times to ensure salt removal (in case salts were not removed, an agarose dialysis step was added to the procedure). DNA was re-suspended in a final volume of 40µl TE.

Ligation efficiency: 3C ligation efficiency was checked by running an aliquot of each tube on 0.8% agarose gels.

**3C quantification:** 3C samples were prepared as described above. Quantification of interactions was done using the ddPCR technology (Biorad) following the manufacturers' instructions with the following modifications in the PCR profile set-up: denaturation 95°C – 10min, followed by 40 cycles of 94°C – 30 sec, 60°C – 30s annealing, 72°C – 30s extension. PCR was stopped with a final 98°C – 10 min step. Plates were then read on the ddPCR droplet reader. All primers were HPLC purified (list available on demand), and a tail probe strategy was used to detect amplicons by adding a short DNA sequence to the SubTel primer<sup>12</sup>, when an internal probe was not available. All PCR pairs used were tested and amplicons were topo-cloned in order to validate the sequences amplified. To quantify and normalized samples, we used the 8 first primer pairs located within the 20Kb limit (Proximity effect) of the fixed primer. An average of the 8 was made and used to normalize 3C target interactions.

**TRF analysis:** Terminal restriction fragment assay was done as previously described<sup>4</sup>.

**Statistical Analysis:** All experiments were repeated at least three times, with three biological replicates (with the exception of human biopsies). Quantitative data are displayed as means  $\pm$  standard error of the mean. Sample sizes as well as the statistical test used of each experiment are described in each corresponding figure legends or methods. Results from each group were treated with the GraphPad prism software for all statistical tests. All tests were two-sided and alpha set at 0.05. Only p-values less than 0.05 were considered statistically significant.

NOTE – References are specific to supplemental methods.

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## Conclusion and new questions

In this work (Robin et al., 2020), we determined that TRF2 expression decreases during early adulthood. We used human myotubes downregulated for TRF2 using a shRNA strategy or a TRF2 KO mouse model using a Cre-lox system under the specific myofiber HSA promoter, thus, TRF2 is specifically depleted in mature myofibers. We expected to induce DNA damage at telomeres, as it is the case in mitotic cells upon TRF2 downregulation. Nonetheless, any DDR was observed upon TRF2 inhibition, neither in human myotubes in vitro, nor in *HSA-Cre<sup>+/-</sup>; Terf2<sup>flox/flox</sup>* mice. We determined a large range-chromatin loop between TRF2 and interstitial telomeric sequences (ITS) within the *SIRT3* locus. TRF2 positively regulates *SIRT3* expression. Moreover, upon TRF2 inhibition, we observed a mitochondrial alteration, ROS accumulation, increased proportion of oxidative fibers and the nuclear translocation of the transcription factor and stress sensor FOXO3a. Altogether, these results suggest TRF2 decline at young ages in human skeletal muscle, leads to an adaptative strategy that could be detrimental at old ages. Therefore, TRF2 loss might trigger a premature aging phenotype.

From this point, two questions were addressed:

How are telomeres protected in myofibers depleted for TRF2?

What are the physiological outcomes of *Terf2* KO in skeletal muscle?

I looked forward to answering these questions. Their results will be explained in the next sections.

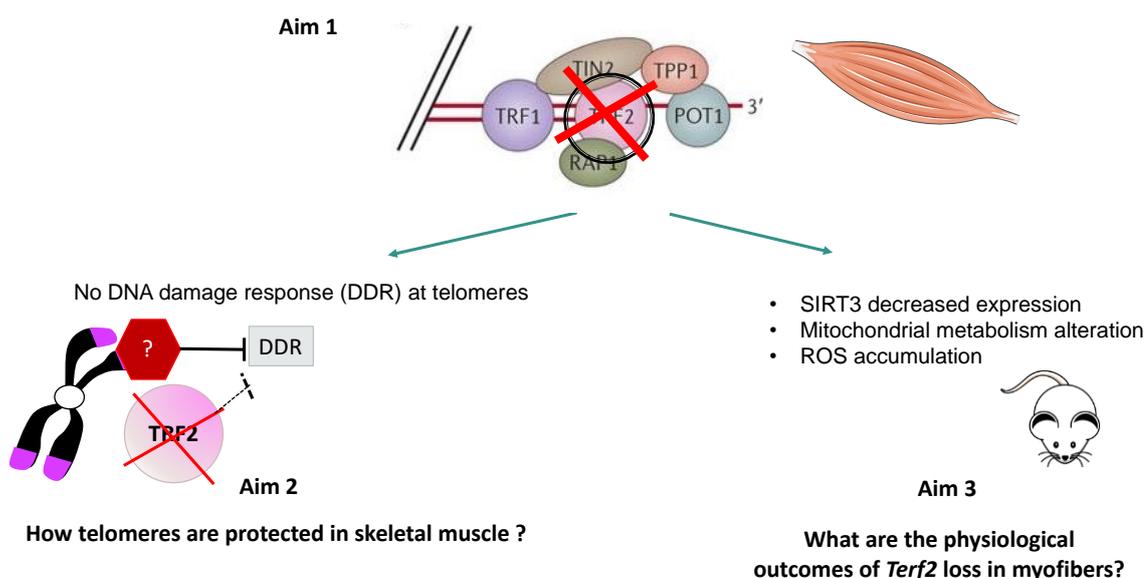


Figure 19: Recapitulative results of TRF2 inhibition in myofibers and new arisen questions

## Second objective: How telomeres are protected in skeletal muscle cells?

We previously observed that TRF2 depletion does not trigger any DDR at telomeres (Robin et al., 2020). Thus, we hypothesized that another factor protects telomeres in skeletal muscle. Since we observed a nuclear translocation of FOXO3a upon TRF2 inhibition, we wanted to determine whether it is involved in telomere protection. To do so, we measured telomere specific damage in human myotubes inhibited for TRF2 and FOXO3a. We showed that telomere protection was lost in these conditions. Hence, FOXO3a is required for telomere protection upon TRF2 decline. Starting from this initial observation, I undertook a study aimed at characterized the function of FOXO3a at telomeres.

# Specific telomere protection ensured by FOXO3a upon genotoxic stress and during aging,

Short title: Specific telomere protection by FOXO3a.

## Authors

Maria Sol Jacome Burbano <sup>1\*</sup>, Jérôme Robin<sup>1\*#</sup>, Serge Bauwens<sup>1</sup>,  
Marjorie Martin<sup>1</sup>, Emma Donati<sup>1</sup>, Lucia Martínez<sup>1</sup>, Sabrina Sacconi<sup>1,4</sup>,  
Frédérique Magdinier<sup>3</sup>, Eric Gilson<sup>1,2</sup>

## Affiliations

1. Côte d'Azur University, CNRS, Inserm, IRCAN, Faculty of Medicine Nice, France;

2. Department of Genetics, CHU; Nice, France.

3. Aix-Marseille Univ-INSERM, MMG, 13005 Marseille, France

4. Peripheral Nervous System, Muscle and ALS, Neuromuscular & ALS Center of Reference, FHU Oncoage, Nice University Hospital, Pasteur 2, Nice, France.

# Present address: Aix-Marseille Univ-INSERM, MMG, 13005 Marseille, France

\*Correspondence: [Eric.gilson@unice.fr](mailto:Eric.gilson@unice.fr)

\* These authors contributed equally to the paper.

## Abstract

Longevity is determined by diverse signaling pathways including telomere protection and homeostasis master regulators like FOXO3a. We previously showed that the telomeric repeat binding factor 2 (TRF2) expression decreases with age in human skeletal muscle and that, surprisingly, its loss in myofibers does not trigger telomere deprotection. We reveal here that in *TERF2*-compromised myotubes, FOXO3a is recruited to telomeres where it acts as a protective factor against ATM-dependent DNA damage activation. Moreover, we show that FOXO3a-telomere association increases with age in human skeletal muscle biopsies. In mitotic fibroblasts, the telomere protective properties of FOXO3a are operative if the cells are treated with bleomycin. The telomere function of FOXO3a does not require its Forkhead DNA binding domain but the CR2C. Overall, these findings demonstrate a

direct connection between two key longevity pathways, FOXO3a and telomere protection. This unveils an unexpected higher level of integration in the regulation of longevity signaling pathway.

## Introduction

Aging can be defined as a progressive failure of homeostasis and organ function that increases susceptibility to several diseases and limits longevity (Singh et al., 2019). An extraordinary diversity of aging trajectories exists in nature (Jones et al., 2014), which can be explained by the effects of natural selection aimed at maximizing fitness in a given environmental context (Partridge and Barton, 1993). Mechanistically, aging is caused by limitation of somatic maintenance and stress response mechanisms, leading to a gradual increase in molecular and cellular damage along with senescent cell accumulation (Gorgoulis et al., 2019; Schumacher et al., 2021). The rate of deterioration is determined by interconnected biological processes, known as aging hallmarks, which are regulated by environmental and genetic factors (López-Otín et al., 2013). Pro-longevity genes, protecting against aging hallmarks, have been identified in human populations through genome-wide association studies. For example, several studies have consistently identified *FOXO3a* as a pro-longevity master transcription factor that serves as a homeostasis regulator (Soerensen et al., 2015; Webb and Brunet, 2014). Meanwhile, the possible existence of one or several developmentally regulated clocks that orchestrate the aging hallmarks remains unclear. In humans and other vertebrates, telomeres may represent such an aging clock, as their DNA length is programmed to shorten in somatic cells during embryonic development (Gilson and Géli, 2007; Wright et al., 1996) and upon stress exposure, accounting in part for the disparate trajectories of aging (Epel et al., 2004; Jacome Burbano and Gilson, 2021). Moreover, dysregulation of telomeres is associated with rare progeroid syndromes (Armanios and Blackburn, 2012) and a broad spectrum of age-related pathologies in the general population (Martínez et al., 2017), while their reinforcement extends the lifespan in mouse models (Muñoz-Lorente et al., 2019). This central position of telomeres as an aging regulator might stem from their interconnection to several aging hallmarks such

as genome stability (de Lange, 2018), senescence (Abdallah et al., 2009), oxidative stress (Barnes et al., 2019), and mitochondrial integrity (Robin et al., 2020; Sahin et al., 2011).

Many telomere functions are performed by a capping protein complex called shelterin. It consists of proteins directly bound to telomeric DNA (in humans, TRF1, TRF2, and POT1) and other proteins that create a bridge between the duplex DNA and the 3' overhang (in humans, TPP1/ACD and TIN2) or are simply bound to other subunits (in humans, RAP1 bound to TRF2). Among shelterin subunits, TRF2 may mediate important regulatory functions during aging, as it interconnects various aging hallmarks (genome stability, senescence, mitochondria, and immunity), is downregulated during aging in multiple tissues (Robin et al., 2020; Wagner et al., 2017), and plays pivotal roles in aging in model organisms (Alder et al., 2015; Kishi et al., 2008; Morgan et al., 2019). The multiple roles of TRF2 rely on its capacity to blunt the DNA damage response (DDR) ataxia–telangiectasia mutated (ATM) checkpoint and non-homologous DNA repair at chromosomal termini, in addition to genome-wide transcriptional functions (Ye et al., 2014). For instance, TRF2 regulates the expression of glyocalyx genes involved in immunosurveillance (Cherfils-Vicini et al., 2019), the *Sirt3* sirtuin gene required for mitochondrial integrity (20) and the *hTERT* telomerase gene (Kim et al., 2016; Sharma et al., 2021).

Strikingly, in skeletal muscle fibers, the absence of TRF2 does not lead to detectable telomere damage, suggesting that an alternative telomere protective mechanism exists in these cells (Robin et al., 2020). Here, we demonstrate that FOXO3a binds to and protects telomeres of post-mitotic skeletal muscle fibers upon *TERF2* downregulation. The role of FOXO3a in telomere protection is conserved in dividing cells upon genotoxic stress. These results reveal a connection between two important longevity pathways.

## Results

### 1. FOXO3a is required for telomere protection upon *TERF2* downregulation in myotubes

In accordance with our previous findings (Robin et al., 2020), *TERF2* knockdown (KD) in human myotubes did not trigger DDR specifically at telomeres, as measured by the ratio of 53BP1 nuclear foci associated with telomeres (designated specific telomere dysfunction-induced foci or sTIF), which led to a high cellular level of reactive oxygen species (ROS) (Fig. 1A–C; Fig. EV1. A-B). Moreover, *TERF2* downregulation in myotubes activates FOXO3a (Robin et al., 2020), a longevity factor involved in the oxidative stress response and muscle homeostasis (Mammucari et al., 2007; Milan et al., 2015; Sandri et al., 2004; Zhao et al., 2007). Therefore, we investigated whether FOXO3a could act at telomeres in *TERF2*-compromised myotubes. Interestingly, downregulation of *FOXO3a* expression in myotubes led to a specific loss of telomere protection against DDR only with simultaneous *TERF2* downregulation (Fig. 1A–C). Further, treatment with chemical inhibitors of the DDR kinases ATM (KU-55933) or ATR (ATR; VE-821) revealed that the telomere-specific damage triggered by double downregulation of *TERF2* and *FOXO3a* in myotubes was ATM dependent (Fig. 1D).

In addition, downregulation of *FOXO3a* expression did not increase the high ROS level induced by inhibition of *TERF2* expression (Fig. EV1 C–F). Similarly, the expression of a panel of oxidative stress-related genes (*NRF2*, *GSS*, *BNIP3*, *PRDX1*, *SOD2*, and *CAT*) was not impaired by *FOXO3a* downregulation in *TERF2*-compromised myotubes (Fig. EV1 G–L). Therefore, telomere deprotection triggered by *FOXO3a* expression inhibition in *TERF2*-compromised myotubes cannot be explained simply by a global increase in ROS or a decrease in oxidative defense.

Next, because telomeres are G-rich and particularly sensitive to oxidation through the formation of 8-oxoguanosine (8-oxoG), we examined whether FOXO3a plays a role in this specific oxidation process. To that end, we quantified colocalization events between telomeric DNA foci marked with a fluorescent PNA probe and 8-oxoG foci revealed with specific antibodies (known as teloxidized telomere foci or TOF). Echoing sTIF, only double downregulation of *TERF2* and *FOXO3* increased the mean number of TOFs per nucleus (Fig. 1E, F).

Our results suggest that FOXO3a is capable of decreasing oxidation and ATM-dependent DDR activation specifically at telomeres in a post-mitotic context (myotubes). As this telomeric role of FOXO3a is revealed in the context of oxidative

stress triggered by *TERF2* downregulation, we hypothesized that FOXO3a provides an alternative strategy for telomere protection in the presence of genotoxic stress.

## **2. FOXO3a localizes to myotube telomeres**

Next, we investigated whether the telomere protective role of FOXO3a is directed through assessment of its putative association with telomeres. On average, 17 FOXO3a foci were detected in untreated myotube nuclei, of which four specific colocalizations with telomeres were observed using a PNA probe (Fig. 1G, H). Upon *TERF2* downregulation, in parallel with nuclear translocation of activated FOXO3a (Fig. EV1 M), we measured a roughly two-fold increase in such colocalization events (Fig. 1G-H). To validate this interaction between telomeres and FOXO3a, we performed a proximity ligation assay (PLA) using TRF1 and FOXO3a as potential partners. Moreover, we assessed FOXO3a enrichment at telomeres through chromatin immunoprecipitation (ChIP) with slot-blotting quantification using a radiolabeled telomeric DNA probe (Fig. EV1 N–Q). We concluded that FOXO3a plays a direct role in telomere protection in myotubes upon *TERF2* downregulation.

Based on these results, we investigated the interaction in an *in vivo* context using our previously published set of muscle biopsies (Robin et al., 2020). We confirmed the presence of FOXO3a at telomeres using co-immunoprecipitation (co-IP) with an antibody against TRF2 based on FOXO3a immunoblotting (Fig. EV1 R). Notably, the level of co-IP increased with age (Fig. EV1 S, T). This result suggests an important role of the FOXO3a–telomere association during skeletal muscle aging. Older muscle biopsies contain lower levels of TRF2 (Robin et al., 2020), and therefore the increase in TRF2–FOXO3a co-IP with age can be interpreted as a high level of oxidative stress triggering the association of FOXO3a with the residual TRF2 present at telomeres, in accordance with observations in *TERF2*-compromised myotubes.

## **3. FOXO3a acts synergistically with TERF2 to protect telomeres in transformed fibroblasts**

Next, we investigated whether the telomeric protection function of FOXO3a is conserved in another cellular context (*i.e.*, mitotic cells). To this end, we used BJ-HELT transformed fibroblasts derived from human foreskin cells expressing *hTERT* and

*SV40 early region* (large-T and small-t antigens) genes. In these cells, downregulation of *FOXO3a* did not lead to specific telomere damage, whereas *TERF2* downregulation caused such damage, as expected (Fig. 2A-B B). Notably, double downregulation of *FOXO3a* and *TERF2* showed synergistic effects, exacerbating telomere damage (Fig. 2A, B). This situation is reminiscent of myotubes, wherein the telomere protective function of *FOXO3a* is revealed only upon *TERF2* downregulation.

Therefore, we explored whether *FOXO3a*-dependent telomere protection is specific to *TERF2*-compromised cells by repeating the previous experiment with sequential downregulation of each shelterin subunit (Fig. EV2 A–I). As expected, individual downregulation of *TERF1*, *TERF2*, *TPP1*, *POT1*, or *TINF2* induced specific telomere damage. When coupled downregulation with *FOXO3a* was tested via RNA interference, only the *TERF2*–*FOXO3a* KD condition increased the level of specific telomere damage. Thus, *FOXO3a* appears to be an alternative protective factor against telomere damage induced by TRF2 loss. This finding is in accordance with the ATM-dependent damage observed with concomitant downregulation of *TERF2* and *FOXO3a* in myotubes (Fig. 1D) and BJ-HELT cells (Fig. 2C-D). In contrast to myotubes, we failed to detect any difference in *FOXO3a* expression or nuclear translocation upon *TERF2* downregulation (Fig. EV2 C, E, J–K) in BJ-HELT cells, indicating that *FOXO3a*-mediated telomere protection in *TERF2*-compromised BJ-HELT fibroblasts does not result from activation due to oxidative stress.

#### **4. FOXO3a specifically protects telomeres from ATM-recognized damage**

To determine the type of telomere damage that *FOXO3a* specifically protects against, we exposed BJ-HELT cells to a set of genotoxic stressors known to cause DNA damage that may be repaired by various processes ( $H_2O_2$ , bleomycin, paraquat, sodium arsenite, and ultraviolet radiation) (Fig. 3A; Fig. EV3 A, B). Only bleomycin-treated cells showed an effect of *FOXO3a* downregulation on telomere protection (Fig. 3A, B). With *TERF2* downregulation, *FOXO3a* specifically protected against ATM-recognized damage in bleomycin-treated BJ-HELT cells (Fig. 3C). These results are reminiscent of previous work reporting that *FOXO3a* protects the genome against instability caused by double-strand breaks (White et al., 2020). We conclude that *FOXO3a* protects against multiple types of ATM-recognized damage at telomeres.

Next, we asked whether, as in myotubes, FOXO3a plays a direct telomere protective role in BJ-HELT cells. We detected TRF2–FOXO3a associations in bleomycin-treated cells using a PLA (Fig. 3D, E; Fig. EV3 C, D). Such associations cannot be attributed only to the partial nuclear translocation of FOXO3a observed upon bleomycin treatment (Fig. EV3 E, F) because starvation, which causes massive localization of FOXO3a in the nucleus, did not increase PLA signals (Fig. 3D, E).

## **5. The CR2C domain of FOXO3a is essential for its role in telomere protection**

Finally, we investigated which domain of the FOXO3a protein is involved in telomere protection. To this end, we constructed a battery of lentiviral vectors expressing various truncated forms of FOXO3a:  $\Delta$ CR1,  $\Delta$ FH,  $\Delta$ CR2C, and  $\Delta$ CR3 (Fig. 4A). Through downregulation of FOXO3a via RNA interference in cells overexpressing either wild-type (WT) or truncated FOXO3a, we obtained cells expressing nearly physiological levels of FOXO3a or its truncated forms (Fig. 4B; Fig. EV4 A-B). Importantly, expression of WT FOXO3a was sufficient to restore telomere protection in bleomycin-treated cells, indicating that the small interfering RNA pool we used to downregulate FOXO3a expression did not cause off-target effects related to telomere protection (Fig. 4C). Notably, expression of the truncated form with deletion of the Forkhead (FH) DNA-binding domain also rescued telomere protection. Among other truncated forms, only the form with deletion of the CR2C domain was unable to rescue telomere protection (Fig. 4C; Fig. EV4 C, D), and this form also reduced FOXO3a telomeric localization (Fig. 4D). We conclude that FOXO3a exhibits telomere protective properties uncoupled from its canonical role as a DNA-binding transcription factor, but that is dependent on the CR2C domain.

The CR2C domain interacts with CBP/p300 and SIRT1 proteins. Interestingly, p300 has been shown to bind and acetylate TRF2 and to be essential for telomere protection in HT1080 cells (Her and Chung, 2013), whereas SIRT1 binds to telomeres and regulates the maintenance of telomere length (De Bonis et al., 2014; Palacios et

al., 2010). Therefore, p300 and SIRT1 are promising candidates that may contribute to the telomere protective properties of FOXO3a. Thus, we downregulated the expression of *p300*, *CBP*, and *SIRT1* in bleomycin-treated cells. Only downregulation of *p300* weakened the TRF2–FOXO3a association (Fig. 4E). Moreover, its downregulation increased telomere-specific damage in bleomycin-treated cells, mimicking the effects of *FOXO3a* downregulation (Fig. 4F; Fig. EV4 E-F). Whereas the TRF2 level was previously reported to decrease upon p300 inhibition (Her and Chung, 2013), we observed no difference in TRF2 protein levels between control and p300-compromised cells (Fig. EV4 G-H).

Overall, our results demonstrate a direct role of FOXO3a in protecting telomeres against ATM-recognized damage through a complex mediated by p300.

## Discussion

Mammalian FOXO transcription factors, homologs of DAF-16, which regulates longevity in *Caenorhabditis elegans*, play a key role in regulating cell homeostasis through upregulation of genes associated with oxidative stress resistance, metabolism, apoptosis, cell cycle arrest, aging, and autophagy (Webb and Brunet, 2014). Here, we reveal a novel function of FOXO3a in providing specific protection to telomeres against a genotoxic insult. Downregulation of *FOXO3a* in unchallenged cells has little or no effect on telomere protection, while FOXO3a uncaps telomeres in cells experiencing genotoxic stress. This conditional protective role of FOXO3a was observed in post-mitotic muscle fibers upon *TERF2* downregulation, leading to mitochondrial dysfunction and telomere oxidation, as well as in transformed fibroblasts upon *TERF2* downregulation combined with treatment with the clastogenic agent bleomycin.

The protective function of FOXO3a on telomeres cannot be explained simply by its general roles in DNA repair and oxidative stress (Brunet et al., 2004; Tran et al., 2002; White et al., 2020), but instead indicates specific properties affecting only telomeres. First, through normalization of the rate of telomere damage to the total amount of nuclear damage, telomeres are better protected than the rest of the genome upon *FOXO3a* downregulation. Second, in *TERF2*-compromised myotubes, the rate of oxidized telomeres increased markedly upon *FOXO3a* downregulation, while the total amount of ROS remained unchanged. Third, a subset of FOXO3a proteins are

associated with telomeres, as revealed through various techniques, including co-localization with telomeric PNA probe foci, PLA foci between FOXO3a and either TRF1 or TRF2, ChIP revealed by telomere slot blotting, and co-IP with TRF2. In myotubes, the telomere–FOXO3a association increased upon *TERF2* downregulation, in parallel with an increase in the number of FOXO3a nuclear foci. In transformed fibroblasts, this association also increased upon bleomycin treatment. Notably, this telomere association may be uncoupled from FOXO3a nuclear translocation. Indeed, starvation, which causes massive translocation of FOXO3a into the nucleus, was not correlated with an increase in telomere association; conversely, bleomycin treatment led to increased recruitment of FOXO3a at telomeres in the absence of detectable nuclear translocation. These findings suggest that FOXO3a must undergo specific post-translational modification in response to genotoxic stress to associate with telomeres.

The telomere protective role of FOXO3a is conserved upon deletion of its FH DNA-binding domain or its C-terminal CR3 transactivation domain. These results rule out the possibility of FOXO3a acting at telomeres through its canonical role as a DNA-binding transcription factor. Moreover, deletion of CR3 increases the capacity of FOXO3a to associate with telomeres. This finding suggests competition between the major transactivation domain of FOXO3a and its telomere-specific functions. Deletion of CR3 may also cause FOXO3a to exhibit an open conformation (Wang et al., 2008) that is more prone to telomere association. The CR3 domain was reported to bind and activate ATM (Adamowicz et al., 2016; Tsai et al., 2008), making a protective role of FOXO3a at telomeres via its interaction with ATM unlikely. In contrast to the FH and CR3 domains, deletion of CR2C impairs the telomere protective role of FOXO3a. CR2C exhibits transactivation activities and mediates the associations of FOXO3a with SIRT1 (Nakae et al., 2006) and with CBP/p300 (Senf et al., 2011; Wang et al., 2012). Among *SIRT1*, *CBP*, and *p300*, only downregulation of *p300* impaired the TRF2–FOXO3a association, as revealed by PLA. In accordance with involvement of a FOXO3a–p300 complex in telomere protection, p300, but not CBP, was associated with telomeres (Cubiles et al., 2018), raising the possibility that acetylation mediated by p300 may promote the repair of damaged telomeres. This model requires substantiation, particularly through determining the targets of FOXO3a–p300 on telomeres. One candidate is TRF2, which contains a p300 binding site (Her and Chung, 2013). These findings raise the question of the type of damage that causes FOXO3a

to be efficiently signaled for telomere repair. Our results suggest that such damage is recognized through the ATM-dependent DDR pathway.

The findings of this study establish a direct link between two key longevity hallmarks, FOXO3a (Martins et al., 2016) and telomere protection (Augereau et al., 2021; Foley et al., 2018; Wirthlin et al., 2018). This connection is likely involved in muscle aging. Indeed, telomere protection relies on FOXO3a in *TERF2* depleted muscle fibers, a situation occurring during muscle aging (Robin et al., 2020). Furthermore, FOXO3a–telomere increased association with age based on human biopsies. Overall, our findings reveal that telomeres are protected in a privileged manner by FOXO3a during aging, supporting their role as central longevity regulators.

## **Materials and Methods**

### **Cell culture:**

Human myoblasts in this study have been previously described (Robin et al., 2020). In brief, cells were plated in dishes coated with 0.1% pigskin gelatin in 4:1 Dulbecco modified Eagle medium/Medium 199 supplemented with 15% FBS, 0.02M HEPES, 1.4mg/l vitamin B12, 0.03mg/l ZnSO<sub>4</sub>, 0.055mg/l dexamethasone, 2.5µg/l hepatocyte growth factor and 10µg/l basic fibroblast growth factor. Cultures were passaged at ~70% confluency. Differentiation into myotubes was started following a change to differentiation medium (2% horse serum in 4:1 Dulbecco modified Eagle medium: Medium 199) when 90% confluent.

For infection of myotubes, myoblasts were seeded in 10 cm dishes, switch to differentiation media (2% Horse Serum) upon confluence (90%) and transduced at least week after. Cells were transduced at a MOI 2 using the different shRNAs.

BJ-HELT cells were immortalized by transduction of hTERT and SV40 ER genes (Biroccio et al., 2013). BjHeLT cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 µg/ml). Cells were cultured at 37°C, 5% CO<sub>2</sub>.

### **Lentivirus infection and siRNA transfection:**

Lentiviruses were produced by transient calcium phosphate transfection of HEK293T cells with the virus packaging plasmids, p8.91 and pVSVg and a pWPIR-GFP lentiviral expression vector or pLKO plasmid, that contained the sequence of interest. We used MISSION shRNAs (pLKO.1; Sigma): *TERF2* (TRNC0000004812), *FOXO3A* (TRNC0000010335, only clone validated by Sigma MISSION shRNAs, to date) and control (SHC002). For upregulation, the pWPIR-GFP backbone was used and modified by Genscript to reach full-length FOXO3a pWPIR-FOXO3aWT-GFP and mutant forms pWPIR- $\Delta$ CR1-GFP, pWPIR- $\Delta$ FH-GFP, pWPIR- $\Delta$ CR2C-GFP, pWPIR- $\Delta$ CR3-GFP. pWPIR-TRF2-GFP and pWPIR-TRF2 $\Delta$ B-GFP vectors have already been validated (Benarroch-Popivker et al., 2016). Transduction efficiency was determined by one-week puromycin treatment and count the number of resistant clones for pLKO.1 vectors and GFP-flow cytometry detection after 3 days for pWPIR-GFP vectors.

siRNA transfections were performed with On-Target Plus SMARTpool (Dharmacon) and Dharmafect1 transfection reagent (T-2001, Dharmacon) for 72 hr.

Efficiency of each shRNA and siRNA was checked routinely by RT-qPCR or Western blotting.

#### **Cellular stressors treatments:**

BJ-HELT cells were treated with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Merk: 95294) for 24h. Bleomycin (Merk B2434), Paraquat (Merk: 36541) and Sodium Arsenite (Merk: S7400) were resuspended in distilled water and added to cell media at 50  $\mu$ g/mL, 1,5 mM and 1  $\mu$ M, respectively for 24h. For UV irradiation, media was removed, cells were washed with PBS and kept in a minimal volume of PBS. Then, cells were irradiated with UVA and UVB at 300 mJ/cm<sup>2</sup> and let to recover for 2 hr before collection or fixation. To induce starvation, BJ-HELT cells were grown in normal conditions up to 70% confluency. Then, FBS containing media was removed, cells were washed with DMEM once and add DMEM without FBS. Cells were incubated under starvation for 1 week.

#### **Human biopsies and Ethic statement:**

The collection of fetal muscle biopsies was approved by the “Agence Française de la Biomedecine” of the Ministry of Health for legal access to the biological material

in full accordance with the law (research protocol number PFS13-006). Samples were obtained after therapeutic abortion. Parents have provided written informed consent for the use of biopsies for medical research in accordance with the Declaration of Helsinki. Muscle biopsies were processed by fetopathologists from fetuses not affected by a muscular pathology. Skeletal muscle biopsies from teens and adults were obtained from the Nice Hospital (CHU l'Archet registered as protocol number DC-2015 2374) and from the Tumorothèque, Assistance Publique des Hôpitaux de Marseille, agreement n°AC-2013-1786, from healthy donors using standardized muscle biopsy protocol.

#### **Immunofluorescence-FISH and Telomere damage Induced Foci (TIFs):**

Cells were grown onto glass coverslips or multichamber slides and fixed for 20 min with 3.7% formaldehyde. Cells were then permeabilized with 0.5% Triton X-100 for 15 min and dehydrated in increasing concentrations of ethanol for 3 min (50%, 70% and 100%). Hybridization of PNA probes was performed for at least 2 hr at RT after denaturation (5 min) in 70% formamide, 10 mM Tris pH 7.2 and 1% blocking solution (Roche) at 80°C. After that, the cells were washed in a 70% formamide, 10 mM Tris pH 7.2 solution for 30 min, followed by washes with 150 mM NaCl and 50 mM Tris pH 7.5 for 15 min. Next, the cells were incubated 1h with blocking buffer (3% BSA and 0,3% Triton X-100) and incubated overnight at 4°C with the desired antibody: FOXO3a, rabbit monoclonal (clone 75D8, 1:200, Cell Signaling Technology), 53BP1, rabbit polyclonal (NB 100-305, 1:200, Novus Biologicals) and 8- Oxoguanine, mouse monoclonal (clone 483.15, 1:50, MAB3560, Millipore). Cells were then washed with 1X PBS and incubated for 1 hr with the corresponding secondary antibody. Finally, slides were prepared with a DAPI containing mounting solution (Vectashield, Vector Laboratories).

#### **Immunofluorescence:**

Cells were grown onto glass coverslips or multichamber slides and fixed for 20 min with 3.7% formaldehyde. Cells were then permeabilized with 0.5% Triton X-100 for 15 min and blocked for 1h with (3% BSA and 0,3% Triton X-100). Next, cells incubated overnight at 4°C with the desired antibody: FOXO3a, rabbit monoclonal (clone 75D8, 1:200, Cell Signaling Technology). Cells were then washed with 1X PBS

and incubated for 1 hr with the corresponding secondary antibody. Finally, slides were prepared with a DAPI containing mounting solution (Vectashield, Vector Laboratories).

### **Reactive Oxygen Species (ROS):**

Cells were grown on cover slides and treated as indicated in the manufacturer's ROS kit instructions (Enzo-51011) and previously described. Transduced differentiated cells were washed twice and 1ml of fresh differentiation media was added, with or without EGCG (10mM) or H<sub>2</sub>O<sub>2</sub> (100μM) and incubated for 30min at 37°C. After additional wash and media renewal (1ml), cells were incubated for 1 hour at 37°C with a solution composed 2X ROS detection and Oxidative stress reagent (5mM; dilution 1:2500). Cells were then washed with 1X PBS and directly mounted using 15μl of vectashield+DAPI, without any fixation. Pictures were taken using a DeltaVision Elite system (GE). An average of 100 stacks and 50 nuclei were taken per conditions. Images were then treated using IMARIS. Intensities of ROS foci and DAPI staining were used for analyses, excluding single-nuclei cells for myotubes analysis.

### **Proximity Ligation Assay (PLA):**

Cells were, fixed with 3,7% formaldehyde, permeabilized with 0.5% Triton X-100 for 15 min, and blocked with Duolink kit Blocking solution (DUO94001, Sigma-Aldrich). Cells were then incubated with primary antibodies to TRF2, mouse monoclonal, (NB100-56506, 1:300, Novus Biologicals), FOXO3a, rabbit monoclonal (clone 75D8, 1:1500, Cell Signaling Technology). Then, cells were incubated for 1h at 37°C with DNA-linked secondary antibodies (PLA probes), analyzed with the Duolink *In Situ* Red Mouse/Rabbit kit assay according to the manufacturer's instructions (DUO94001, Sigma-Aldrich). In brief, the ligation and amplifications steps were performed for 30 min and 2 hr, respectively, at 37°C. Finally, cells were fixed again with formaldehyde 37°C. Images were taken with Deltavision Elite® and analyzed with FIJI software. PLA spots per cell were manually counted. Only nuclear spots were considered for analysis. Each antibody used was first tested by immunofluorescence. PLA negative controls were performed to test each couple of antibodies: each antibody alone or without any antibody but with mouse/rabbit PLA probes.

### **Chromatin-Immunoprecipitation (ChIP):**

Samples were crosslinked for 10 min at RT and 20 min at 4°C with 0.8% formaldehyde (methanol free, ultrapure EM grade, Polysciences, Inc; Warrington PA). Reaction was stop at RT for 10 min with the addition of Glycine to a final concentration of 0.125 M. Cells were rinsed twice with ice-cold 1X PBS, scraped from the dish and pelleted after centrifugation (800g, 5min at 4°C). For sonication, we used a total processing time of 15min per sample in a Bioruptor (Diagenode) using the following settings: 14 cycles; 30 Sec ON/30 Sec OFF on High power. Sonicated DNA was controlled on a 2% agarose gel, adequate sonication is achieved when a smear ranging from 200-700bp is obtained. IPs were processed using a 4°C O/N incubation with TRF2 antibody at 1.5µg; Novus: NB100-56506); 1µl of each preparation: IP, IgG, Rabbit non-immune Serum. Next, magnetic beads (Dynabeads, Life Technologies) were added for 3 h. Samples were washed with a low salt buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS), a high salt buffer (500 mM NaCl, 1% Triton X-100, 0.1% SDS) and a lithium salt buffer (0.25 M LiCl, 1% NP40, 1% deoxycholic acid). Chromatin was eluted (1% SDS, 0.1 M NaHCO<sub>3</sub> solution), and the cross-linked chromatin was reversed at 65°C overnight. The DNA was treated with RNaseA and proteinase K, followed by phenol–chloroform purification. The DNA obtained from ChIP was denatured and blotted onto nylon membranes using a slot blot apparatus, cross-linked, and hybridized with telomere and Alu repeats radioactively labeled probes. The membranes were exposed onto phosphorimager screens, and the signal intensity was quantified with ImageQuant software.

#### **Co-Immunoprecipitation :**

Skeletal muscle human biopsies were lysed in 50 mM Tris-HCl, 150 mM NaCl and 0,1% NP40; complemented with phosphatase inhibitors (Roche). Samples were sonicated using a Bioruptor (Diagenode) for 5 minutes (30 sec ON/ 30 sec OFF on High Power). Then, tubes were centrifugated 5 min at 4°C, 12 000 g. 10% of each sample was used for Input. Immunoprecipitations were processed using a TRF2 antibody (Novus: NB100-56506) at 4°C, O/N incubation. Next, magnetic beads (Dynabeads Protein G, Thermofisher, 10004D) were added for 1h at 4°C. Samples were washed with 50 mM Tris-HCl, 150 mM NaCl and 0,1% NP40; complemented with phosphatase inhibitors (Roche), five times. Resuspend samples in 2X Laemmli. Finally, samples were denaturated at 95°C for min and load then on a SDS-PAGE gel for Western Blot running.

**Western Blot:**

Cells were collected in 1X PBS and spin down (250g, 5min) and pellet stored at -80°C for further use. Whole cell lysates were prepared from cells by adding cell lysis RIPA buffer, complemented with phosphatase inhibitors (Roche) and kept 30 min on ice. For each sample, 30µg of protein was resolved in a 4-15% gradient mini-protean precast polyacrylamide gels (BioRad) and transferred to nitrocellulose membranes (Whatman, GE Healthcare) for 1h at 4°C. After blocking for 1 hour with 5% skim milk in PBST (0.1% Tween-20 in PBS), the membranes were incubated overnight at 4°C with primary antibodies diluted in 5% milk. The following primary antibodies were used: TRF2, mouse monoclonal, (NB100-56506, 1:1000, Novus Biologicals); TRF2, rabbit monoclonal, (NB110-57130, 1:5000, Novus Biologicals); FOXO3a, rabbit monoclonal (clone 75D8, 1:1500, Cell Signaling Technology); GAPDH, rabbit polyclonal (1:2000, NB100-56875, Novus Biologicals). The membranes were then rinsed three times in PBST for 10min and incubated 1 hour at room temperature with appropriate secondary antibodies diluted (1:5000) in 5% PBST-milk (e.g. anti-mouse HRP IgG and anti-rabbit HRP IgG, Vector Laboratories). Membranes were developed using the Luminata Forte HRP substrate (Millipore) and exposed in the Fusion Solo apparatus (Vilbert Lourmat).

**RT-qPCR:**

Myotubes RNA was extracted by Tri Reagent (Trizol; Sigma T9424), following manufacturer's instructions. BJ-HELT and myoblasts were lysed (RNeasy plus kit (Qiagen: 74034)). Total RNA purified according to the manufacturer's instructions and was quantified on a Nanodrop 1000 spectrophotometer (Thermo Scientific). For Reverse Transcription (RT) 500ng RNA was reverse transcribed High-Capacity RNA-to-cDNA Kit (Thermo Scientific). Each qPCR contained 5X diluted cDNA, 0.2 µM primers, and SYBR Green Master Mix (Roche, 4913914 001). Quantitative RT-PCR (qRT-PCR) was performed in triplicates using FastStart universal SYBR Green master Mix (Roche). Melting curves were analyzed (SYBR green) to exclude non-specific amplification products. We confirmed amplicon size at least once on agarose gels. Crossing-threshold (Ct) values were normalized by subtracting the geometric mean of two housekeeping genes (GAPDH and HPRT).

**Statistical Analysis:**

Statistical analysis was performed using the Prism 7 software (GraphPad). Quantitative data are displayed as means  $\pm$  standard error of the mean. For comparison of two groups, we used two-tailed Mann–Whitney U-test or Student's t-test, and for multiple groups, the Kruskal–Wallis test was used.

$p$ -value  $< 0.05$  were considered significant (\* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

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### **Author contributions:**

The project was design by EG, MSJB and JDR. Investigation and validation were performed by MSJB and JDR. SB, MM, ED and LM provided technical help. FM and SS provided human skeletal muscle biopsies. MSJB and EG wrote and edit the original draft. EG supervised this work. All authors read and commented on the manuscript.

### **Competing interests:**

Authors declare that they have no competing interests

### **Data and materials availability:**

All data are available in the main text or the supplementary materials.

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**Fig. 1: FOXO3a directly protects telomeres upon TRF2 downregulation in myotubes.**

**(A)** Telomere Induced damage Foci assay in transduced myotubes (10 days), using a telomeric PNA probe (green) and 53BP1 (red) staining. Colocalizations pointed by an arrow, indicate DDR activation at telomeres. 30-40 nuclei were analyzed per replicate and per condition, n=4.

**(B)** Western Blotting showing *TERF2* and *FOXO3a* downregulation. In *shTERF2* myotubes, *FOXO3a* expression is increased.

**(C)** Ratio between the number of TIFs and the total number of 53BP1 within each nucleus, corresponding to the specific telomere damage (sTIF). *shScramble* vs *shTERF2-shFOXO3a*, *p-value* <0.000001.

**(D)** Telomeric specific damage (TIFs/53BP1 ratio) in myotubes inhibited for ATM and/ or ATR. 5 days after differentiation and 5 days before harvesting, ATM or ATR was inhibited using either KU-55933 (10mM) or VE-821 (10mM), respectively. Fresh media with chemical inhibitors was added every 2 days. Bars represent SEM of two biological replicates. *p-value* <0.000001.

**(E)** Immunofluorescence detection of 8-Oxoguanosine (red), combined with telomeric FISH probe (green) in transduced myotubes and quantified in **(F)**. Oxidated telomeres (TOFs) were analyzed by colocalization of the green and red signals. Bars represent SEM of two biological replicates (approximately 35 nuclei). *shScramble* vs *shTERF2-shFOXO3a*, *p-value* <0.000001.

**(G)** Immunofluorescence detection of *FOXO3a* (red), combined with telomeric FISH probe (green) in myotubes transduced with *shTERF2*. Colocalization are shown by a white arrow and a zoom is shown at the left. Quantification of *FOXO3*-Telomere foci per nucleus in H). *p-value*= 0,0008.

Statistical analyses were performed using Kruskal-Wallis' multiple comparisons test (\**p* < 0.05, \*\**p* < 0.001, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001).

**Fig. 2: FOXO3a protects telomeres upon TERF2 KD in BJ-HELT fibroblasts.**

**(A)** Telomere Induced damage Foci assay in transfected BJ-HELT (72h siRNA), using a telomeric PNA probe (green) and 53BP1 (red) staining. 53BP1-Telomeres colocalizations are pointed by a white arrow.

**(B)** Quantification of sTIF observed in **(A)**. 30-40 nuclei were analyzed in three biological replicates. siCtrl vs siTRF2 *p-value*= 0,008304 and siCtrl vs siTRF2-siFOXO3a *p-value* <0.000001.

**(C)** TIF assay in siTRF2-siFOXO3a transfected BJ-HELT cells (72h siRNA, PNA-telomere probe in green and 53BP1 in red), inhibited for ATM or ATR. Inhibitors were added 24h before cell harvest: KU-55933 (10mM) or VE-821 (10mM), respectively. Zoom at the left.

**(D)** sTIF quantification. Bars represent SEM of two biological replicates. DMSO vs KU-55933 *p-value* =0,0112.

Statistical analysis were performed using Kruskal-Wallis' multiple comparisons test;  $\alpha=0.05$ )

**Fig. 3: FOXO3a specifically protects telomeres from ATM-recognized damages.**

**(A)** Telomere specific damage (sTIF) in transfected BJ-HELT cells with siControl (black circles) or siFOXO3a (pink circles) and exposed to different stressors. Statistical analyses were performed using Kruskal-Wallis' multiple comparisons test ( $*p < 0.05$ ,  $**p < 0.001$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ) in biological triplicates.

**(B)** Representative images of TIFs in non-treated or bleomycin exposed BJ-HELT cells downregulated or not for FOXO3a. Immunofluorescence of 53BP1 (red) and telomeric PNA-FISH probe (green).

**(C)** sTIF in siFOXO3a-BJ-HELT cells, treated with bleomycin (50  $\mu\text{g}/\text{mL}$ , 24hr) and inhibited for ATM (KU-55933) or ATR (VE-821). Two biological replicates analyzed with a Kruskal-Wallis' multiple comparisons test (DMSO vs KU-55933, *p-value*= 0,0464).

**(D)** PLA showing TRF2-FOXO3a association in non-treated BJ-HELT or exposed to bleomycin (50  $\mu\text{g}/\text{mL}$ , 24hr) or under caloric restriction. NT vs Bleo (*p-value* <0.000001) and NT vs Starvation (*p-value*=0,007385) were analyzed using a Kruskal-Wallis' multiple comparisons in biological triplicates.

**(E)** Representative images of TRF2-FOXO3a PLA in E). PLA red foci are pointed by white arrows.

**Fig. 4: FOXO3a CR2C domain is required for telomere protection**

**(A)** FOXO3a full-length structure (top) showing relevant domains and truncated mutant forms ( $\Delta$ CR1,  $\Delta$ FH,  $\Delta$ CR2C and  $\Delta$ CR3). FOXO3a is composed by three conserved regions: CR1, CR2 and CR3 known to interact with several proteins. CR2 is divided in three subregions: CR2A, CR2B and CR2C. FOXO3a contains a Forkhead (FH) or DNA binding domain, that recognizes a TGTTTAC consensus DNA sequence, followed by a nuclear localization sequence (NLS).

**(B)** Western blotting showing endogen FOXO3a downregulation, FOXO3a full-length rescue and expression of FOXO3a truncated proteins. FOXO3a-Cell Signaling Technologies #2497 monoclonal antibody does not recognize  $\Delta$ CR1 form because its epitope surrounds Glu50 (N-terminal). Cells were transduced 5 days with lentiviruses and the last 3 days transfected with siRNA. GAPDH was used as loading control.

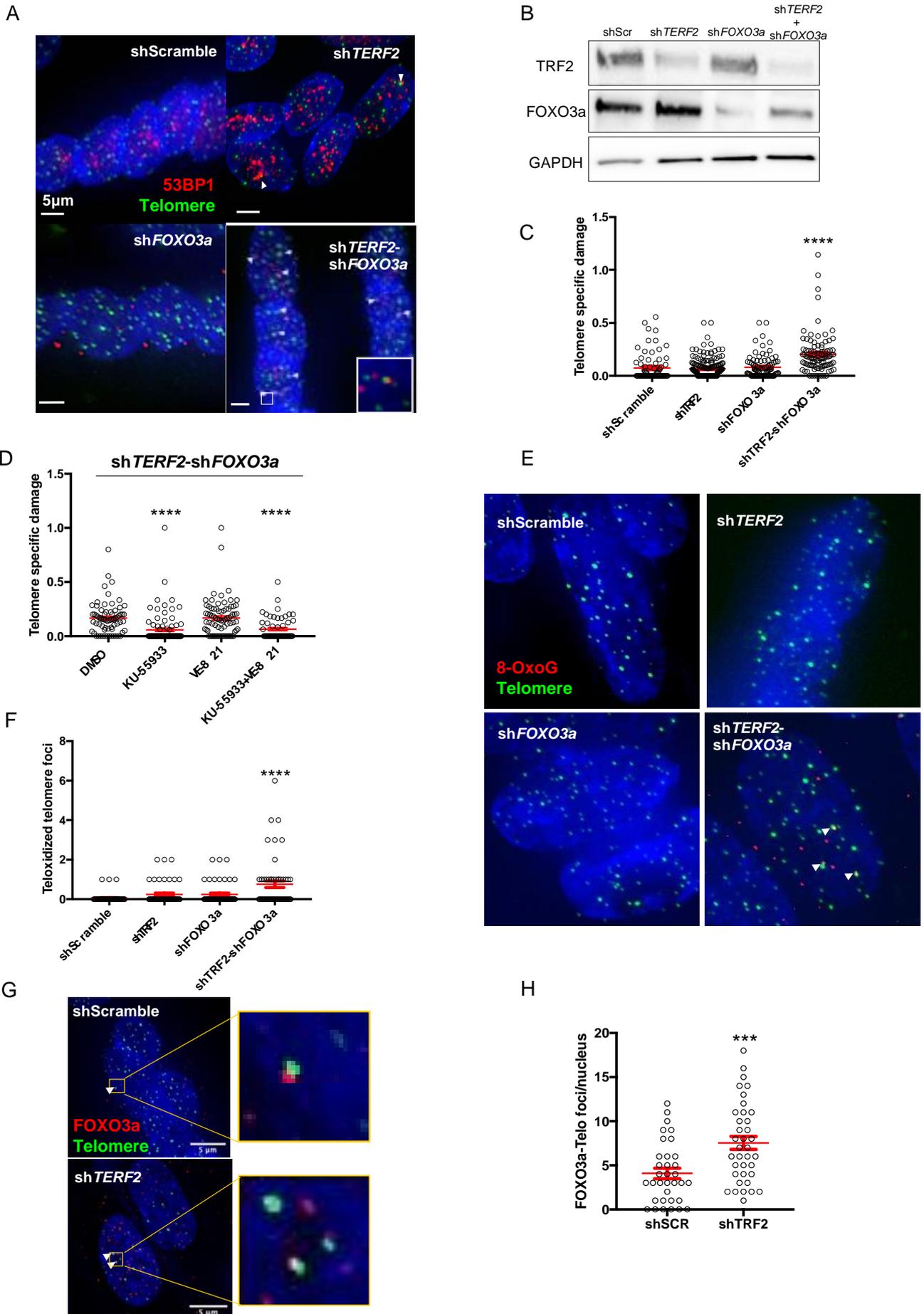
**(C)** sTIF quantification in siFOXO3a + truncated FOXO3a overexpression in BJ-HELT treated with bleomycin (50  $\mu$ g/mL, 24hr), n=3. Conditions were compared to siFOXO3a-FOXO3a+Bleo using a Kruskal-Wallis' multiple comparisons test. siFOXO3a-FOXO3a+Bleo vs siFOXO3a- $\Delta$ CR2C+Bleo, *p-value*=0,0345.

**(D)** PLA showing TRF2-FOXO3a association in siFOXO3a + truncated FOXO3a overexpression in BJ-HELT treated with bleomycin (50  $\mu$ g/mL, 24hr), n=3.

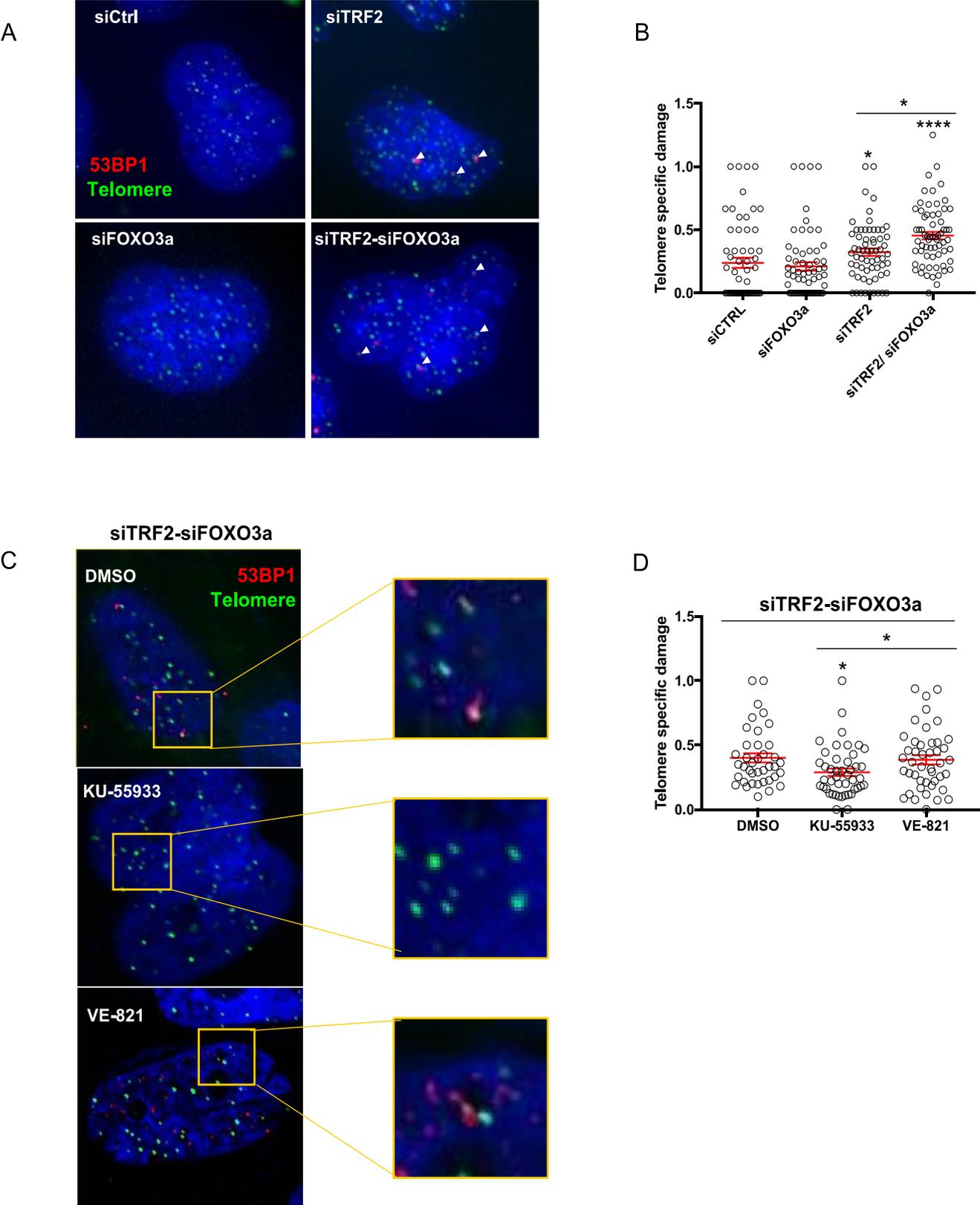
**(E)** TRF2-FOXO3a PLA in transfected BJ-HELT cells treated with bleomycin, n=2. Statistical analyses were performed using Kruskal-Wallis' multiple comparisons test (\**p* < 0.05, \*\**p* < 0.001, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001).

**(F)** Telomere specific damage upon p300 inhibition. siCtrl and sip300 transfected BJ-HELT cells were treated with bleomycin. Means  $\pm$  SEM of two replicated are shown, Man-Whitney U test (*p-value*= 0,0346).

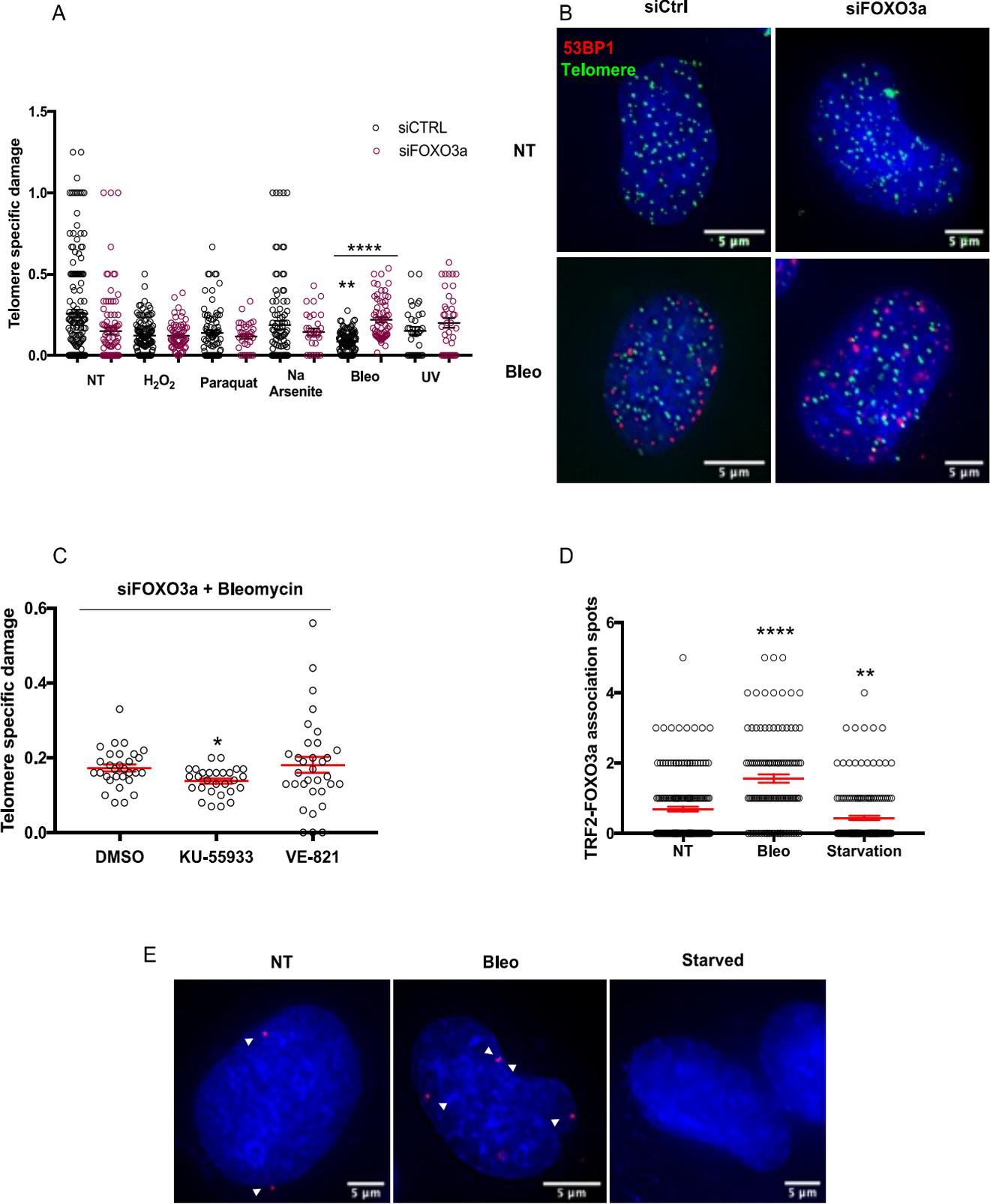
**Figure 1**



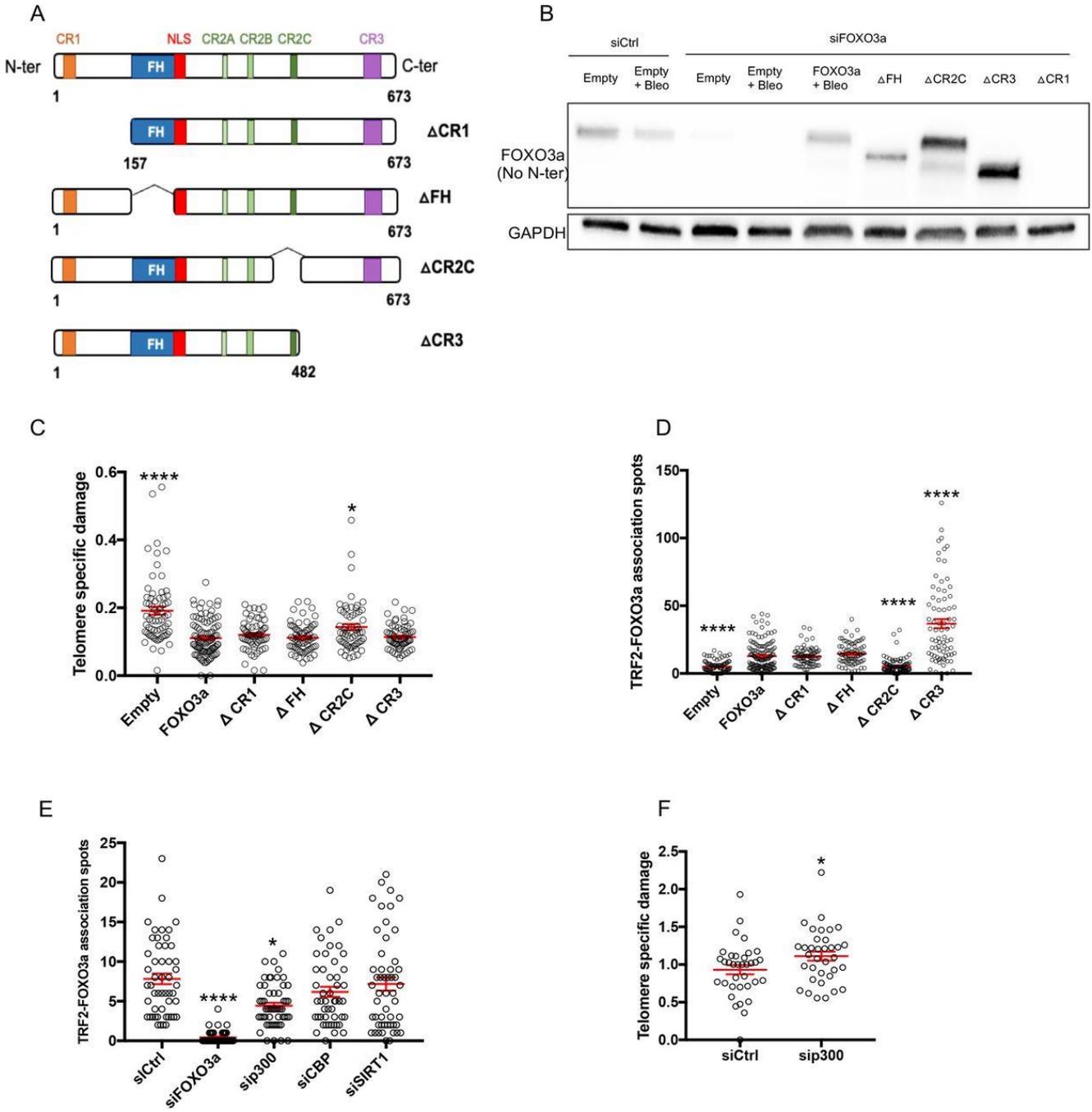
**Figure 2**



**Figure 3**



**Figure 4**



Expanded view legends

## EV1.

### **a) Telomere damage is not due to FOXO3a inhibition induced oxidative stress.**

(A) Telomere damage Induced Foci (TIF) and 53BP1.

(B) quantification. Means  $\pm$  SEM are shown. Only foci within multinucleated cells, that is, corresponding to postmitotic myotubes were counted. 30-40 nuclei were analyzed per replicate and per condition,  $n=4$ .

(C) Representative images of ROS foci in transduced myotubes.

(D) DAPI and ROS (E) intensities quantification in transduced myotubes represented in (C). On average,  $n>50$  nuclei per conditions, means  $\pm$  SEM are shown.

(F) Total number of ROS foci normalized to the number of nuclei. Down-regulation of TERF2 significantly increases ROS. Statistical analyses were performed using one-way ANOVA test ( $*p < 0.05$ ,  $**p < 0.001$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ).

(G) to L) Gene expression quantified by RT-qPCR in transduced myotubes. Each measure represents the average fold-change expression of six independent repetitions (Biological triplicate in technical RT duplicate) normalized to two housekeeping genes (HKG: HPRT and GAPDH); Pfaffl method). Means  $\pm$  SEM are shown.

### **b) FOXO3a directly binds to telomeres in myotubes, upon TRF2 downregulation.**

(M) FOXO3a nuclear Foci quantification in transduced myotubes.  $p$ -value  $< 0.0001$ , Mann-Whitney test.

(N) PLA TRF1-FOXO3a (red spots) in myotubes downregulated for TRF2. White arrows point association spots. Zoom at the left.

(O) Quantification of two biological replicates.  $p$ -value = 0,0295.

(P) and (Q) Slot blot of ChIP samples using a Telomeric (right) and Alu (left) probe in transduced myotubes and associated quantification normalized to Alu repeats. IgG shown as control.  $n=3$  per condition; means  $\pm$  SEM are shown. FOXO3A localizes to telomeres upon *TERF2* down-regulation (shScramble vs. *shTERF2*,  $p=0.0286$ ; Man-Whitney test,  $\alpha=0.05$ ).

(R) to (T) Immunoprecipitation (IP) using TRF2 antibody in human skeletal muscle biopsies, using TRF2 antibody for precipitation and FOXO3A for revelation. The TRF2-FOXO3A interaction enhances with ageing. Correlation and goodness of fit associated ( $R^2$ ), 95% interval confidence (blue lines) and means  $\pm$  SEM are shown.

## **EV2.**

### **FOXO3a protects telomeres specifically upon *TERF2* inhibition in BJ-HELT cells.**

- (A) Western Blotting showing FOXO3a downregulation in BJ-HELT cells.
- (B) Shelterin mRNA expression upon siRNA transfection, measured by RT-qPCR(n=3).
- (C) Western Blotting showing FOXO3a protein levels in BJ-HELT cells downregulated for TRF2.
- (D) Downregulation of *TERF2* was also assessed by RT-qPCR. *p-values* of n=5 were obtained using the Mann-Whitney test (\*  $p < 0.05$ ).
- (E) FOXO3a expression measured by RT-qPCR upon TRF2 inhibition.
- (F) TIFs in BJ-HELT fibroblasts transfected with siRNA against each shelterin protein (TRF1, TRF2, POT1, RAP1, TIN2 and TPP1) individually or combined to siFOXO3a. (G) 53BP1 foci per nucleus in conditions described in F). Statistical analysis was performed between single vs couple siRNA (example: siTRF1 vs siTRF1-siFOXO3a), using Mann–Whitney U-test (\* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.001$ ).
- (H) Telomere specific damage of A and B (TIFs/ 53BP1). (I) Heat map of (H) statistical analysis using Mann–Whitney U-test. Conditions labelled in blue are significantly different, the threshold  $\alpha = 0.05$  *p-value* is represented in grey, non-significant conditions correspond to the yellow staining.
- (J) and (K) FOXO3a immunofluorescence and qualitative determination of FOXO3a cellular localization: mostly nuclear, mostly cytoplasmic, nuclear and cytoplasmic. Approximately 60 cells were analyzed per condition in biological triplicates.

## **EV3.**

### **FOXO3a-mediated telomere protection upon cellular stress.**

- (A) TIFs and 53BP1 (B) in transfected BJ-HELT cells with siControl (black circles) or siFOXO3a (pink circles) and exposed to different stressors. Statistical

analyses were performed using Kruskal-Wallis' multiple comparisons test ( $*p < 0.05$ ,  $**p < 0.001$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ) in biological triplicates.

(C) Representative images of PLA negative controls: FOXO3a antibody alone + PLA probe, TRF2 antibody alone + PLA probe, no antibodies but PLA probe alone.

(D) PLA negative control quantification, compared to PLA (TRF2, FOXO3a antibodies and PLA probes added). Kruskal-Wallis' multiple comparisons test ( $****p < 0.0001$ ) in three biological replicates.

(E) FOXO3a cellular localization upon different kind of stress. FOXO3a pattern was qualitatively determined as mostly nuclear, mostly cytoplasmic, nuclear and cytoplasmic in (F). Approximately 30 cells were analyzed per condition in biological duplicates.

#### **EV4.**

##### **p300 is involved in the FOXO3a-dependent telomere protection.**

(A) FOXO3a truncated mutant overexpression and endogen FOXO3a downregulation technical strategy. Cells were plated at day 0 and transduced at MOI 2 with lentiviral FOXO3a full-length or truncated forms and Empty vector at day 1. At day 3, each condition was transfected with siControl or siFOXO3a. At day 5, bleomycin (50  $\mu\text{g}/\text{mL}$ ) was added and cells were fixed or harvest 24hr later.

(B) Western blotting showing endogen FOXO3a downregulation, FOXO3a full-length rescue and expression of FOXO3a truncated proteins. FOXO3a-Abcam ab12162 polyclonal antibody recognizes 653-668 aa of human FOXO3a (C-terminal), does not recognizes  $\Delta\text{CR3}$ . Cells were transduced 5 days with lentiviruses and the last 3 days transfected with siRNA.

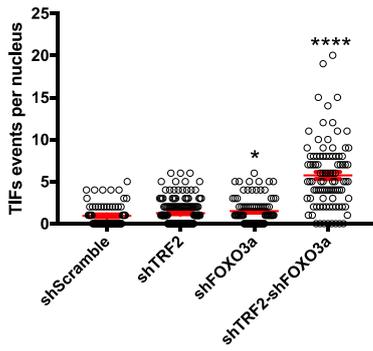
(C) TIFs and (D) 53BP1 in BJ-HELT cells transfected with siFOXO3a and transduced with the truncated FOXO3a overexpression. All conditions were treated with bleomycin (50  $\mu\text{g}/\text{mL}$ , 24hr),  $n=3$ . Conditions were compared to siFOXO3a-FOXO3a+Bleo using a Kruskal-Wallis' multiple comparisons test ( $*p < 0.05$ ,  $**p < 0.001$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ).

(E) TIFs and (F) 53BP1 in BJ-HELT cells transfected with sip300 and treated with bleomycin. Man-Whitney U test ( $p\text{-value}= 0,0011$  and  $0,0368$ , respectively). (G) p300 expression measured by RT-qPCR to control p300 inhibition by siRNA ( $n=3$ ).

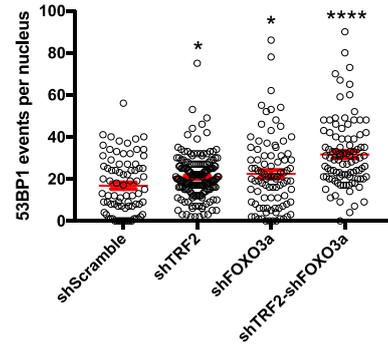
(H) Western blotting showing that neither TRF2 nor FOXO3a expression are affected by p300 KD.

# EV1 a

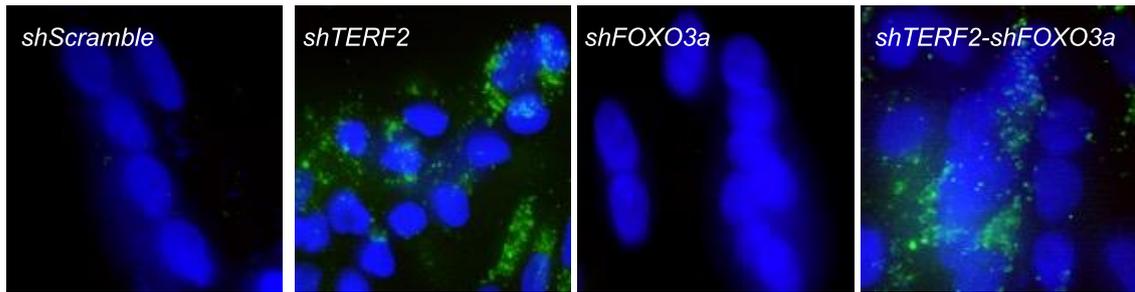
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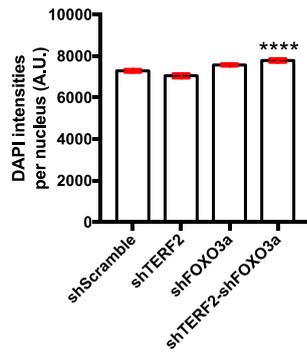
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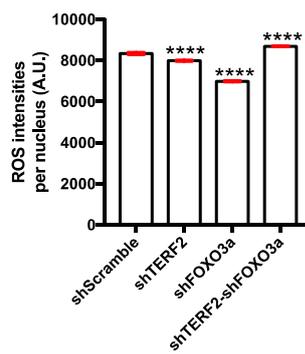
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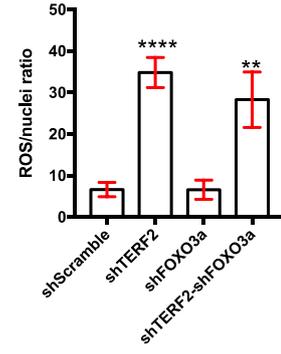
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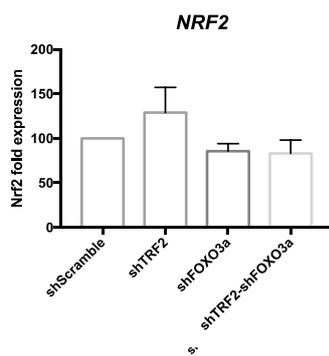
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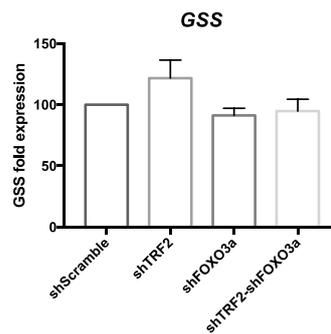
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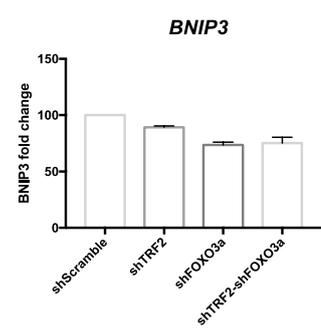
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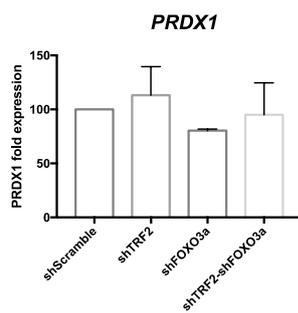
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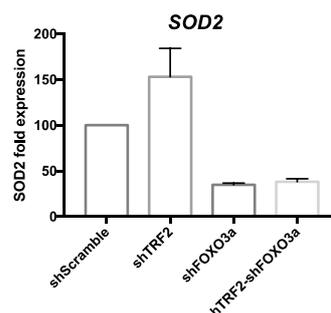
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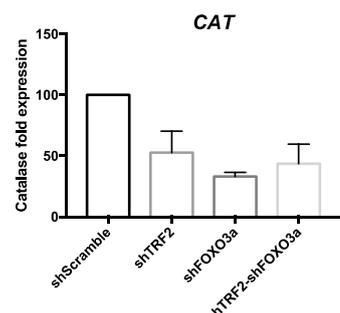
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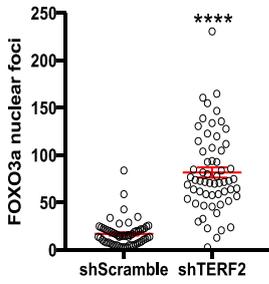


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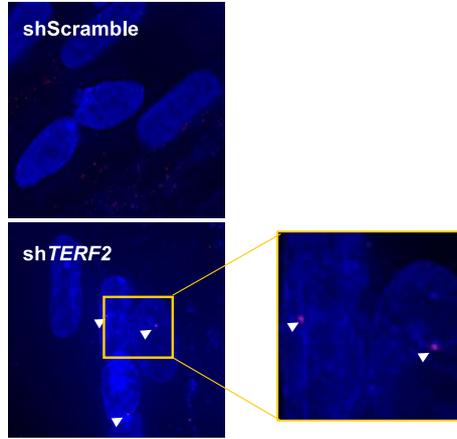


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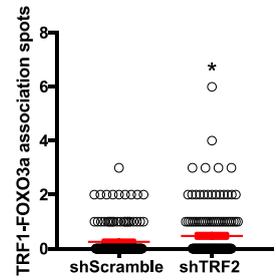
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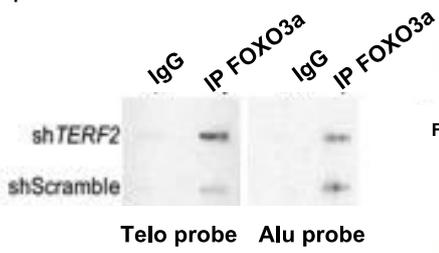
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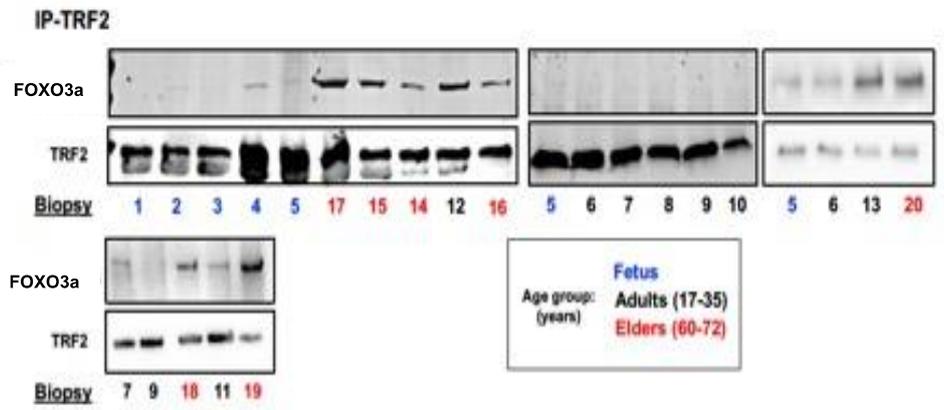
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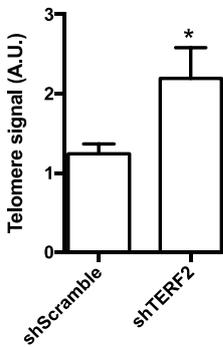
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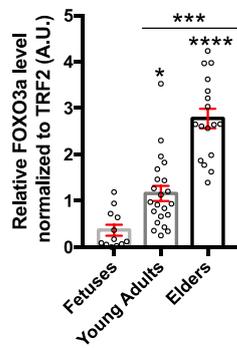
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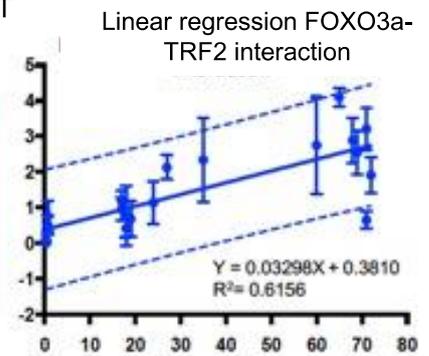
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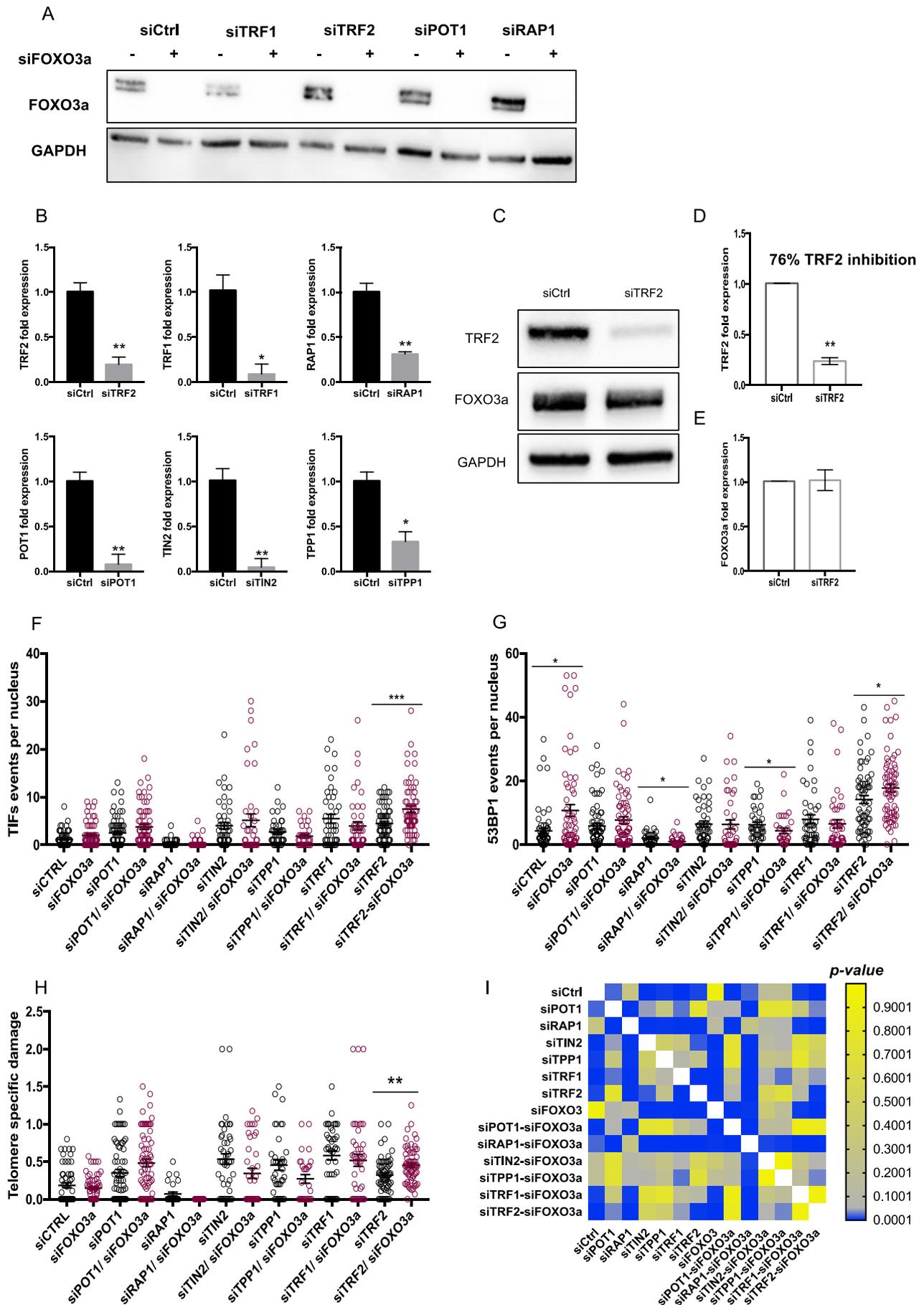
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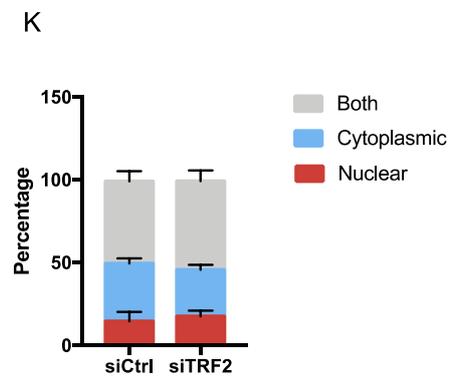
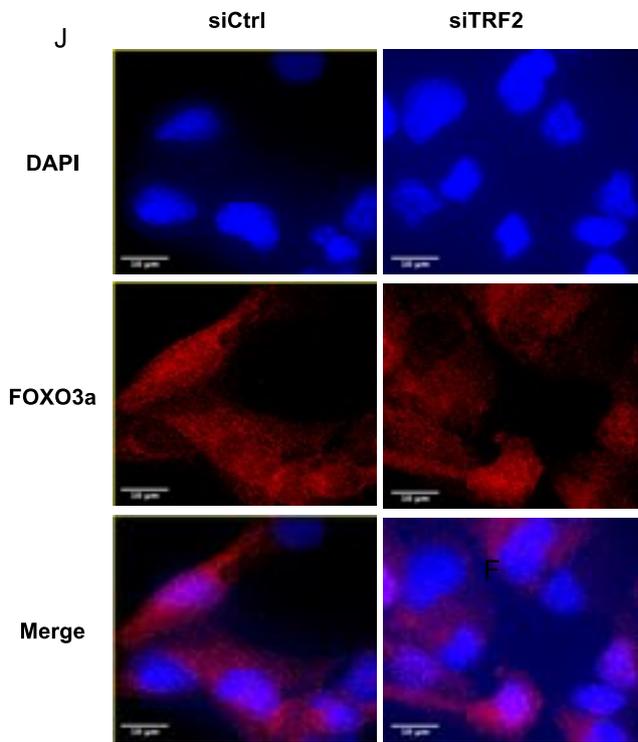
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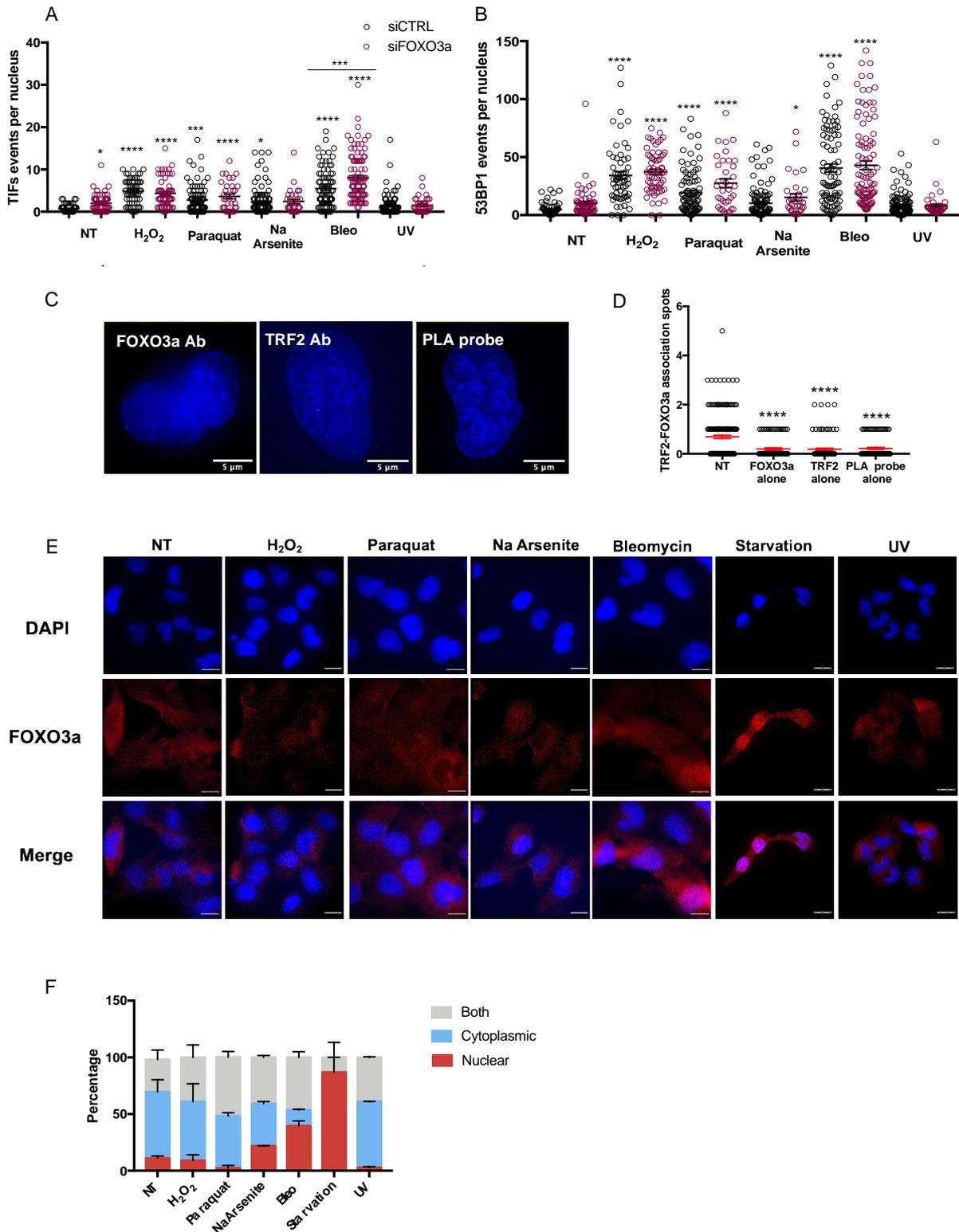
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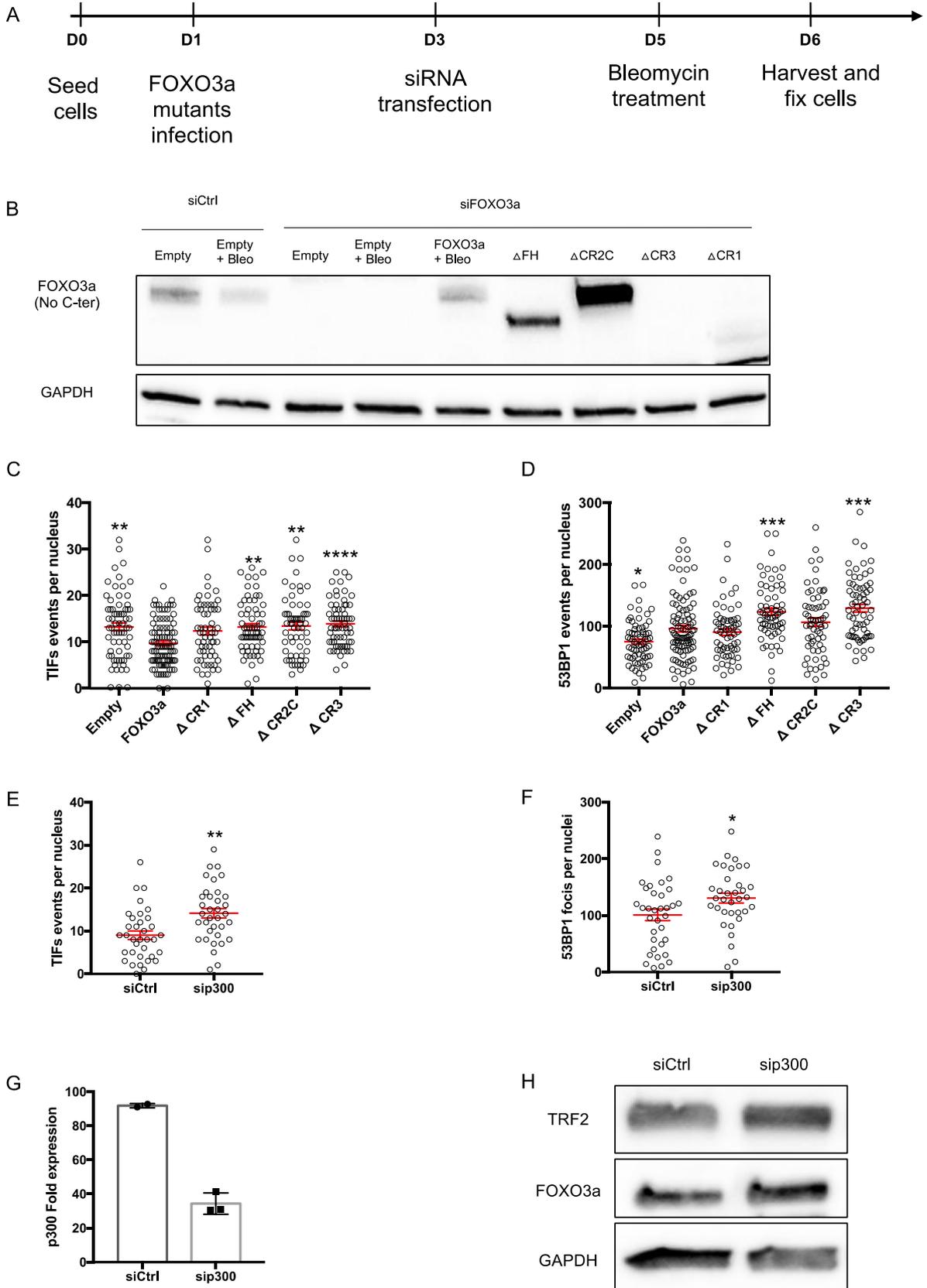
# EV2



# EV3



# EV4



### Third objective: What are the physiological outcomes of TRF2 loss in skeletal muscle during aging?

In order to directly address the question of the role of TRF2 in skeletal muscle aging, I characterized the phenotype of *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice, in which TRF2 expression is depleted specifically in myofibers. To this end, I used physiological and lifespan assays, supported by -omics approach.

## Specific telomeric repeat factor 2 (*Terf2*) knock-out induces hormesis in mouse myofibers

Maria Sol Jacome Burbano<sup>1\*</sup>, Jerome Robin<sup>3\*</sup>, Marjorie Martin<sup>1</sup>, Julien Gondin<sup>4</sup>, Mélanie Pousse<sup>1</sup>, Jean-Luc Thomas<sup>4</sup>, Serge Bauwens<sup>1</sup>, Emmanuelle Girard<sup>4</sup>, Peggy Del Carmine<sup>4</sup>, Edwige Belotti<sup>4</sup>, Pauline Billard<sup>4</sup>, Tiphaine Dorel<sup>6</sup>, Laurent Schaeffer<sup>4,5</sup>, Eric Gilson<sup>1,2</sup>.

1. Côte d'Azur University, CNRS, Inserm, IRCAN, Faculty of Medicine Nice, France.

2. Department of Genetics, CHU; Nice, France.

3. Aix-Marseille Univ-INSERM, MMG, 13005 Marseille, France.

4. INMG, Inserm U1217, CNRS UMR5310, Université Lyon 1, Université De Lyon, Lyon, France.

5. Centre De Biotechnologie Cellulaire, Hospices Civils De Lyon, Lyon, France.

6. Plateau de Biologie Expérimentale de la Souris (PBES), Ecole Normale Supérieure de Lyon, Université Lyon1, CNRS UMS3444 INSERM US8, 69007, Lyon, France.

Correspondence: [Eric.gilson@unice.fr](mailto:Eric.gilson@unice.fr)

\* These authors contributed equally to the paper.

### **Abstract**

Among the hallmarks of aging, only telomere attrition is known to be programmed at the end of embryogenesis. Whether or not this early pro-aging process triggers the other aging hallmarks in a development-aging continuum remains to be explored. Previously, we created a mouse strain by knocking-out the *Terf2* shelterin gene, specifically in mature myofibers. Unexpectedly, this telomeric chromatin damage did not induce telomere uncapping but instead mitochondrial dysfunctions leading to a mild increase in reactive oxygen species (ROS). Here we characterized myofiber *Terf2* KO mice physiological phenotype during aging. We found any regeneration mark, nor myofiber atrophy in old mice. Upon aging, myofiber *Terf2* KO females exhibit a slight increase in muscle strength as compared to age-matched controls, an altered mitochondrial respiratory chain and extended lifespan. Surprisingly, these results

suggest an anti-aging strategy triggered by *Terf2* loss in post-mitotic myofibers. Altogether, these findings advocate for a hormetic effect of telomere changes programmed during skeletal muscle development.

## **Introduction**

Skeletal muscle is the main tissue among the whole muscular mass that also includes cardiac and smooth muscle (Chal and Pourquié, 2017). Skeletal muscles are responsible for volunteer movement and represents around 40% of our body mass. Therefore, its maintenance and good health are critical for daily performance and mobility (Tieland et al., 2018). Ageing is characterized by the overall physiological decline, altered response to stress and tissue homeostasis maintaining. Applied to skeletal muscle aging, or sarcopenia, it reflects 30-50% muscle mass loss, starting at approximately 40 years old, reduced muscle strength (Cohen et al., 2015; Larsson et al., 2019; Volpi et al., 2004). It results in a decrease in physical activity (sedentary life), that could be a major impediment to accomplish daily life simple tasks or carrying weight.

At the cellular level, myotubes, as well as neurons are long-lived post-mitotic cells (LLPMCs) that are rarely replaced during lifetime (Jacome Burbano and Gilson, 2020). Although LLPMCs do not exhibit clear marks of senescence, they undergo a functional decline during aging, as observed by Reactive Oxygen Species (ROS) accumulation, altered proteostasis, dysfunctional autophagy, metabolic and transcriptional changes (Bua et al., 2006; Fritzen et al., 2016; Glatigny et al., 2019; Linnane et al., 1989; Loos et al., 2017; Miljkovic et al., 2015; Robin et al., 2020; Sakuma et al., 2016; Terman et al., 2010). However, the age-dependent mechanism that leads to these cellular changes, as well as the link of this LLPMC aging process to the senescence pathways are still largely unknown.

Among the aging hallmarks, only telomere attrition is known to be a developmentally programmed biological clock for mitotic cells senescence. Nonetheless, telomeric DNA length (TL) changes in LLPMCs are a matter of debate. It was generally accepted that telomere homeostasis does not play an important role in LLPMC aging given that TL is considered to shorten only in dividing cells. Interestingly, some publications report age-dependent TL shortening in post-mitotic or low proliferative tissues (Ain et al., 2018; Aix et al., 2016; Anderson et al., 2019; Baek

et al., 2019; Carneiro et al., 2016; Chahine et al., 2019; Daniali et al., 2013; Decary et al., 1997; Jacome Burbano and Gilson, 2020; Ludlow et al., 2014; Magi et al., 2018; Quinn et al., 2013; Robin et al., 2020; Wang et al., 2016). Telomere dynamics do not depend only on TL modulation but also on telomeric protein composition and high order chromatin structure.

Previously, we unveiled that among the six telomeric proteins of the shelterin complex, only the Telomere Repeat Factor 2 (TRF2) expression decreases during human skeletal muscle aging. TRF2 is known to prevent undesired DNA damage response (DDR) activation at telomeres of mitotic cells (Ancelin et al., 1998; Benarroch-Popivker et al., 2016; Buscemi et al., 2009; Okamoto et al., 2013; Poulet et al., 2009; Ye et al., 2010). Strikingly, we detect any DDR activation at telomeres using *in vitro* myotubes downregulated for TRF2 or in a myofiber specific *Terf2* gene knock-out mouse model (*HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup>) (Robin et al., 2020). However, TRF2 inhibition results in altered mitochondrial metabolism, the mitochondrial sirtuin SIRT3 reduced expression, ROS and mitochondrial DNA (mtDNA) copies accumulation. Our mouse model exhibited a switch on *soleus* fiber-type proportion toward oxidative metabolism. Altogether, these results argued for a premature aging phenotype.

Here we characterize the *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice physiological phenotype during aging. We found any regeneration mark, nor myofiber atrophy in old *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice. Upon aging, the *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females exhibit a slight increase in muscle strength as compared to age-matched controls, an altered mitochondrial respiratory chain and extended lifespan. These results suggest an hormetic effect of telomere changes programmed during skeletal muscle development.

## **Results and discussion**

### **1) Expression of TRF2 decreases in skeletal muscles of old mice**

Since we previously observed in human skeletal muscle biopsies a decreased expression of TRF2 starting early in the adulthood, we measured, in mice of different ages, the TRF2 expression by Western blotting. In two types of muscles (*tibia anteralis* (TA) and *gastrocnemius-plantaris-soleus* (GPS)), we observed an initial phase of increased TRF2 expression during the first year of life followed by a decrease, detectable when the mice become old (15 month) (Figure 1A-B, Supplemental Figure 1A-B). This downregulation of TRF2 is likely to result from the aging process *per se* since late generations (G6) telomerase KO mice exhibited a decreased TRF2 expression as

compared to their G4 counterparts (Figure 1C, Supplemental Figure 1C), known to have longer telomeres and younger biological age. We conclude that, in mice, TRF2 expression starts to be downregulated in skeletal muscles when they become old. Therefore, we explored the physiological consequences in old mice of the muscle specific *Terf2* knock-out (KO) in *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice.

## **2) *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> muscle do not exhibit signs of regeneration during aging**

First, we studied the regeneration process by using myofiber nuclei position as a marker of ongoing (Roman and Gomes, 2018) and past regeneration (Meyer, 2018). At young ages (1-2 months old), *HSA-Cre*<sup>+</sup>, *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> or *Terf2*<sup>flox/flox</sup> *gastrocnemius* muscles contain mostly peripheral nuclei (Figure 2A-B, Supplementary Figure 2A). At old ages (15 months old), *HSA-Cre*<sup>+/-</sup> and *Terf2*<sup>flox/flox</sup> had 5% of central nuclei, indicating regeneration events most likely due to age-related muscle injuries. In contrast, *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice do not show an increased rate of central nuclei during aging (Figure 2A-B). This result could result from either an absence of age-related injuries in TRF2-compromised muscles or an impaired regenerative capacities of satellite cells. The later possibility could reflect a change in muscle stem cell niche triggered by TRF2-compromised myofibers.

## **3) The diameter of *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> myofibers does not decrease during aging**

To further investigate the muscle physiology consequences of specific *Terf2* KO, we measured the diameters of *tibia anterioralis* (TA) and *gastrocnemius* fiber size. As expected, the diameter of TA and *gastrocnemius* myofibers decreased with age in *HSA-Cre*<sup>+/-</sup> and *Terf2*<sup>flox/flox</sup> mice (Figure 2C, Supplemental Figure 2B-E). Surprisingly, myofiber diameters of *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice remain constant upon aging (Figure 2C). This differential effect between *Terf2* KO mice and the other control genotypes was observed in type I, IIa and IIb myofibers (Supplementary Figure 2B-E). These findings suggest that TRF2-compromised muscles are protected against age-related muscle atrophy, at least at the early stages of aging (15 months). Nonetheless, muscle fiber atrophy is characteristic of sarcopenia, suggesting that *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice have healthier and “younger-like” muscle phenotype.

#### 4) Old females *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> exhibit faster and more intense muscle strength

Skeletal muscle aging is accompanied by a strength decline. We investigated whether myofiber specific *Terf2* KO affect muscle strength. To this end, we performed an *in vivo* isometric *gastrocnemius-plantaris-soleus* (GPS) force measurement in 15 months old mice. Considering absolute force, *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females were slightly stronger than *HSA-Cre*<sup>+/-</sup> females (*p-value*= 0.0484) (Supplemental Figure 3A). We did not detect any difference in males between the two genotypes or comparing males vs females of the *HSA-Cre*<sup>+/-</sup> genotype (Supplemental Figure 3A and 3B). Next, mice were subjected to a fatigue protocol, which consist in 180 stimulations performed at 30 Hz. The *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females showed a stronger response during the first 55 contractions than *HSA-Cre*<sup>+/-</sup> females (*p-value*=0,0283) and *HSA-Cre*<sup>+/-</sup> *Terf2*<sup>ff</sup> males (*p-value*= 0.0089) (Figure 3A and Supplemental Figure 3C). However, *HSA-Cre*<sup>+/-</sup> females were more resistant to fatigue by the end of the protocol, as compared to *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females and *HSA-Cre*<sup>+/-</sup> males (*p-value* < 0.0001) (Figure 3A and Supplemental Figure 3C). Overall, our findings suggest that 15 months old females invalidated for *Terf2* in myofibers have a fast response muscle strength advantage but more sensitive to muscle tiredness. This phenotype is not observed in the males of the same genotype.

Throughout skeletal muscle aging, there is a type I-low twitch-oxidative fiber accumulation at the expense of type II-fast twitch- glycolytic myofibers. Whereas oxidative fibers are prone to a slow but long-lasting response activity (several hours), glycolytic fibers induce a faster response but shorter in time. Here, we observed that *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females have a more powerful force response to stimuli but reach muscular fatigue rapidly. All together, these physiological results support the hypothesis that *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice, at least females, possess a younger skeletal muscle.

#### 5) Transcriptional changes induced by *Terf2* invalidation in skeletal muscle

In order to understand the apparent advantage conferred by *Terf2* invalidation in skeletal muscle, we performed transcriptomic (RNA-sequencing) analyses of GPS muscle tissues of 8 months old mice. Principal component analysis (PCA) and heatmap compiling the differentially expressed genes (DEG) showed that *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> and *HSA-Cre*<sup>+/-</sup> mice have different transcriptional profiles (Supplemental Figure 4A-B). We found 78 DEG in female GPS and 428 DEGs in male GPS (Supplementary Tables 1

and 2). Despite the elevated number of differentially expressed genes in males, there were a few significantly enriched Reactome pathways (Figure 4B). Noteworthy, the circadian clock was the prevalent pathway, a result confirmed by RT-qPCR of circadian clock genes (*Per2*, *Npas2*, *Nrip1* and *Arntl*) (Supplemental Figure 4C). Differently from the male results, the female DEG profile is dominated by mitochondrial respiratory chain pathways (Figure 4A, Supplemental Figure 4bis and Supplemental Table 3). This result was supported by other enriched pathway analysis methods (KEGG and Wikipathway) and was confirmed by RT-qPCR analysis of *Cox5a*, *Atp5k*, *Ndufa5*, genes (Supplemental Figure 4C and Supplemental Figure 4bis B). It is in agreement with our previous work (Robin et al., 2020) showing mitochondrial dysfunctions, reduced Complex I activity and increased ROS in human and mouse TRF2-compromised myofibers. Overall, the muscle strength advantage of *Terf2* invalidated females appears to be associated to mitochondrial alterations.

Recently, a study unveiled the 15-hydroxyprostaglandin dehydrogenase (15-PGDH), responsible for prostaglandin PGE2 catabolism, as a new marker of aged skeletal muscle (Palla et al., 2021). 15-PGDH inhibition in aged muscles results in PGE2 increased expression, higher muscle mass and strength, as well as enhanced mitochondrial activity and biogenesis. A tempting hypothesis would be that myofiber TRF2 loss results in reduced 15-PGDH. Nevertheless, our transcriptomics results in 8 months old mice showed any difference in 15-PGDH expression. We cannot exclude that *Terf2* KO alters the prostaglandin pathway later in life, at an age in which we observe the beneficial phenotype of *Terf2* depletion (15 months old mice).

## **6) The invalidation of *Terf2* specifically in skeletal muscle extends female median lifespan**

Then, we investigated whether *Terf2* inhibition in mouse skeletal muscle has a general impact on the aging phenotype. We followed two colonies of *Terf2*<sup>flox/flox</sup> and *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice over lifetime. We did not detect any difference between the males survival curves of the two genotypes (median lifespan around 25 months old) (Figure 5). In contrast, *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females median lifespan was extended by 11% as compared to *Terf2*<sup>flox/flox</sup> females (Figure 5). When the male vs female lifespans were studied per genotype, *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females median lifespan was extended of 15% compared to males, but it was not the case for the *Terf2*<sup>flox/flox</sup> mouse colony (Supplemental Figure 5). These results indicate that the loss of TRF2 in myofiber during

embryogenesis gives a slight but detectable longevity advantage specific to females. Strikingly, its effects involved the whole organism even though *Terf2* KO occurs only in mature myofibers.

Overall, we found unexpected beneficial physiological outcomes induced by specific *Terf2* KO in myofibers. These results suggest that female skeletal muscle *Terf2* depletion during early development, delays sarcopenia or at least protects against sarcopenic-mediated stress. Interestingly, any difference was detected in young KO and control mice neither in regenerative marks, nor myofiber diameter. Therefore, *Terf2* depletion associated consequences embraces an “anti-aging” response. We hypothesize that TRF2 loss results in a telomere-mediated hormesis, dubbed telo-hormesis.

Further studies are required to understand why this advantage is female specific. Does it depend on female hormones? Are circulating factors involved in this advantageous phenotype? Does specific myofiber *Terf2* KO result in systemic effect, altering other organs homeostasis?

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### **Author contributions**

MSJB and JR executed the experiments. MSJB helped design and wrote the manuscript. LS, EG and JR helped design. MM, JG, JT, SB, PC and EB provided technical help; MP assisted and performed the bioinformatics analysis of the RNA-Seq results. TD, PB and E.Girard help with mouse carrying. LS helped project coordination. EG designed the project and wrote the manuscript.

## Material and Methods

### Mice – *Transgenic model*:

Specific skeletal muscle TRF2 KO mice (*HSA-Cre*<sup>+</sup> / - ; *Terf2*<sup>flox/flox</sup>) were generated as previously described (Robin et al., 2020). First, the previously characterized TRF2 floxed mice from the Jackson laboratory (strain B6; 129P2-*Terf2*<sup>tm1Tdl/J</sup>) (Celli and de Lange, 2005) were backcrossed with C57BL/6 strain for 6 generations. Next, mice were crossed with the *HSA-Cre*<sup>+/+</sup> strain expressing the Cre-recombinase under the control of the Human Skeletal Actin (*HSA*) gene promoter specifically expressed in striated muscle fibers. The colony and protocols were controlled and approved by the Comité Institutionnel d'Éthique Pour l'Animal de Laboratoire (CIEPAL) and the Ministry of Education and Scientific Research under the number APAFIS#10444-2017052414468355v2 and APAFIS#31428-2021032913431387 v2. All experiments were performed at the ANIPHY core (ENS, Lyon), agreement n°C693880803 and at the PLATEAU DE BIOLOGIE EXPERIMENTALE (PBES) core (ENS-Lyon), agreement n°D691230303.

1, 3, 12 and 15 months old C57BL6/J mice were directly ordered to Charles River and maintain for 1 week adaptation within the IRCAN Experimental Core Facility (Nice) before organ collection.

### Western Blot:

Mouse skeletal muscles (*gastrocnemius-plantaris-soleus* and *tibia anterioralis*) were snap frozen in liquid nitrogen and preserved at -80°C. Tissues were crush in liquid nitrogen to obtain a powder. Next, tissues were lysed using RIPA cell lysis buffer, complemented with phosphatase inhibitors (Roche) and kept 30 min on ice. For each sample, 30µg of protein was resolved in a 4-15% gradient mini-protean precast polyacrylamide gels (BioRad) and transferred to nitrocellulose membranes (Whatman, GE Healthcare) for 1h at 4°C. After blocking for 1 hour with 5% skim milk in PBST (0.1% Tween-20 in PBS), the membranes were incubated overnight at 4°C with primary antibodies diluted in 5% milk. The following primary antibodies were used: TRF2, rabbit monoclonal, (NB110-57130, 1:5000, Novus Biologicals) and GAPDH, rabbit polyclonal (1:2000, NB100-56875, Novus Biologicals). The membranes were then rinsed three times in PBST for 10min and incubated 1 hour at room temperature

with appropriate secondary antibodies diluted (1:5000) in 5% PBST-milk (e.g., anti-mouse HRP IgG and anti-rabbit HRP IgG, Vector Laboratories). Membranes were developed using the Luminata Forte HRP substrate (Millipore) and exposed in the Fusion Solo apparatus (Vilbert Lourmat). TRF2 expression was quantified using FIJI software, by correcting with background measure and normalized by GAPDH.

### **Fiber size and nucleus position:**

Mice were sacrificed, fresh muscle were dissected: *gastrocnemius-plantaris-soleus* (GPS) and *tibia anterioralis* (TA) and then flash frozen in cold isopentane steam (dry-ice) in an OCT block. Transverse frozen sections (10 $\mu$ m) of each muscle mice were prepared and kept at -80°C for storage.

For staining, muscle slides were melted at room temperature for 30min and fixed for 10min with 4% paraformaldehyde. Next, slides were washed three times in PBS 1X, 3 min and permeabilized in 0.5% Triton X-100 in PBS for one hour and washed again in PBS 1X. After 1 hour blocking using a solution of M.O.M IgG diluent (Vector Laboratories, Burlingame, USA) at room temperature, slides were washed twice in PBS 1X for 5 min and incubated with M.O.M protein diluent solution for 5min. Next, slides were incubated with primary antibodies in M.O.M protein diluent (1:20) over night at 4°C. Each slide was treated simultaneously with 4 conditions separated using a Dako pen (Agilent); MyHC-I (Isoform BA-D5, DSHB, University of Iowa), MyHCIIa (Isoform SC-71, DSHB, University of Iowa) specific to type I and type IIa fibers, respectively, as well as combined MyHC-I + MyHC-IIa and no primary antibody. After three washes of 5 min each in PBS 1X, slides were incubated for 40min in M.O.M protein diluent containing the secondary antibody (Alexa 488 Donkey anti-mouse; 1:200) and washed three times 5min in PBS 1X. Last, slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, USA). Images were taken using an Axio Imager 2 and a 20X lens (Zeiss).

Muscular fibers size was determine using Imaris software. More than 1000 fibers were analyzed per mice, and 3 to 5 mice per genotype and age.

For Peripheral or central nucleus position, 100 fibers were counted per mice (3 to 6 mice per genotype and age).

### ***In vivo* isometric force measurement.**

Mice were maintained under isoflurane anesthesia in supine position. Then, the right back leg was shaved and a conductor gel was applied. Each mouse was then placed in a cradle allowing for a standardization of the animal positioning. The right foot was positioned and firmly immobilized through a rigid slipper on a pedal of an ergometer (NIMPHEA\_Research, All Biomedical SAS). The pedal of the ergometer was adjusted to position the ankle joint at 20° of plantar flexion (where 0° corresponding to the pedal of the ergometer perpendicular to the tibia). The right knee was also firmly maintained using a rigid fixation in order to optimize isometric force recordings. Two 27G needle electrodes (745 12-100/24, Ambu® Neuroline) were positioned subcutaneously on the proximal and distal parts of the *gastrocnemius*. Monophasic rectangular pulses of 0.2 ms were delivered using a constant-current stimulator (Digitimer DS7AH, Hertfordshire, UK; maximal voltage: 400 V). The force-frequency curves were determined by stepwise increasing stimulation frequency, with resting periods > 30 s between stimuli in order to avoid effects due to fatigue. Force signal was sampled at 1000 Hz using a Powerlab system and Labchart software (ADInstruments).

### **RNA sequencing**

Mouse skeletal muscles were snap frozen in liquid nitrogen and preserved at -80°C. Total RNA was extracted using Tris Reagent (T9424 - Merck), according to manufacturer's instructions. In brief, muscles were incubated in Tris Reagent in ceramic beads tubes (KT03961-1-003.2 Precellys) and dissociated with a Precellys tissue homogenizer. Add 200 µL chloroform and centrifuge 15 min at 12000g (4°C). Aqueous phase was transferred, RNA pellet was precipitated with 500 µL isopropanol and centrifuge again. Next, RNA pellet was washed twice in ethanol 75%. RNA quantification was determined by Nanodrop 1000 spectrophotometer (Thermo Scientific) and RNA quality with an Agilent 2100 Bioanalyser. Paired-end sequencing (read length: 2x 150 bp) was performed by NovoGene using an Illumina NovaSeq 6000 sequencer.

### **qPCR**

After total RNA extraction, Reverse Transcription (RT) was performed with 500 ng RNA using High-Capacity RNA-to-cDNA Kit (Thermo Scientific). Each qPCR contained 5X diluted cDNA, 0.2 µM primers, and SYBR Green Master Mix (Roche,

4913914 001). Quantitative RT-PCR (qRT-PCR) was performed in triplicates using FastStart universal SYBR Green master Mix (Roche). Melting curves were analyzed (SYBR green) to exclude non-specific amplification products. We confirmed amplicon size at least once on agarose gels. Crossing-threshold (Ct) values were normalized to 36B4 housekeeping gene.

### Statistical analysis

GraphPad Prism 7 software was used to generate graphs and to perform statistical analysis. p-values were obtained using either Mann-Whitney U test or the Kruskal-Wallis test. Differences were considered statistically significant when  $p < 0.05$ , as it follows: \* $p < 0.01$ , \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ . Absence of statistical annotation indicates non-significance (ns).

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## Figure Legends

### Figure 1: TRF2 expression decreases in skeletal muscle of old

A) Quantification of TRF2 Western Blots in *tibia anterioralis* and *gastrocnemius-plantaris-soleus* (GPS) mice muscles (B). TRF2 expression increase during young adulthood: 1 to 12 months old and then it decreases (15 months). For GPS: n=5 females C57BL6/J per age. For TA: n=4 for 1- and 12-months old mice, n=5 for 3- and 15-months old mice. In technical triplicates. Each point represents each mice average expression.

C) Western Blot quantification of TRF2 expression in the GPS of 8 months old TERT KO females G4 vs G6 mice, n=5 G4 and n=3 G6, *p-value*=0,0357.

TRF2 expression was normalized to GAPDH. Mann–Whitney U-test was performed to compare TRF2 expression at each time point.

### Figure 2: *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> mice do not undergo skeletal muscle fiber aging-related switch.

A) Representative images of *gastrocnemius* fiber type characterization (type I: green, type IIa: yellow, type IIb: pink) and nuclei staining (in blue). Young (1,5 months) vs old (15 months) mice were compared for three different genotypes: *HSA-Cre*<sup>+/-</sup> (young: n=3, old n=10), *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> (young: n=4, old n=12) and *Terf2*<sup>ff</sup> (young: n=2, old n=12). Nucleus position was determined as peripheral or central.

B) Quantification of the percentage of nuclei with central localization in *gastrocnemius* type II fibers in young (Y) and old (O) mice. Skeletal muscle nuclei in old *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> mice are not central compared to old *HSA-Cre*<sup>+/-</sup> and *Terf2*<sup>ff</sup>. Statistical analyses were performed using Kruskal-Wallis' multiple comparisons test (\**p* < 0.05, \*\**p* < 0.001).

*HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> vs *Terf2*<sup>ff</sup>: *p-value*=0,0327 and *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> vs *HSA-Cre*<sup>+/-</sup>: *p-value*=0,0083.

C) Quantification of mice fiber size repartition (all types of fibers were included) of images in C. Fibers were categorized according to their surface area (<500 to >5000 μm<sup>2</sup>). More than 500 fibers were counted per mice: *HSA-Cre*<sup>+/-</sup> (young: n=3, old n=3), *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> (young: n=4, old n=5) and *Terf2*<sup>ff</sup> (young: n=3, old n=5). T-test student test was performed at each size range *HSA-Cre*<sup>+/-</sup> vs *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> (\*) and *Terf2*<sup>ff</sup> vs *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> (#) (\**p* < 0.05, \*\**p* < 0.001, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001).

### Figure 3: Female *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> mice are stronger

Contraction-induced muscle fatigue at 30 Hz, represented on percentage of the maximal force. Left panel: males (*HSA-Cre*<sup>+/-</sup> n=5 and *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> n=6 and right panel: females (*HSA-Cre*<sup>+/-</sup> n=6 and *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> n=8). Each contraction point was analyzed using a t-Test (\**p* < 0.05). To calculate the area under the curves, data was divided into subgroups, before and after the curves crossing intersection. For females it corresponds to 55 contractions and around 32% of maximal force. Areas under the curve were calculated and then compared using Mann–Whitney U-test, *p*-value= 0.0283 for the first part (left) and *p*-value <0.0001 for the second part (right). *Terf2* myofiber inhibited females are stronger at the beginning of the muscular activity, but their muscle reach tiredness faster compared to *HSA-Cre*<sup>+/-</sup> females.

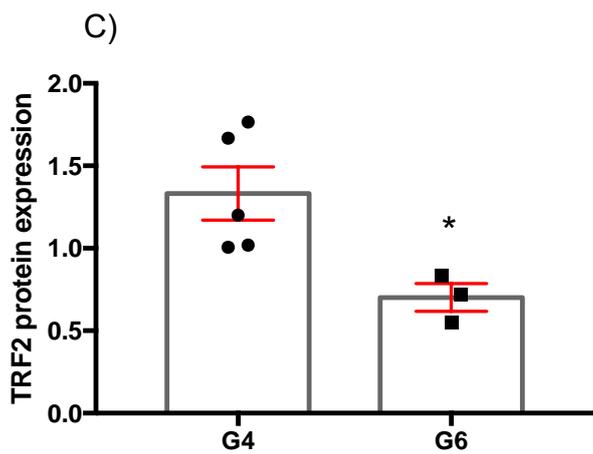
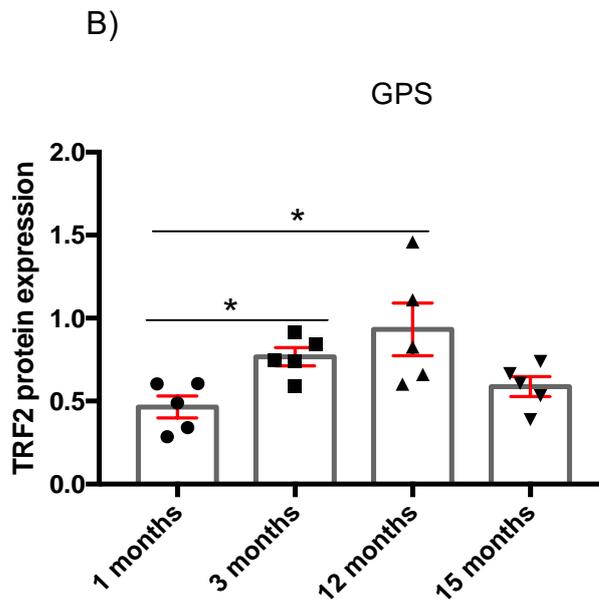
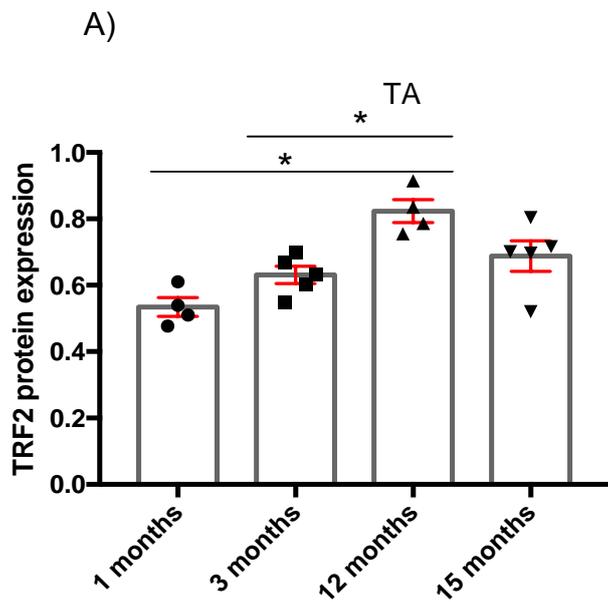
### Figure 4: *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> mice show transcriptional differences

Reactome pathway analysis of RNA-seq differential expression data of 8 months old females (A) and males (B) *HSA-Cre*<sup>+/-</sup> vs *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> GPS. Grey box hides the not significantly differentially expressed pathways. For females the top 10 significant pathways are shown. 3 *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> and 5 *HSA-Cre*<sup>+/-</sup> females and 4 *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> and 5 *HSA-Cre*<sup>+/-</sup> males were analyzed.

### Figure 5: *Terf2* specific myofiber inhibition prolongs female median lifespan

Kaplan-Meier survival curves of males *Terf2*<sup>ff</sup> vs *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup>, n=11 and 19, respectively (left panel). Lifespan extension was observed in *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>ff</sup> female mice: 3 months median survival extension (11%) compare to *Terf2*<sup>ff</sup> female mice (right panel). n= 27 *Terf2*<sup>ff</sup> and 23 KO females (log-rank – Mantel-Cox, *c*<sup>2</sup>= 11.47 and *p*-value= 0,0007).

Figure 1



**Figure 2**

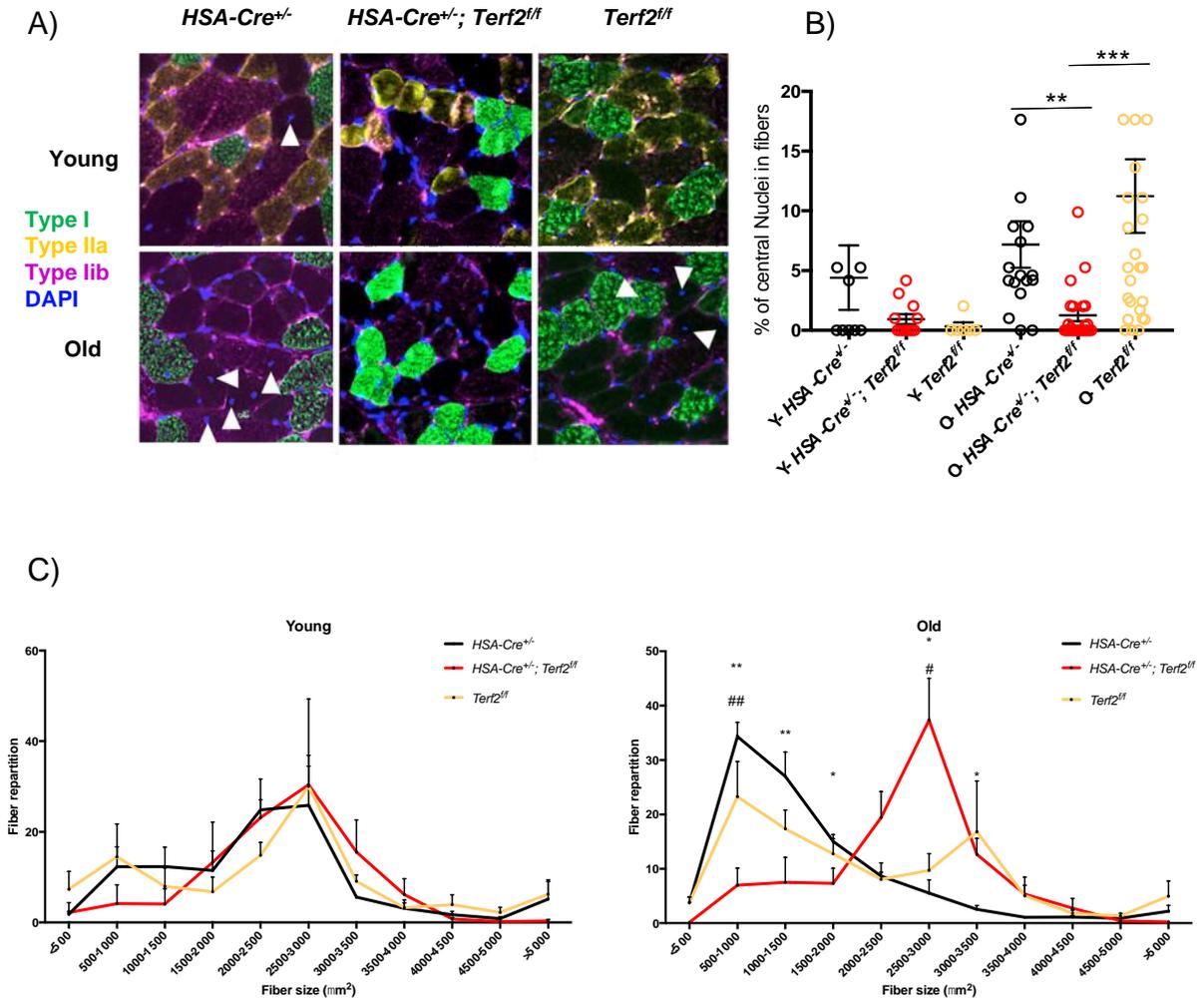
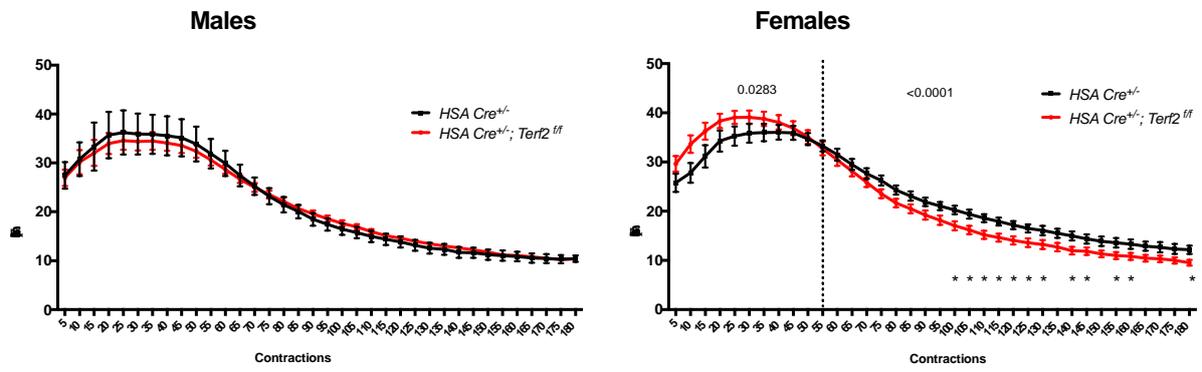
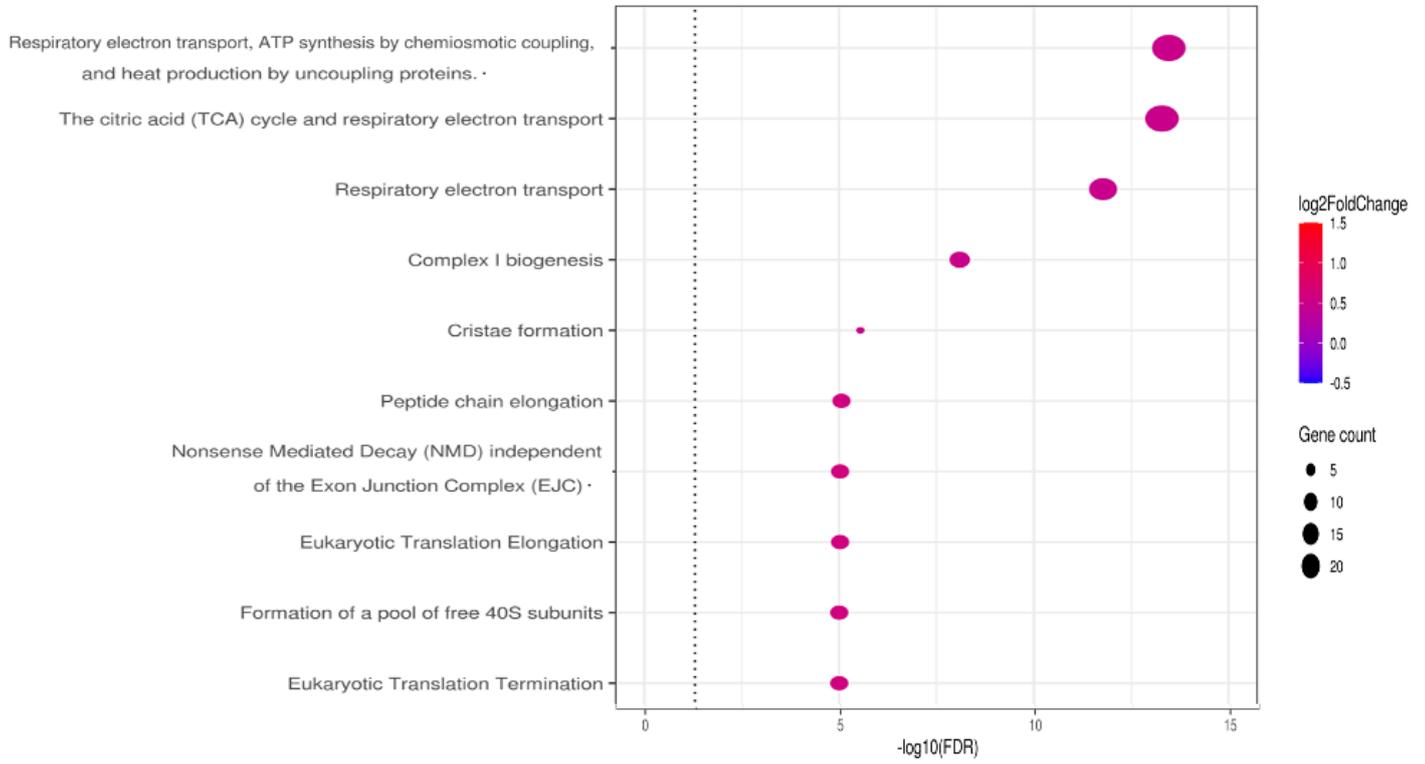


Figure 3



# Figure 4

A)



B)

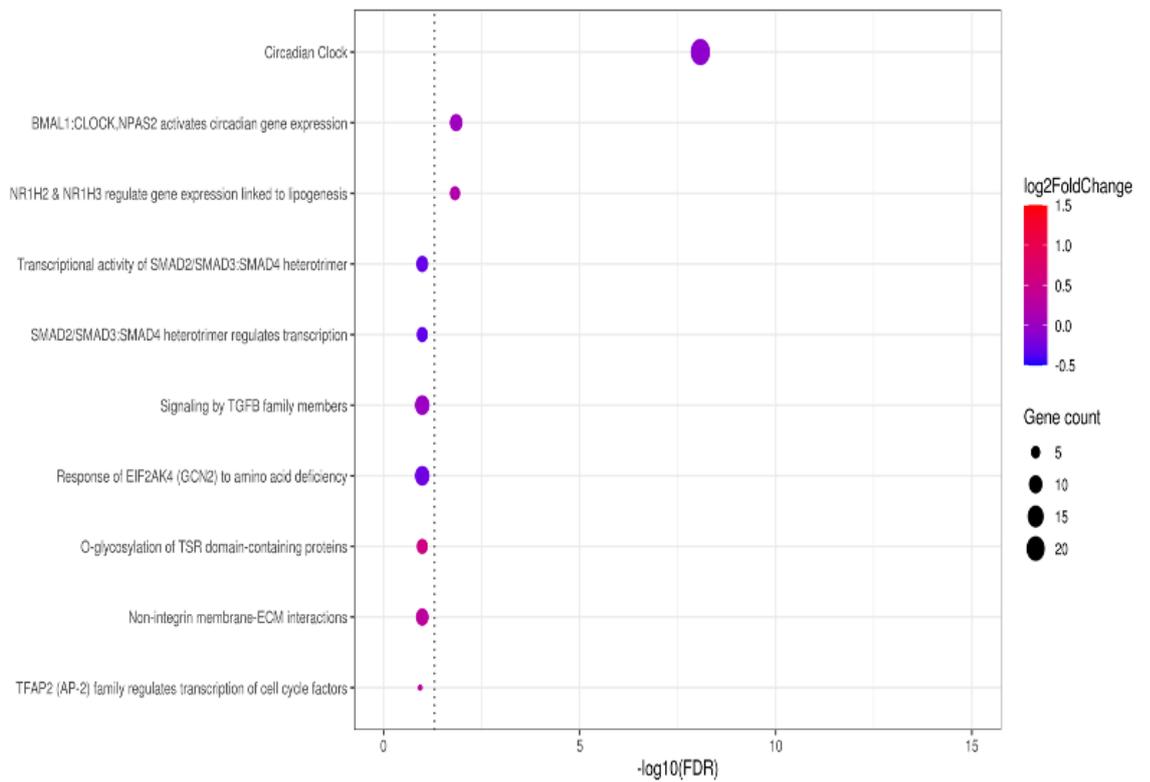
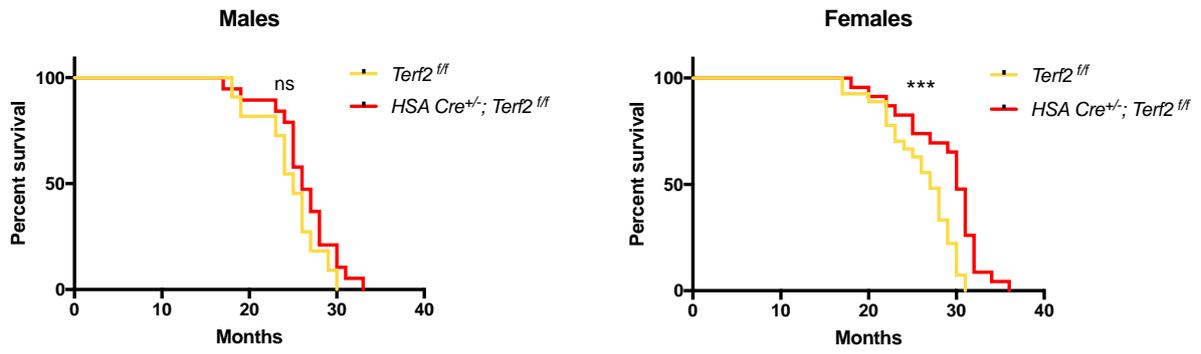


Figure 5



**Supplemental Figure 1:** TRF2 expression in skeletal muscle during aging and in pre-mature aging mouse model

A) TRF2 and GAPDH Western Blots in *tibia anterioralis* and *gastrocnemius-plantaris-soleus* (GPS) mice muscles (B). C57BL6/J females 1-, 3-, 12- and 15-months old. C) TRF2 and GAPDH Western Blot in TERT KO 8 months old females GPS.

**Supplemental figure 2:** *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* skeletal muscle are not atrophied with age.

A) Quantification of the percentage of nuclei with central localization in gastrocnemius type I, IIa and IIb fibers in young (Y) and old (O) mice. Statistical analyses were performed using Kruskal-Wallis' multiple comparisons test (\**p* < 0.05, \*\**p* < 0.001).

B) to D) Quantification of mice skeletal muscle type I (B), type IIa (C), type IIb (D) fiber size repartition in *tibia anterioralis* of young (left panel) and old mice (right panel). Fibers were categorized according to their surface area (<500 to >5000  $\mu\text{m}^2$ ). More than 50 fibers were counted per mice.

*HSA-Cre<sup>+/-</sup>* (young: n=3, old n=4), *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* (young: n=4, old n=5) and *Terf2<sup>ff</sup>* (young: n=3, old n=6)

E) Quantification of mice skeletal muscle fiber size repartition (all types included) in *gastrocnemius* of young (left panel) and old mice (right panel). Fibers were categorized according to their surface area (<500 to >5000  $\mu\text{m}^2$ ). More than 300 fibers were analyzed per mice *HSA-Cre<sup>+/-</sup>* (young: n=3, old n=4), *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* (young: n=4, old n=4) *Terf2<sup>ff</sup>* (young: n=4, old n=4). Mann–Whitney U-test was performed at each size range *HSA-Cre<sup>+/-</sup>* vs *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* (\*) and *Terf2<sup>ff</sup>* vs *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* (#) (\**p* < 0.05, \*\**p* < 0.001, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001).

**Supplemental figure 3:** Gender specific muscular force

A) Absolute GPS (*gastrocnemius-plantaris-soleus*) muscle strength measurement of 15 months old mice, in response to different frequency stimulations. Males: left panel (*HSA-Cre<sup>+/-</sup>* n=5 and *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* n=6, females: right panel (*HSA-Cre<sup>+/-</sup>* n=6 and *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* n=8). Area under the curve was calculated for each curve and compared between genotypes (*HSA-Cre<sup>+/-</sup>* vs *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>*) using Mann–Whitney U-test (\**p* < 0.05).

B) Absolute GPS force of 15 months old mice. *HSA-Cre<sup>+/-</sup>* (left panel) and *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* (right panel), females vs males.

C) Contraction-induced muscle fatigue at 30 Hz, represented on percentage of the maximal force. Left panel: *HSA-Cre<sup>+/-</sup>* and right panel: *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* females vs males. Each contraction point was analyzed using a t-Test (\**p* < 0.05). To calculate the area under the curves, data was divided into subgroups, before and after the curves

crossing intersection. It corresponds to 55 contractions. Areas under the curve were compared using Mann–Whitney U-test.

**Supplemental figure 4:** Validation of RNA-sequencing data

- A) Principal component analysis (PCA) of GPS RNA-sequencing data, females: left panel and males: right panel.
- B) GPS RNA-sequencing data differentially expressed genes heat-map. 8 months old *HSA-Cre<sup>+/-</sup>* vs *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* mice, females (left) and males (right).
- C) qPCR validation results of genes differentially expressed in GPS of males or females *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* compared to *HSA-Cre<sup>ff</sup>*. Female samples: n=3 *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* mice and n=5 *HSA-Cre<sup>+/-</sup>* mice. For males, n=5 mice per genotype. Crossing-threshold (Ct) values were normalized to *36B4* housekeeping gene. The RNAseq column corresponds to the downregulated (Down) or upregulated (Up) gene expression detected by RNA-sequencing.

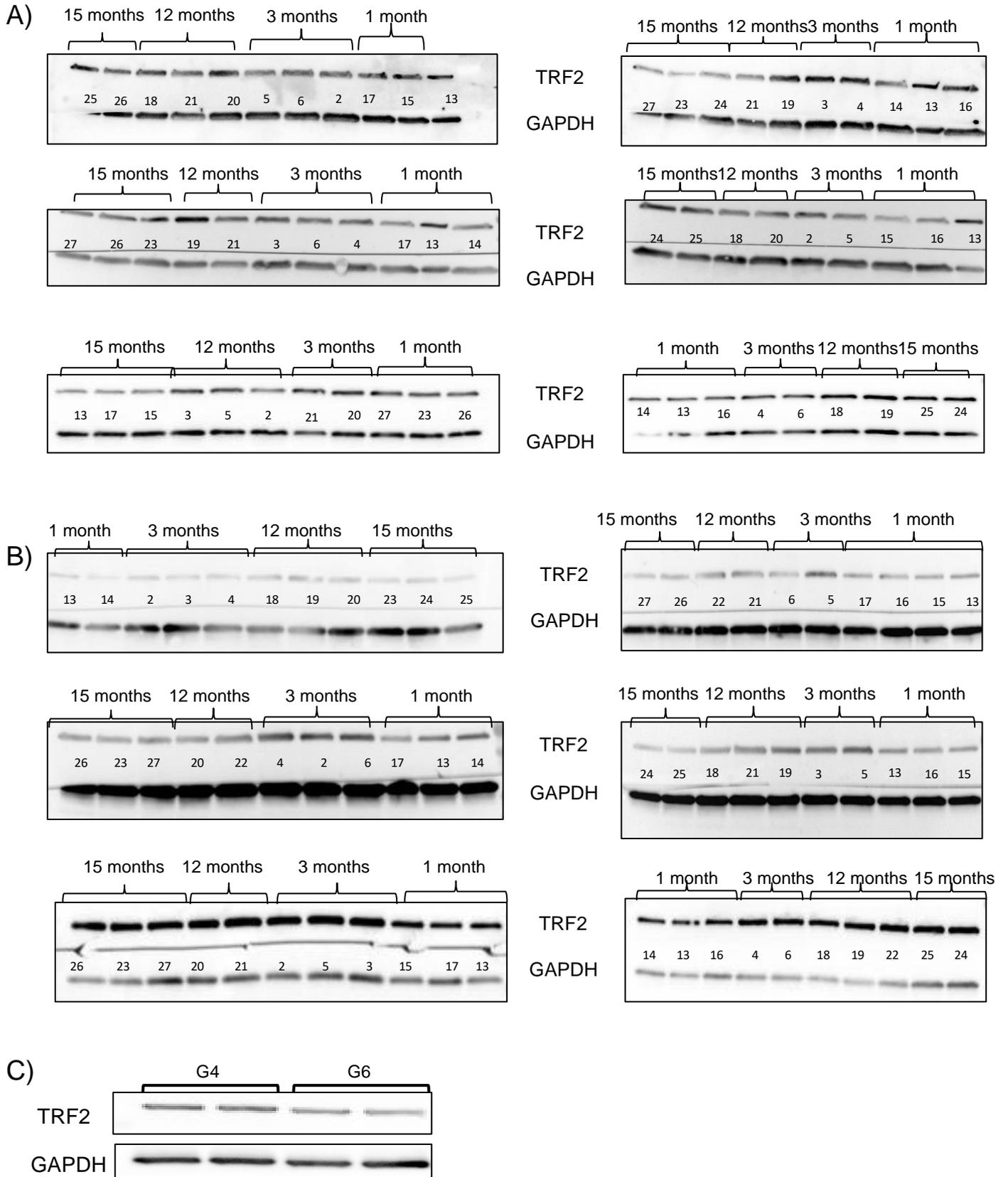
**Supplemental figure 4 bis:** Validation of RNA-sequencing data

- A) KEGG data base oxidative phosphorylation pathway scheme showing differentially expressed genes in GPS from 8 months old females *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* compared to *HSA-Cre<sup>+/-</sup>* (highlighted in red).
- B) Most differentially enriched pathways between 8 months old females *HSA-Cre<sup>+/-</sup>* vs *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* GPS RNA-sequencing, using WebGestalt – KEGG (top) or WebGestalt – Wikipathway (bottom) data bases.

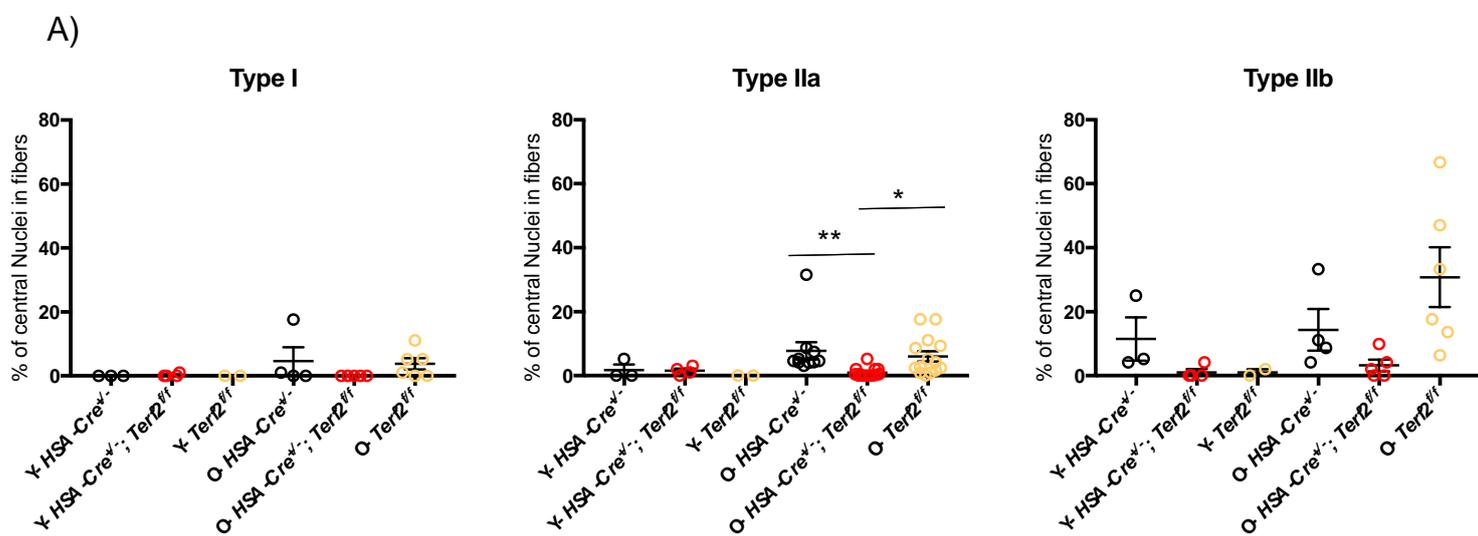
**Supplemental figure 5:** *Terf2<sup>ff</sup>* and *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* survival curves: females vs males

Kaplan-Meier survival curves of *Terf2<sup>ff</sup>* (A) and *HSA-Cre<sup>+/-</sup>; Terf2<sup>ff</sup>* (B), females vs males. Statistical analysis was performed using log-rank – Mantel-Cox, *p-value*= 0,0093.

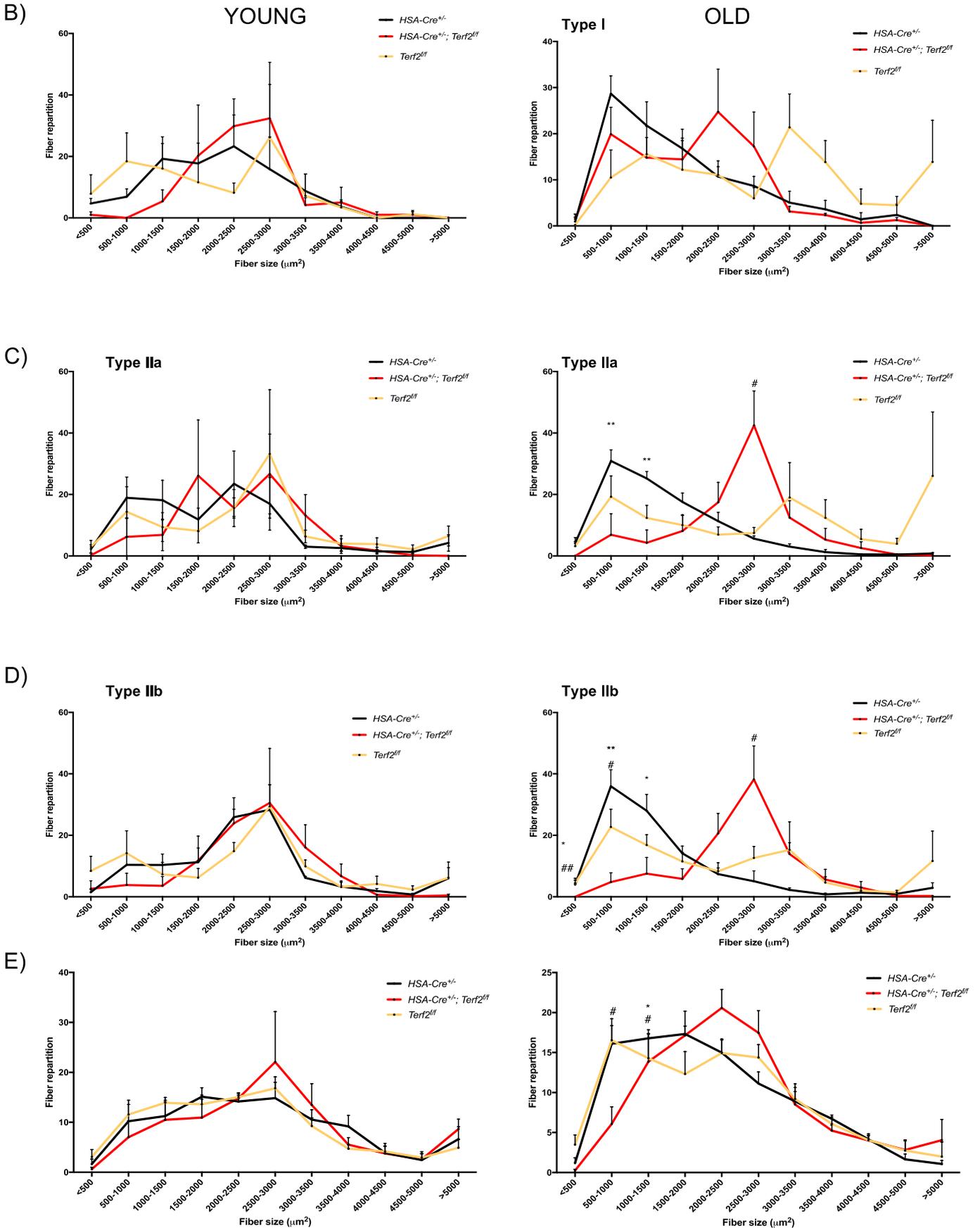
# Supplemental Figure 1



## Supplemental Figure 2

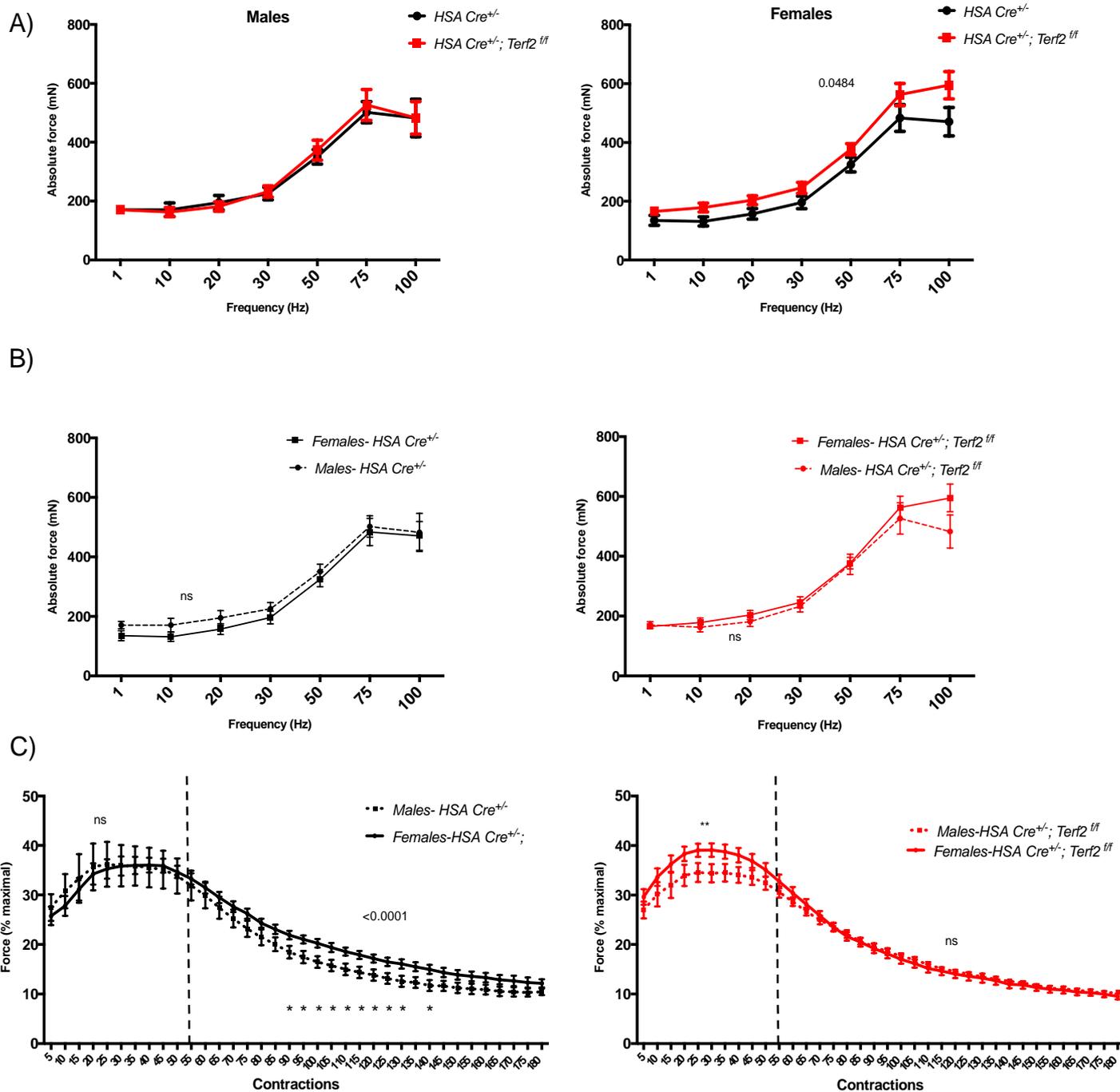


# Supplemental Figure 2

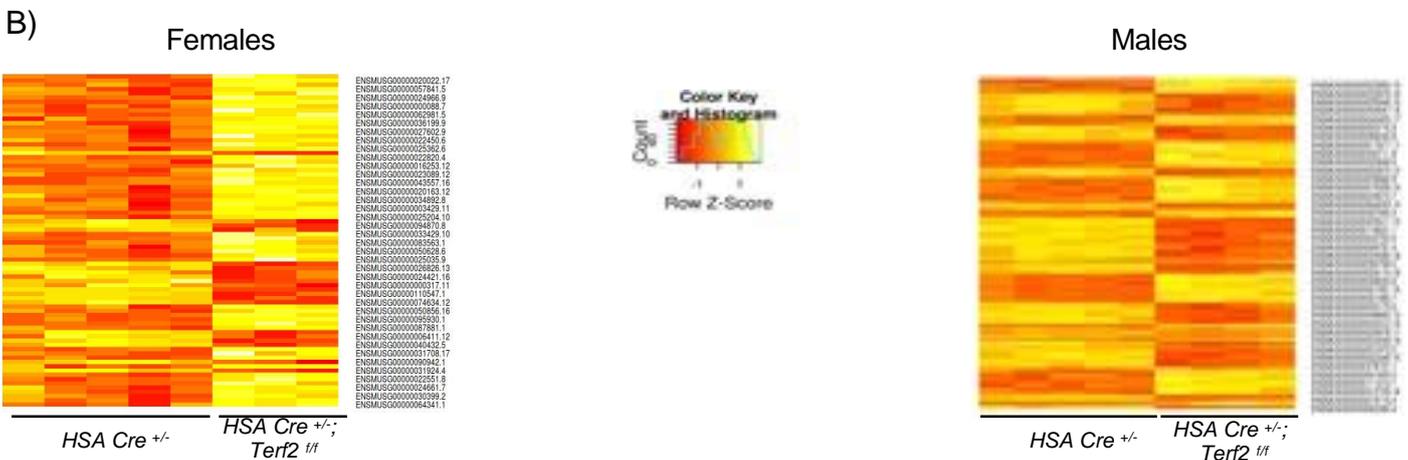
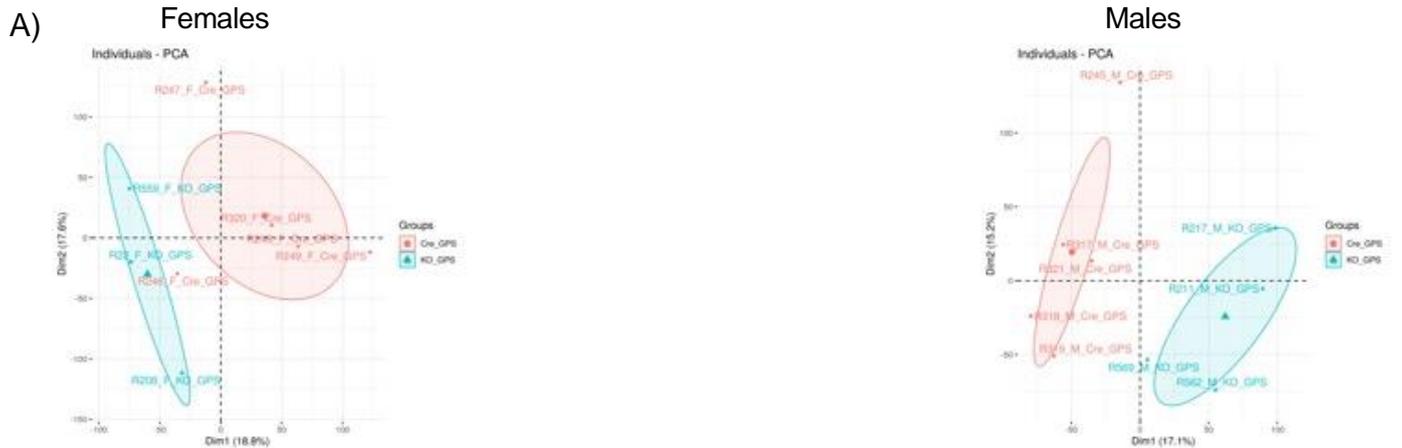


*gastrocnemius*

### Supplemental Figure 3



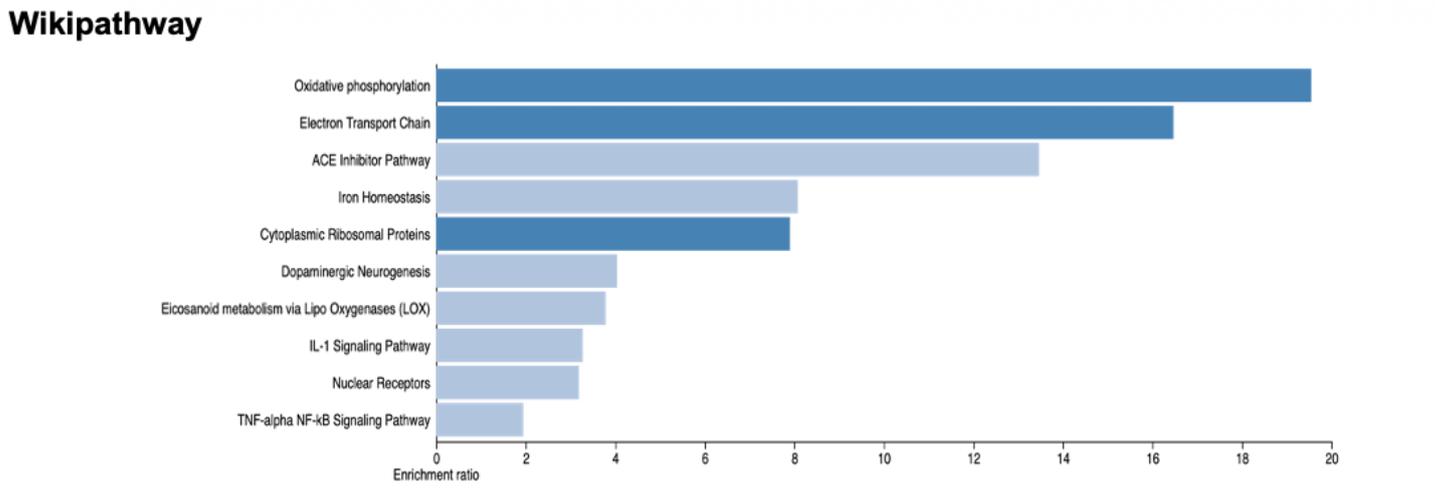
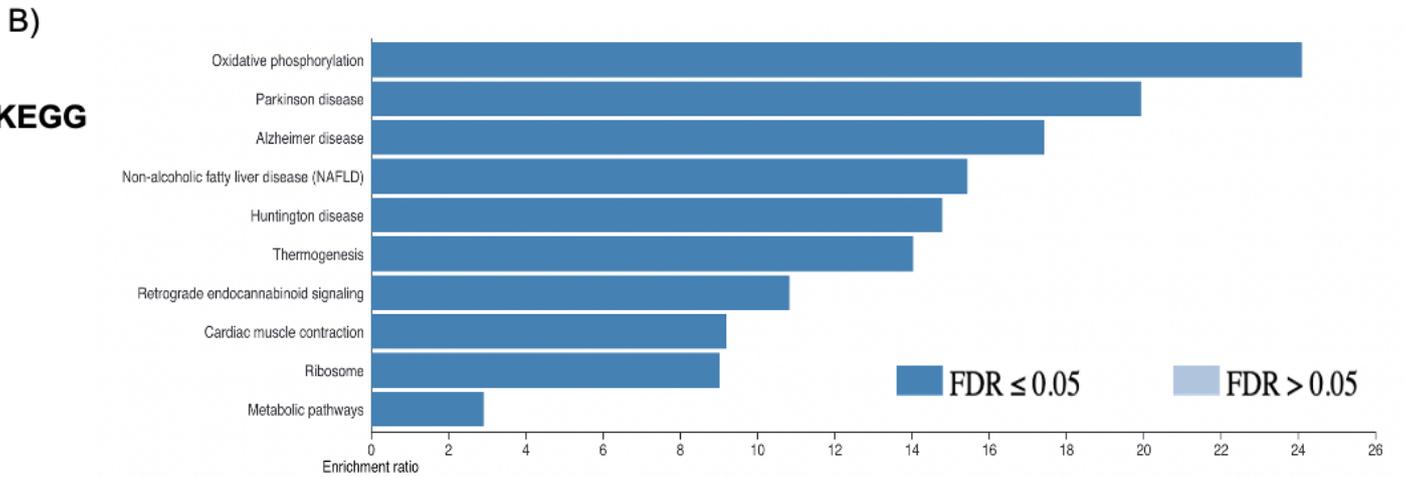
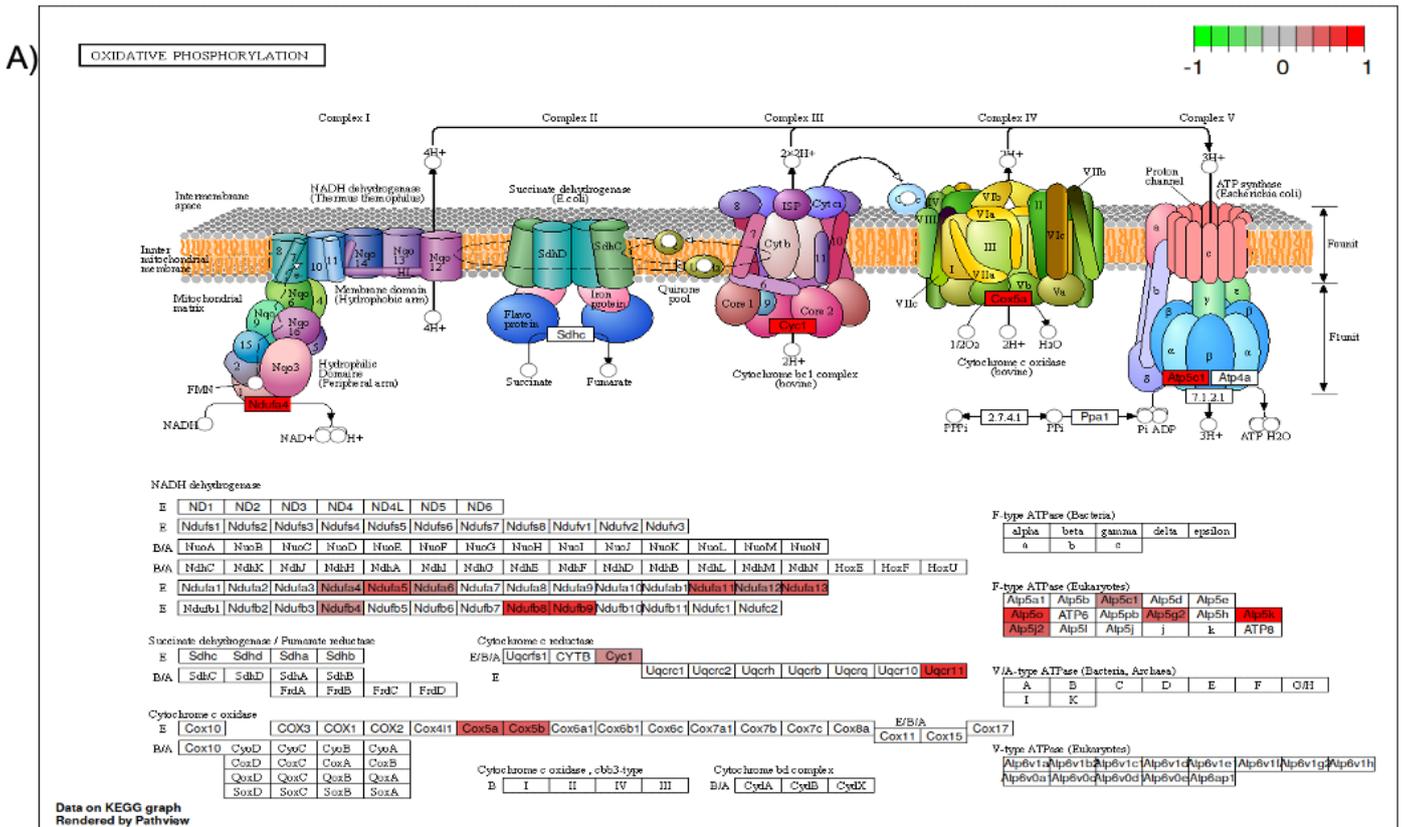
# Supplemental Figure 4



**C)**

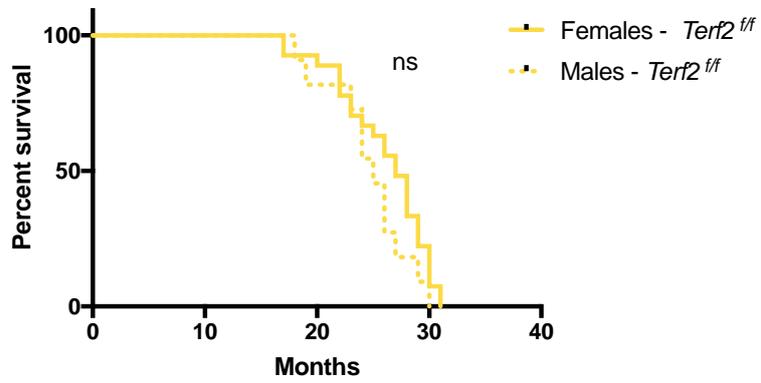
Gene tested	Reference gene	Sample	log2 (Fold Change) by qPCR	RNAseq
Cox5a	36B4	Females-GPS	0,041746562	Up
Atp5k	36B4	Females-GPS	0,140385428	Up
Ndufa5	36B4	Females-GPS	0,192154838	Up
Per2	36B4	Males-GPS	-2,283206097	Down
Npas2	36B4	Males-GPS	1,959889787	Up
Nrip1	36B4	Males-GPS	-0,924051147	Down
Arntl	36B4	Males-GPS	1,685566442	Up

# Supplemental Figure 4 bis

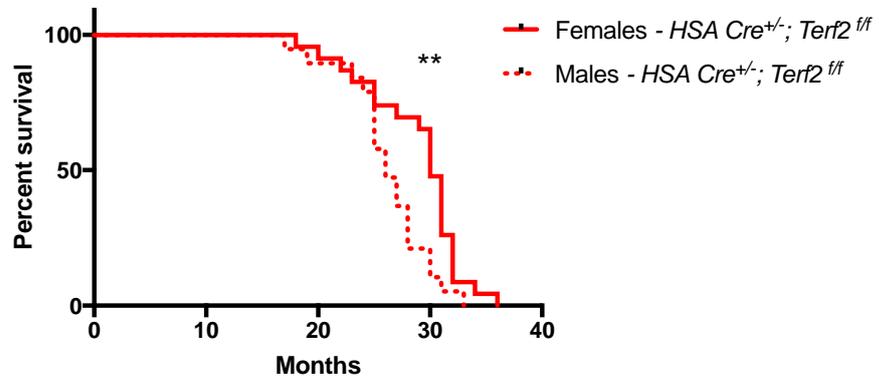


## Supplemental Figure 5

A)



B)





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## *Discussion & perspectives*

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Telomeres are nucleoprotein complexes at the extremity of linear chromosomes. Telomeres have two critical functions: i) to preserve genome integrity despite the end-replication problem, and thus, termini erosion; ii) to protect them against undesirable DNA damage response and recombination. Telomerase inhibition in somatic cells induces progressive telomere shortening, a developmentally regulated process, driver of replicative senescence in mitotic cells.

Nonetheless, the post-mitotic cells (neurons, skeletal muscle, bones, fat tissues, etc) also exhibit functional decline during lifetime. Noteworthy, senescence markers are not always observed in these aging cells, raising the possibility of cellular aging mechanisms that do not rely on classical cellular senescence. The mechanisms and drivers of the LLPMC aging/ senescence remain undetermined.

During my PhD, I studied the broad question of telomere implications on long-lived post-mitotic cells aging, using skeletal muscle as a model for our research. Following on from several minor questions were addressed and new questions appeared at the issue of this work.

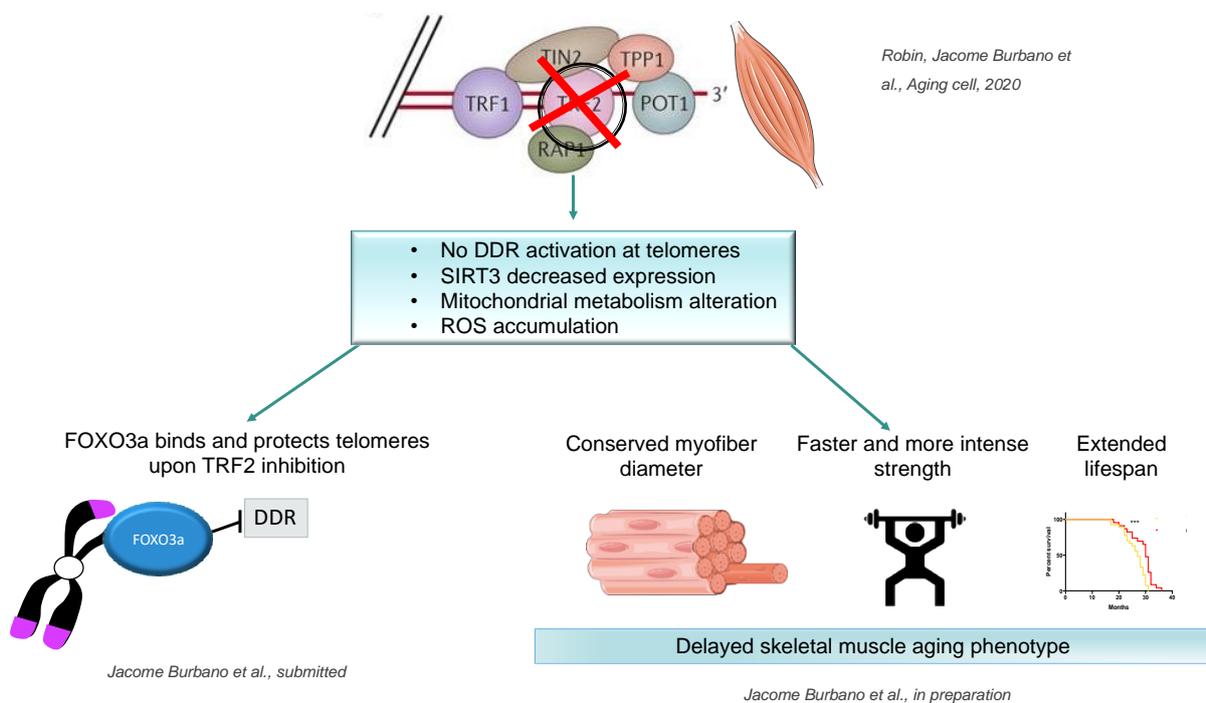


Figure 20: Schematic representation of the processes studied during my PhD work.

## Is there a link between FOXO3a and telomeres?

As mentioned in the introduction of this manuscript, telomeres use peculiar structures (t-loop) and proteins to ensure their terminal protection function. Indeed, some of the telomeric proteins directly prevent DDR activation, NHEJ and recombination at telomeres. Besides, shelterin complex, or at least part of it, is required to preserve telomere homeostasis. For instance, in a study using Cas9 system to knock out one-by-one each protein of the shelterin complex in HeLa cells, the number of damage foci at telomeres was highly increased upon each shelterin KO, except for RAP1. Furthermore, several studies revealed TRF2 importance for telomere protection (Ancelin et al., 1998; Benarroch-Popivker et al., 2016; Fujita et al., 2010; Schmutz et al., 2017; Ye et al., 2010b; Zhang et al., 2006).

In my PhD project, I found that among the shelterin proteins, only TRF2 expression decreases with age in human skeletal muscle biopsies from fetuses (14 weeks) to seniors (72 years old). This downregulation is rapid during lifetime beginning during young adulthood and goes along with progressive telomeric DNA shortening (Robin et al., 2020). Nevertheless, clear skeletal muscle aging phenotype is usually evidenced a couple of decades later. How skeletal muscle perceives the decline of TRF2 expression? Are skeletal muscle telomeres unprotected rapidly during lifetime? If it is the case, do myofibers compensate this phenotype? What are the pathways involved in TRF2 expression regulation? Is the decline of TRF2 expression a driver, a consequence or a compensation of skeletal muscle phenotype changes during young-middle adulthood?

Telomere dynamics observations in human samples are not always consistent with other studies.

First, telomere shortening in post-mitotic or low proliferative cells, during lifetime, is a matter of debate between findings showing progressive TL shortening in skeletal muscle, brain, cardiomyocytes, oocytes (Ain et al., 2018; Aix et al., 2016; Anderson et al., 2019; Baek et al., 2019; Carneiro et al., 2016; Daniali et al., 2013; Ge et al., 2019; Magi et al., 2018; Nakamura et al., 2007) and those suggesting there is any TL variation in these cells or tissues (Allsopp et al., 1995; Decary et al., 1997, 2000; Hiam et al., 2020; Ponsot et al., 2008). These discrepancies could be due to the technique

used to measure TL (qPCR, qFISH, TRF, etc). As well, the telomere shortening rate seems to be important given that highly proliferative cells, such as leukocytes, should show higher TL variation than low proliferative cells, simply because they divide more often. Nonetheless, Daniali and colleagues investigate TL in 19 to 77 human samples by Telomere Restriction Fraction (TRF) and noticed that telomeres in skeletal muscle, leukocytes, skin and subcutaneous fat shorten at a similar rate. However, at old ages, telomeres were much longer in leukocytes than in skeletal muscle, suggesting that tissue specific TL variations might occur during early life (Daniali et al., 2013). A ground-breaking analysis of telomere length in more than 900 people and 6000 samples from the Genotype-Tissue Expression project consortium (GTEx) have recently been published (Demanelis et al., 2020). Despite it uses post-mortem samples, it is, so far, the major study of relative telomere length (RTL) in humans. Whereas no age-mediated RTL difference was observed in cerebellum, skeletal muscle, ovary, vagina, testis and thyroid, RTL decreases with aging in mitotic and post-mitotic tissues such as cortex and hippocampus (Demanelis et al., 2020). Therefore, so far, publications are not in agreement with each other. For the GTEx dataset, RTL was measured using a Luminex-based assay with a small amount of DNA hybridized to telomere specific and probes specific to a reference gene. Then both, telomere and reference gene, signals are amplified and quantified using Luminex equipment (Kibriya et al., 2014). Whereas Luminex-based TL quantification assay has been compared to other methods measuring TL (qPCR, Southern blot), it is mainly used by the same research team. Authors mentioned that the Luminex protocol need to be performed in other labs in order to confirm it reproducibility (Pierce et al., 2016). Hence, further studies are required settle TL changes during LLPMCs aging.

Second, shelterin protein expression does not vary equally during aging through the tree of life. For example, mRNA quantification in zebrafish suggested that the 6 shelterin mRNA decreases upon aging in several tissues, including muscle and brain (Wagner et al., 2017). As well, TRF1 was shown to decrease in old mice (Derevyanko et al., 2017), and we found that TRF2 expression decreases in mouse skeletal muscle starting at middle-adulthood. Therefore, telomere status might be different in each studied specie at different relative ages, perhaps for different physiological demand.

TRF2 is one of the key telomeric proteins. It binds to DSB telomeric repeats, promotes t-loop formation and avoids ATM undesired activation, NHEJ and HR processes at telomeres (Ancelin et al., 1998; Benarroch-Popivker et al., 2016; Bilaud et al., 1997; Buscemi et al., 2009; Ghilain et al., 2021; Okamoto et al., 2013; Poulet et al., 2009; Saint-Léger et al., 2014, 2014; Schmutz et al., 2017; Ye et al., 2010b). Therefore, it was generally admitted that TRF2 downregulation activates the DDR pathways at telomeres. During my PhD, we looked forward to quantifying DDR at telomeres in conditions mimicking human skeletal muscle aging. To this end, we used in vitro human myotubes downregulated for TRF2 and *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice. In contrast to all our expectations, TRF2 depletion triggers any DDR activation at telomeres but nuclear translocation of FOXO3a. Hence, my work broke the previous conviction that telomere of somatic cells depleted for TRF2 are inevitably damaged leading to genomic instability and telomere end-to-end fusions. This might be a particularity of mature myofibers. Whether telomeres of other types of LLPMCs can be protected in a TRF2-independent manner is unknown. Thus, my work opens invite to study telomeres in other post-mitotic tissues and cells such as neurons, bones, etc.

Since FOXO3a is a longevity associated transcription factor and a stress sensor involved in several cellular pathways (Martins et al., 2016; Webb and Brunet, 2014), I wondered whether it could be involved in telomere protection upon TRF2 inhibition. Interestingly, I determined that FOXO3a binds to telomeres in order to protect them from ATM-recognized damage. FOXO3a-mediated telomere protection was true in TRF2-compromised myotubes but also in human BJ-HELT fibroblasts downregulated for TRF2 or exposed to bleomycin. We used BJ-HELT as a simpler model to characterize this mechanism. Hence, my work revealed a novel function of FOXO3a in providing specific protection to telomeres against a genotoxic insult, whereas little or any effect was detected in unstressed cells upon FOXO3a inhibition. An interesting result in TRF2 downregulated myotubes, is that despite ROS accumulation telomeres are “oxidized” (observed by 8-oxoG presence at telomeres) only when FOXO3a is also inhibited (Jacome Burbano et al., 2021b- Figure 1E-F). TRF2 inhibition or a double FOXO3a and TRF2 KD result in ROS accumulation in a similar manner. Indeed, we detected any FOXO3a KD-associated ROS production. Therefore, the observed teloxidation triggered by *FOXO3a* expression inhibition in *TERF2*-compromised myotubes cannot be explained simply by a global increase in ROS or a decrease in

oxidative defense. Hence, these results suggest that FOXO3a provides an alternative strategy for specific telomere protection upon cellular stress. In line with this hypothesis, FOXO3a promotes DNA repair in an oxidative environment (Tran et al., 2002) and is involved on the DDR ATM pathway activation (Adamowicz et al., 2016; Chung et al., 2012; Tsai et al., 2008). Moreover, a recent publication, show that FOXO3a is required to prevent genomic instability (HR, NHEJ) after bleomycin treatment in human and mouse cells (White et al., 2020).

My work also determined that FOXO3a is found associated to the telomeric protein complex. I characterized this association in myotubes by confocal microscopy colocalization, proximity ligation assay (PLA) between TRF1 and FOXO3a in cells depleted for TRF2, FOXO3a ChIP-Slot Blot revealed with a telomeric probe and FOXO3a co-immunoprecipitation with TRF2 in human skeletal muscle biopsies. In BJ-HELT, we observed the TRF2-FOXO3a association by PLA. The detected association show reproducible and significant results but the amplitude of FOXO3a association to telomeres is reduced. One can imagine, that FOXO3a telomeric localization is sporadic but sufficient to control telomere protection. This could explain why FOXO3a has not been observed yet as a telomeric protein in proteomics of isolated chromatin segments (PICH) or quantitative telomeric chromatin isolation protocol (QTIP) assays (Déjardin and Kingston, 2009; Grolimund et al., 2013).

We expect that FOXO3a binding to telomeres increases progressively during aging. It would be interesting to perform PLA or ChIP in human skeletal muscle biopsies to study FOXO3a telomeric localization during lifetime. These experiments should be completed by FOXO3a protein expression and post-translational status as well as cellular localization in the same biopsies, in order to determine how FOXO3a expression and functional state is linked to its telomeric functions.

Besides, it would be important to perform a FOXO3a ChIP-sequencing in myotubes downregulated for TRF2, as well, in BJ-HELT fibroblasts exposed to bleomycin. Does FOXO3a binds to the same regions in both conditions? Does it bind to the same telomeres? Previous studies observed that FOXO3a binds mostly to intergenic regions (Eijkelenboom et al., 2013). Is important to take into consideration that despite telomeres are repeated sequences, they are not in the ENCODE “blacklist”, a list used to filter complicated regions within the genome known to give an unspecific signal (Amemiya et al., 2019).

Moreover, I discovered that the conserved region 2C (CR2C) (461-482 aa) is required for FOXO3a telomeric association and protection. This domain, along with CR3 (483-673) are the two FOXO3a transactivation domains. I observed that p300 is required for the FOXO3a mediated telomere protection. Importantly, p300 is known to bind to the CR2C and CR3 FOXO3a domains (Wang et al., 2012c). Therefore, I expected that both CR2C and CR3 were required for FOXO3a-mediated telomere protection because CR3 was the first domain to be described to interact with p300 and AMPK phosphorylation within CR3 enhances the FOXO3-p300 association (Wang et al., 2012c). As well, the CR3 domain binds to ATM. Counterintuitively, we observed that  $\Delta$ CR3 mutant expression, increases the TRF2-FOXO3a association. Besides, the Forkhead (FH)/ DNA binding domain was not required neither for FOXO3a-telomere association neither for telomere protection. Thus, my work suggests a non-canonical FOXO3a function independent from its DNA binding domain. We could hypothesize that p300 mediated FOXO3a post-translation modifications are important for its telomeric functions. Nevertheless, p300/CBP acetylates FOXO3a at residues K242, K245 and K259, resulting in reduced DNA binding. These residues are within the FH domain and the Nuclear Localization Sequence (NLS) (van der Heide and Smidt, 2005; Shiota et al., 2010). Thus, according to our results, p300 mediated acetylation is not likely to be implicated in FOXO3a telomeric functions. One can imagine that p300 recruits FOXO3a to telomeres given that p300 binds to the basic domain of TRF2 and acetylates it at K239 (hinge domain). We can also study whether the TRF2-p300 association or p300 mediated TRF2 acetylation is required for FOXO3a-mediated telomere protection.

To validate our results in a muscle context, it would be interesting to express FOXO3a  $\Delta$ CR2C mutant form in myotubes depleted for TRF2 and endogenous FOXO3a. One can anticipate that in this context, the FOXO3a dependent telomere protection effect will be lost, or at least reduced.

Finally, the *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mouse model would be useful to study whether the FOXO3a telomere binding is conserved in other organisms. To this end, FOXO3a protein expression in skeletal muscle should be quantified, followed by co-immunoprecipitation, PLA, ChIP-Slot blots at different aging time points. According to the results, it would be of interest to characterize whether this binding takes place in all kinds of skeletal muscles or whether it is specific of oxidative or glycolytic myofibers.

Next, if results are promising, it would be attractive to create *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> and *FOXO3a* knockout and knock in mouse strains to examine FOXO3a mediated telomere protection role in an entire organism, during lifetime.

## The paradoxical outcomes of TRF2 loss in skeletal muscle

As mentioned before, the TRF2 KD/KO models (shTRF2-myotubes and TRF2 KO in mouse mature myofibers) do not show any DDR at telomeres. However, we observed an accumulation of ROS, higher mitochondrial respiratory chain Complex I activity but reduced for Complex IV, mtDNA higher copy number and reduced expression of SIRT3. We have shown that telomeres create a long-distance chromatin loop triggering telomeric TRF2 to the subtelomeric *SIRT3* locus and positively regulates its expression (Robin et al., 2020). In TRF2 KD myotubes, the mitochondrial altered phenotype was rescued upon SIRT3 overexpression, establishing that a TRF2-SIRT3 axis modulates the oxidative metabolism in skeletal muscle fibers.

SIRT3, along with SIRT4 and SIRT5, are mitochondrial sirtuins involved in mitochondrial metabolism. However, SIRT3 appears to be a major regulator of mitochondrial acetylation (Hebert et al., 2013; Onyango et al., 2002). It regulates mitochondrial bioenergetics, some complexes of the respiratory chain and it is involved in ROS detoxification (Benigni et al., 2019; Finley et al., 2011; Y et al., 2018).

Recently, it was shown that *SIRT3*<sup>-/-</sup> mice have a shorter lifespan and exhibit heart dysfunction, correlated with mitochondrial cristae disorganization and impaired bioenergetics (Benigni et al., 2019). It is not clear whether skeletal muscle was also analyzed. Altogether, these findings and our discovery of TRF2 as an upstream regulator of SIRT3 expression, reinforce our hypothesis that telomeres are involved in post-mitotic cells aging, in a telomeric DNA length-independent manner.

Next, I looked forward to characterizing the *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mouse strain during aging. I have been facing to unexpected results: i) 15 months old *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice do not show signs of ongoing or past regeneration events. ii)

*HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice do not atrophy skeletal muscle fibers at old age (15 months), but WT mice do, whereas there was no difference at young age. iii) *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females, exhibit a more powerful strength in response to stimuli but reach fatigue quicker. Any distinction was observed in *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> males compared to age-mated WT mice. iv) 8 months old *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice show a different transcriptional profile, suggesting that TRF2 regulates several genes expression. In KO females there was a clear mitochondrial respiratory chain alteration and in KO males, the circadian rhythm associated pathways seem to be involved. v) *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females have a longer median lifespan in relation to WT females. No difference was detected in male lifespan. Overall, these results suggest that *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice tend to keep a younger muscle phenotype at old ages, resulting in an extended lifespan. Hence, to our greater surprise, TRF2 KD in mouse skeletal muscle seems to be beneficial.

In order to complete our 8 months old RNA-seq data, it would be of interest to perform RNA-sequencing in young (1-2 months) and old (15 months) mice and even in newborn mice. When during development, the TRF2 loss-mediated transcriptional changes are them detected? Is the mitochondrial phenotype also altered at young age? Are the same pathways that are affected at different ages?

I observed that TRF2 expression decreased in C57BL6/J mice skeletal muscle starting at 12 months. Therefore, one can imagine transcriptional alterations might be attenuated after 12 months of age. Nonetheless, we can also hypothesize differences induced by TRF2 loss earlier during lifetime in *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice will be accumulated leading to a different phenotype. Metabolic cages assays (monitoring mouse activity, food consumption, etc) at different ages would give some insights when during lifetime TRF2 KD-mediated differences are the most emphasized.

As explained in the introduction of this manuscript, skeletal muscles are secretory organs with autocrine, paracrine and endocrine effects. Secreted myokines regulate muscle homeostasis but also carry cross-talks and impacts other organs (Saini et al., 2021; Scisciola et al., 2021), In order to detect whether skeletal muscle TRF2 loss induces or prevents an aging/ anti-aging systemic effect, it would be interesting to measure circulating secreted factors and muscular factors in *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> and WT mice at different time points. Does skeletal muscle TRF2 loss lead to a systemic effect that impacts other organs homeostasis? Is the anti-aging

*HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mouse phenotype only due to skeletal muscle metabolic alterations? To complete this perspective data, other organs should be collected in WT and *Terf2* KO mice to analyze aging related markers, such as fibrosis. I expect that *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> mice, or at least females, show reduced aging markers in skeletal muscle but also in other organs, including the post-mitotic brain. Moreover, hormones or other factors quantification should give some answers to elucidate why there is an anti-aging effect regarding muscle strength and lifespan in *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females but no variations are observed in males as compared to WT males.

## The Telo-hormesis hypothesis

We previously observed a fiber-type switch from type II-glycolytic to type I-oxidative metabolism in *soleus* of 8 months old *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> (Robin et al., 2020). A hallmark of skeletal muscle aging is an increased proportion of type I-slow twitch at expenses of type II-fast twitch fibers (Lexell et al., 1988; Verdijk et al., 2014). Therefore, our first remarks on *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> fiber type characterization suggested a premature aging phenotype. Nonetheless, my PhD work confronted our first hypothesis by revealing anti-aging effects upon myofiber *Terf2* KO. As well, the pro-aging initial hypothesis was supported by an altered mitochondrial metabolism, ROS accumulation, SIRT3 downregulation and the simple fact of inducing telomere changes. All of them advocate that TRF2 decreased expression in skeletal muscle might be harmful. The contradictory advantageous effect, unveiled during the third aim of my project, led me to propose a model based on an hormetic effect of TRF2 downregulation during muscle development and aging.

The concept of hormesis can be defined as a dose response process in which low and high doses of stressors have opposite effects (Calabrese et al., 2007). Applied to telomeres, a hormetic effect could be beneficial in, a priori, two types of situations: i) a low level of dysfunction (shortening, chromatin and shelterin changes) leading to a beneficial effect, for instance by triggering a redox signaling or changing gene expression, while a higher dose (critical shortening or profound chromatin destruction)

would trigger senescence, chromosomal instability and aging; ii) a low level of senescence, triggered by telomere dysfunction, being beneficial (for instance to limit the proliferation of pre-cancerous cells) while too many senescent cells would favor aging and even cancer. Pioneer data from Seiichi Yokoo and colleagues, suggested telo-hormesis effect on human keratinocytes exposed to low doses of hydrogen peroxide (Yokoo et al., 2004). H<sub>2</sub>O<sub>2</sub> effects regarding the number of population doublings, ROS amounts and TL were better than those observed on keratinocytes exposed to antioxidant agents. Later, this concept was discussed in a study on *cdc13-1* dysfunctional budding yeast, which found that pre-exposure to a low dose of telomere stress led to more resilient yeast under acute stress (Markiewicz-Potoczny and Lydall, 2016). Besides, repeated hyperbaric oxygen therapy (HBOT) in 66-68 years old individuals triggers telomere elongation in cytotoxic T cells, T-helpers, NK and B cells (Hachmo et al., 2020). HBOT consists of breathing pure (100%) oxygen in a pressurized environment, increasing the amount of oxygen that could be carried by blood. HBOT is used as a medical therapy and has shown beneficial effects on wound healing, ischemia/reperfusion and inflammatory injuries and induces cognitive enhancement in healthy adults over 64 years old (Amir et al., 2020; Thom, 2009). Fluctuations of oxygen (hypoxia/ hyperoxia) triggers adaptative metabolic changes. Intriguingly, it has been observed that intermittent high oxygen exposure induces some pathways and mechanisms usually observed during hypoxia. It seems that decreased free oxygen after hyperoxia is felt as a lack of oxygen, leading to hypoxia-related pathways activation (Cimino et al., 2012; Hadanny and Efrati, 2020). This is the hypoxia/hyperoxia paradox. Although hyperoxia, such as HBOT, induces an increase of ROS production, it also triggers higher antioxidants expression (Godman et al., 2010; Thom, 2009). Hence, HBOT might act as exercise or caloric restriction, known for ROS mild increase, in which a single session could be harmful but regular repeated sessions could lead to a greater antioxidant activity and protection (Godman et al., 2010; Hachmo et al., 2020; Hadanny and Efrati, 2020). Thus, HBOT might be considered as a model of the hypoxia/hyperoxia mediated hormesis and telo-hormesis (dubbed for hormetic effect at telomeres).

A recent report showed that TL was shorter in mammals living in the wild than in domesticated species (Pepke and Eisenberg, 2021), suggesting that possessing short telomeres in a wild habitat confers a selective advantage. Since a wild habitat

forces organisms to cope with both resource scarcity and fluctuating environments, we can postulate that short telomeres may improve survival and fitness during exposure to stress. Nonetheless, it is noteworthy to highlight that telomere alterations do not only concern TL variation but also higher chromatin order modifications, telomeric proteins expression and its binding to telomeres. Changes in these factors might affect telomere integrity, triggering an hormetic or a detrimental effect. The telo-hormesis hypothesis here postulated is supported by publish data showing or suggesting beneficial consequences of telomere alterations. Telo-hormesis has been observed in prevention of tumorigenesis, telomere position effect transcriptional regulation and paired with mitochondrial hormesis (mito-hormesis). These points are detailed in our review (Jacome Burbano and Gilson, 2021). Here, I will focus on telomere and the mito-hormesis mechanisms.

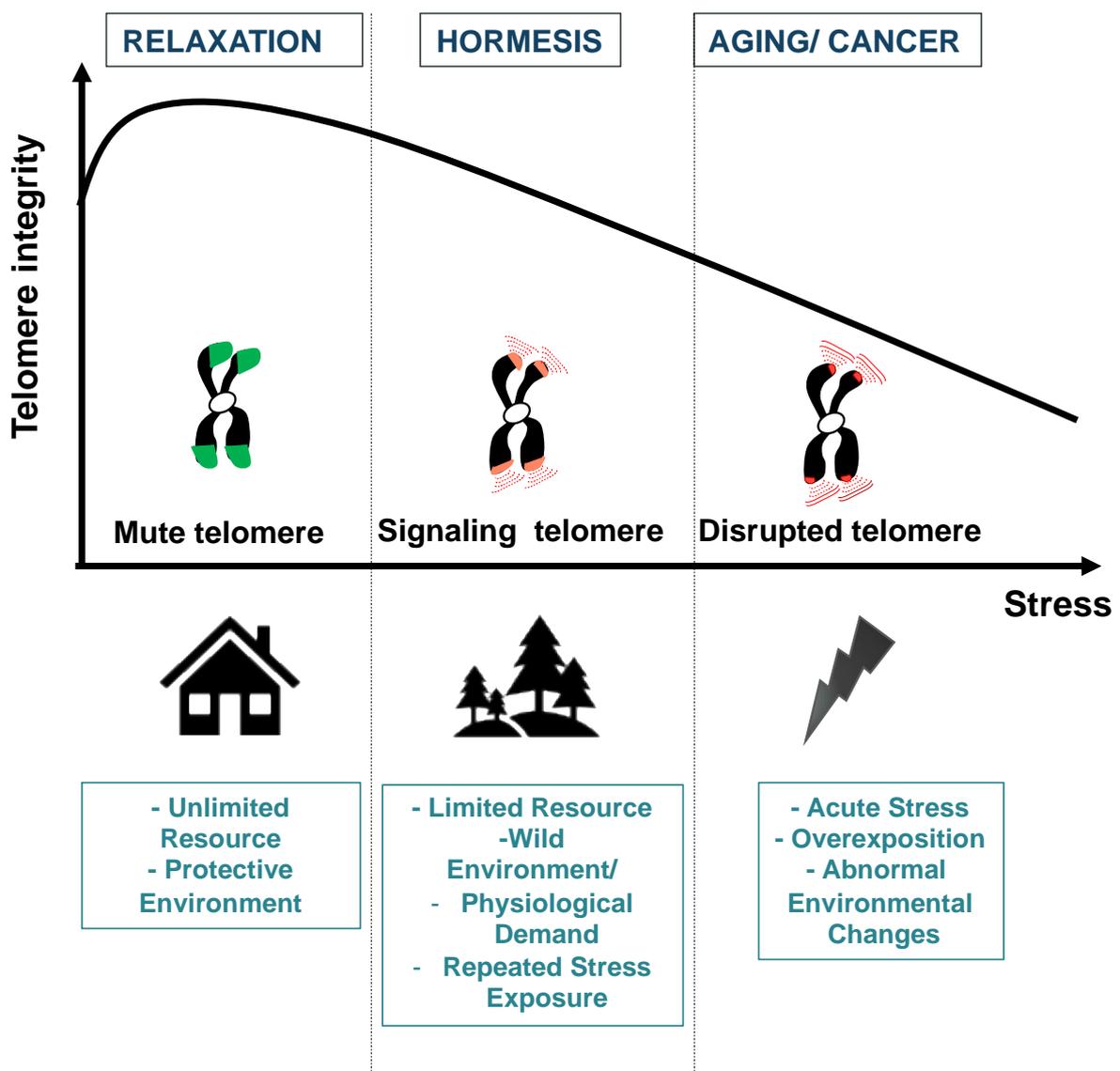


Figure 21: The telo-hormesis hypothesis

Telomere integrity encompasses telomeric DNA length but also telomeric protein expression and telomeric binding, as well as high order chromatin organization. Under exposure to predicted stresses (such as regular seasonal fluctuations and limited resource) or normal/physiological demands, telomere integrity might be affected in order to trigger a hormetic signal through transcriptional changes and mito-hormesis. To stop an early oncogenic event, this hormetic signaling can also result in cellular senescence. Under unexpected, overexposure and acute stresses, telomere integrity can be dramatically disrupted leading to detrimental effects with an excessive chromosome instability resulting in premature aging and cancer development. In contrast, in protective, unchallenged conditions, one can speculate that telomeres are in a “mute” state that just protect chromosome ends but without leading to any beneficial or detrimental signaling, resulting in the “relaxation” of the TL regulation processes, possibly leading to heterogenous and long telomeres, such as those present in laboratory mice. Image adapted from (Jacome Burbano and Gilson, 2021).

The most studied pathway of hormesis is mito-hormesis, defined as mitochondrial stress leading to better health and viability (C et al., 2018). Mitochondrial dysfunction is signaled by the nucleus through a retrograde communication pathway triggering a differential gene expression response. Among the mito-hormesis signals of stressed mitochondria, the major factors are ROS, but there are also metabolites and the mitochondrial unfolded protein response (UPR).

There is a wealth of data showing a strong link between telomeres and mitochondria. Much data indicate that mitochondrial dysfunction leads to telomere stress and *vice versa*. Telomeric repeats are preferential targets of oxidative stress-induced DNA damage, due to its G-rich sequences (Barnes et al., 2019a), a process that we dubbed teloxidation (Jacome Burbano et al., 2021a). Telomere shortening is exacerbated by oxidative stress (von Zglinicki, 2002) and enhanced antioxidant contexts tend to lengthen TL (Serra et al., 2003; Stauffer et al., 2018). Some antioxidant proteins are associated with telomeres (PRDX1 and MTH1) (Aeby et al., 2016; Ahmed and Lingner, 2018). Recently, it was shown that the ROS secreted by activated neutrophils leads to teloxidation of neighboring cells and senescence (Lagnado et al., 2021). Besides, extra telomeric functions of the telomerase subunits TERT and *TERC* have been related to modulating mitochondrial metabolism. First, upon stress, TERT is exported from the nucleus to the mitochondria, where it protects from DNA damage, apoptosis and oxidative stress (Ahmed et al., 2008; Haendeler et al., 2009; Miwa et al., 2016; Spilsbury et al., 2015; Zheng et al., 2019). Second, the telomerase RNA *TERC* is processed within the mitochondria to be later released again to the cytoplasm (Cheng et al., 2018b). It is likely that cleaved *TERC* inform the nucleus

about mitochondrial functional state and activity (Zheng et al., 2019). In telomerase-deficient mice, short telomeres induce p53 activation, which in turn directly represses *PGC-1 $\alpha$*  and *PGC-1 $\beta$* , increasing ROS production. Since ROS signaling is the main mechanism of mito-hormesis and since dysfunctional telomeres could trigger mitochondrial instability and ROS production, telomere-induced oxidative metabolism changes could have a hormetic effect.

Moreover, KO of each shelterin subunit show that they are all, except TRF1, involved in mitochondrial metabolism (Kim et al., 2017). Specially, the glycolysis and the pentose phosphate pathways were modulated upon shelterin KO. This suggests that telomere dysfunction influences metabolism to better adapt the energy consumption upon stress. Interestingly, TIN2 can be localized within the mitochondria and affects its activity and ROS production (Chen et al., 2012). TIN2 specific KO affects metabolites of the tricarboxylic acid cycle (TAC), supporting its role in mitochondrial function.

Additionally, our results revealed, in human and mouse skeletal muscle, the expression of the mitochondrial sirtuin *SIRT3*, a subtelomeric gene, is activated by TRF2 fixation at its locus through a long-distance chromatin loop. *SIRT3* is downregulated upon TRF2 inhibition, leading to mitochondrial alteration, characterized by higher mitochondrial DNA content and ROS levels (Robin et al., 2020). Accordingly, our data unveiled that the most affected pathway upon TRF2 inhibition is the mitochondrial respiratory chain. Whereas at the beginning of my PhD we expected a premature aging phenotype, latter, we observed an advantageous effect leading to a boosted resistance to aging-induced atrophy and frailty, as well as elongated lifespan. Therefore, telomere alteration could induce mitochondrial-mediated mild stress, keeping skeletal muscle “young” and equipped with an enhanced response to stress strategy. If the anti-aging outcomes observed in this project were due to mito-hormesis, I hypothesize that upon antioxidant treatment or factors to restore mitochondrial normal function, the positive effect should be lost or at least decreased. It would be of interest to compare *HSA-Cre*<sup>+/-</sup>; *Terf2*<sup>flox/flox</sup> females treated with N-Acetyl Cysteine (NAC), a fast anti-aging antioxidant, to non-treated mice by using skeletal muscle characterization (type of fibers, myofiber diameter, muscle force), mobility activity observation (metabolic cages), transcriptional, metabolic approaches (RNA-sequencing and metabolomics) and lifespan assays.

Overall, telomere and mitochondria are functionally interconnected in skeletal muscle cells and telomeric changes could trigger in some circumstances a mitohormesis response during muscle aging. Further studies are required to reveal the mechanisms of this telomere/mitochondria cost/benefit balance and its implications in response to stress.

A combination of anti-aging responses have been observed in the transcriptomic profiles of mouse strains showing either anticipated aging (Nucleotide Excision Repair: NER mice *Csb<sup>m/m</sup>; Xpa<sup>-/-</sup>, Ercc1<sup>-/-</sup>, Ercc1<sup>Δ-7</sup>*) or long-lived strains (somatotropic mutants: *Snell, Ames, Ghr<sup>-/-</sup>*) or under caloric restriction known to extend longevity (Schumacher et al., 2008). Indeed, NER progeroid and long-lived mutant or starved mice share a similar signature of transcriptional changes, suggesting that premature or delayed aging activates, at least partially, the same pathways but in contrasting physiological contexts (Schumacher, 2009; Schumacher et al., 2008). Genes upregulated in the biological pathways common to NER progeroid and long-lived mice are involved in oxidative stress response, apoptosis and DNA repair, whereas downregulated genes are associated to the GH (growth hormone)/IGF-1 or somatotropic axis, oxidative metabolism and electron transport chain (Garinis et al., 2009; Schumacher et al., 2008). Hence, DNA repair-deficient mutants induce stress response and survival pathways. The somatotropic axis reduction was also observed in natural mouse aging but at a lesser extent (Garinis et al., 2009), it could be a strategy to prevent cancer formation. Therefore, low levels of DNA damage could trigger a stress response activation leading to an hormetic effect and resulting in an extended longevity. Although, DNA repair mutants also induce a similar stress response pathway, the excessive load in DNA mutations is detrimental, resulting in premature aging.

It is important to highlight that among the common pathways in NER progeria and delayed-aging mice a central factor is the transcription factor FOXO3a. As previously mentioned, FOXO3a is a major regulator of survival and stress response pathways, activated by inhibition of the IGF-1 pathway and required for oxidative stress response. Although, Schumacher and colleagues found significant FOXO3a downregulation only in liver of *Csb<sup>m/m</sup>; Xpa<sup>-/-</sup>* mice, in naturally aged WT mice lungs and upregulation only in *Ghr<sup>-/-</sup>* mice liver, we cannot exclude FOXO3a expression is

modulated in other organs nor that FOXO3a activation could change in these conditions. FOXO3a might be a central factor of the premature/delayed aging hormesis.

Adapted to my PhD, FOXO3a association to telomeres and its protective functions might be a hormetic response to a mild stress induced by TRF2 inhibition that is observed in young adults in human and later in life for mice. We can propose a model in which at young age, TRF2 efficiently protects telomeres, activates SIRT3 expression and maintains balanced mitochondrial metabolism in skeletal muscle. Then, during early and middle adulthood in humans and mice, respectively, TRF2 expression decreased resulting in FOXO3a binding and protection of telomeres, SIRT3 reduced expression and mitochondrial alteration leading to the beneficial effects of ROS signaling. At older age, one can imagine that a greater accumulation of damage and/or changes in FOXO3a regulation and functions lead to a detrimental phenotype, higher oxidative metabolism and muscle force weakening. In this hypothesis, one can interpret our mouse model of mature myofiber specific TRF2 KO as an early induction of mitochondrial respiratory chain alteration and ROS signaling, leading to a mild anticipated stress. This condition could induce an hormetic effect, resulting in a “young-like” muscle phenotype in old mice.

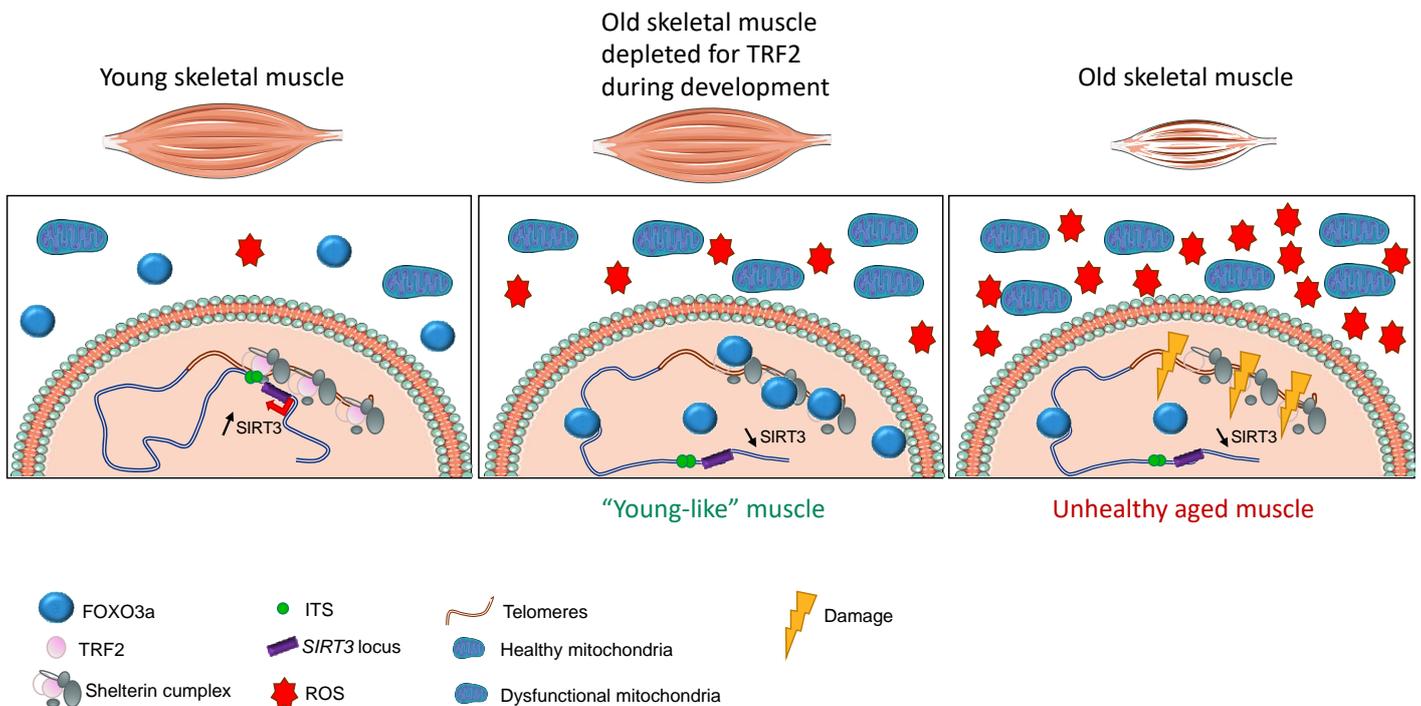


Figure 22: Telo-hormesis hypothesis model in skeletal muscle during normal aging or in TRF2 depletion conditions.



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## *Appendix*

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# Mechanisms of Ageing and Development

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## Long-lived post-mitotic cell aging: is a telomere clock at play?

Maria Sol Jacome Burbano<sup>a</sup>, Eric Gilson<sup>a, b, \*</sup>

<sup>a</sup> Université Côte d'Azur, Nice, France CNRS, Inserm, Institut for Research on Cancer and Aging, Nice (IRCAN), France

<sup>b</sup> Department of Medical Genetics, Archet 2 Hospital, FHU Oncoage, CHU of Nice, Nice, France

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### ABSTRACT

Senescence is a cellular response to stress for both dividing and post-mitotic cells. Noteworthy, long-lived post-mitotic cells (collectively named LLPMCs), which can live for decades in the organism, can exhibit a distinct type of cellular aging characterized by a progressive functional decline not associated to an overt senescence phenotype. The age-related drivers of senescence and aging in LLPMCs remain largely unknown. There is evidence that an increased production of reactive oxygen species (ROS) due to dysfunctional mitochondria, coupled with an inherent inability of cellular-degradation mechanisms to remove damaged molecules, is responsible for senescence and aging in LLPMC. Although telomeric DNA shortening, by nature linked to cell division, is generally not considered as a driver of LLPMC aging and senescence, we discuss recent reports revealing the existence of age-related telomere changes in LLPMC. These findings reveal unexpected roles for telomeres in LLPMC function and invite us to consider the hypothesis of a complex telomere clock involved in both dividing and non-dividing cell aging.

### 1. Introduction

Aging is a multifactorial process that results in progressive loss of regenerative capacity and tissue function, while simultaneously favoring development of a wide range of age-related diseases. Evidence suggests that one or more basic aging processes are pivotal drivers of these changes (López-Otín et al., 2013). There is a growing consensus that cellular senescence is one such basic aging process. Cellular senescence can be defined as a permanent non-dividing cellular state triggered by numerous types of stress and certain physiological signals leading to the activation of the tumor suppressing pathways p53/ p21<sup>WAF1</sup> and p16<sup>INK4a</sup>/pRB, as well as profound changes in cellular morphology, chromatin structure, metabolism and gene expression, including a senescence-associated secretory phenotype (SASP)—the expression and secretion of inflammatory cytokines, growth factors, proteases and other molecules (Gorgoulis et al., 2019). Cellular senescence is distinct from other non-dividing cellular states such as the quiescence that is a reversible absence of proliferation and the terminal differentiation corresponding to the acquisition of a functional specialization by cells that are often irreversibly cell-cycle arrested and therefore named post-mitotic.

An emerging paradigm stipulates that the accumulation of senescent cells is a major contributor to physiological and pathological aging and

that their elimination improves health (Anderson et al., 2019; Baker et al., 2016, 2011). Both dividing and non-dividing cells can enter into senescence in response to stress (Anderson et al., 2019; Farr et al., 2016; Jurk et al., 2014; Ohashi et al., 2018; Oubaha et al., 2016; Vettorelli et al., 2019) (Fig. 1A), although most of our knowledge on cellular senescence stems from dividing cells.

Another type of aging process at the cellular level concerns terminally differentiated long-lived post-mitotic cells (LLPMCs), such as myofibers and neurons, whose function can decline with age with no overt sign of cellular senescence. For instance, aging neurons progressively show a decline in axonal transport, focal accumulations of cytoplasmic and membrane proteins, synaptic transmission alteration, impaired synaptic plasticity, and altered calcium homeostasis (Milde et al., 2015; Vagnoni and Bullock, 2018; Vagnoni et al., 2016), which correlates with profound transcriptional changes (Mertens et al., 2015). Also, aging myofibers exhibit progressive dismantlement of neuromuscular synapses, alterations in muscle-specific transcriptional mechanisms, and the progressive loss of the most powerful fast fibers (Fry et al., 2015).

If stem cell division can be considered a rejuvenation mechanism, the situation is different for LLPMCs, which mostly rely on their intracellular capacities of repair and renewal (e.g., autophagy and mitophagy). It is generally accepted that an increase in reactive oxygen species (ROS)

\* Corresponding author at: 28 Avenue de Valombrose, 06107, Nice Cedex, IRCAN, France.  
E-mail address: [Eric.GILSON@univ-cotedazur.fr](mailto:Eric.GILSON@univ-cotedazur.fr) (E. Gilson).

production by dysfunctional mitochondria and the age-dependent decrease in the efficiency of the cellular mechanisms renewing, removing and repairing damaged molecules are responsible for the functional decline observed in LLPMCs during aging (Bua et al., 2006; Fritzen et al., 2016; Kravtsov et al., 2006; Linnane et al., 1989; Loos et al., 2017; Rubinsztein et al., 2011; Sakuma et al., 2015) (Terman et al., 2010) (Fig. 1B). However, the cascade of age-related events that triggers these changes and its link to LLPMC senescence is unclear.

Among the known clocks of aging, only the unrelenting telomeric DNA (TL) shortening is a developmental process that can act as a driver of senescence in dividing cells (Gilson and Géli, 2007). The “clock” starts ticking when telomerase is shut down during mid-embryogenesis in most cells of the human body. Therefore, telomeric DNA becomes progressively shorter upon each round of somatic replication, ultimately leading to replicative senescence. Hence, it is believed that telomeric DNA shortening, by nature linked to cell division, is not involved in LLPMC aging.

In this review, we discuss the recent reports of telomere changes during LLPMC senescence and aging and their links to mitochondrial dysfunction and cell differentiation. These findings reveal unexpected roles for telomeres in cell function and invite us to consider the hypothesis of a complex telomere clock involved in both dividing and non-dividing cell aging.

## 2. Mammalian telomere basics

Telomeres adopt peculiar chromatin conformations involving telomeric DNA looping (T-loop), specific protective factors, and non-coding RNA (named TERRA) (Giraud-Panis et al., 2010). In mammals, the shelterin protective protein complex binds to telomeric DNA to prevent unwanted DDR activation. This complex 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 (Palm and de Lange, 2008). The formation of T-loops, which is facilitated by the shelterin subunit TRF2, contributes to a blunt DDR at telomeres and to avoidance of non-homologous end-joining (NHEJ) repair, leading to end-to-end fusion and karyotype instability (Benarroch-Popivker et al., 2016; Doksan et al., 2013). Finally, the single-stranded DNA-binding trimeric complex CST (CTC1-STN1-TEN1) participates in multiple aspects of telomere replication (Giraud-Panis et al., 2010).

TL shortening drives rare progeroid syndromes such as Dyskeratosis congenita (Armanios and Blackburn, 2012) and is associated with a broad spectrum of age-related pathologies, including cardiovascular and neurodegenerative diseases, type II diabetes, pulmonary fibrosis, and arthritis (Martinez and Blasco, 2017; Turner et al., 2019). It is important to understand how age-related telomere changes contribute to these pathologies.

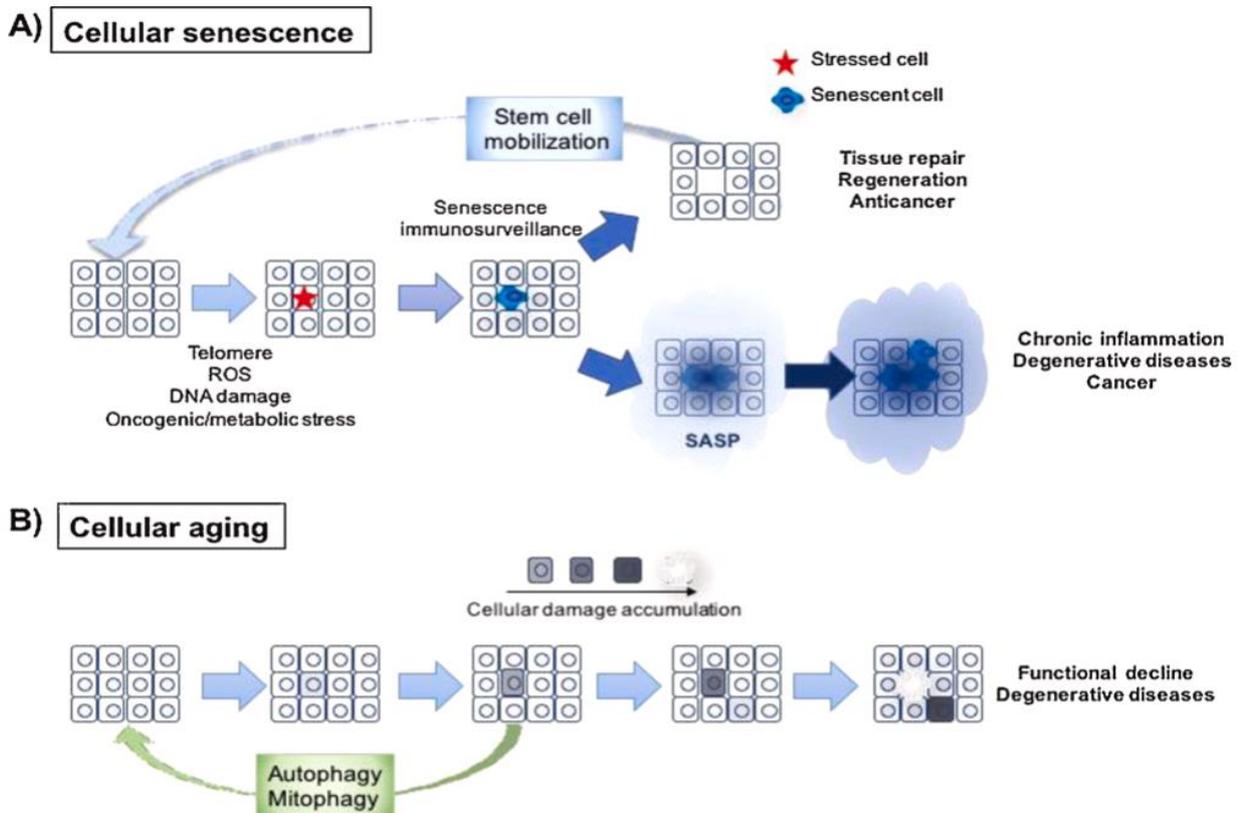


Fig. 1. Two mechanisms of cellular aging.

A) Over time, cells are exposed to different types of stress, leading to cellular senescence. The immune system is mobilized to eliminate senescent cells. In order to fill empty spaces left by eliminated senescent cells, stem cells must undergo differentiation to preserve tissue integrity. If senescent cells escape to immunosurveillance and accumulate in tissues, a chronic pro-inflammatory environment is created through SASP. B) In the course of time, the function of long-lived post-mitotic cell (LLPMC) can progressively decline without developing a clear senescence phenotype, most likely as a consequence of the progressive inability of these cells to repair and renew damaged molecules.

### 3. Telomeric DNA length changes during LLPMC aging

In agreement with the view that TL shortening is intrinsically linked to cell division and therefore mainly concerns tissues with a high level of cell renewal, TL was shown to decrease in peripheral blood leukocytes and no TL change was detected during the aging of low-proliferative tissues, which are mostly composed of LLPMCs, such as skeletal muscle (Decary et al., 2000, 1997; Hiam et al., 2020; Ponsot et al., 2008) and brain (Allsopp et al., 1995). Accordingly, telomeric DNA is longer in low as compared to highly proliferative tissues (Daniali et al., 2013).

However, this view is challenged by several recent reports of age-dependent TL shortening in low proliferative tissues. Carneiro et al. showed that TL decreases in several zebrafish tissues, including muscle, but at different attrition rates (Carneiro et al., 2016). A decrease in TL was also observed during the aging of various mouse low-proliferative cell types and tissues, such as the retina (Baek et al., 2019), brain (Ain et al., 2018), cardiomyocytes (Aix et al., 2016; Anderson et al., 2019) and oocytes (Ge et al., 2019; Yamada-Fukunaga et al., 2013). Also in human samples, TL was reported to shorten in adipocytes (Monickaraj et al., 2013, 2012) and in aging fat and skeletal muscle tissues (Daniali et al., 2013; Magi et al., 2018; Robin et al., 2020) as well as in cerebral white matter (Nakamura et al., 2007).

How to explain these seemingly disparate results regarding TL dynamics in post-mitotic tissues? First, most of these studies were performed at the whole-tissue level, making it difficult to distinguish the TL of LLPMC from those of other proliferative cells, such as leukocytes. Second, various methods are used to determine TL (telo-quantitative PCR (telo-qPCR), Terminal Restriction Fragment (TRF) analysis, slot blotting, and quantitative fluorescence *in situ* hybridization (qFISH)), which might account for experimental differences. Third, in many tissues, LLPMC can be renewed, even at a low rate, from resident progenitor or stem cells (see e.g. Spalding et al., 2013), in particular upon injury or disease (Decary et al., 1997).

Overall, if there is no definitive evidence that the TL of LLPMCs decreases over their lifetime, these various publications (summarized in Table 1) invite us to seriously consider the existence of age-regulated telomere maintenance-mechanisms specific to LLPMCs. In agreement with this view, yeast telomeres undergo a profound reorganization when the cells enter into quiescence (Guidi et al., 2015; Maestroni et al., 2017). Moreover, as described below, our knowledge of the mechanisms

of telomere maintenance suggests that different replication-independent scenarios could be the basis of TL shortening in LLPMCs (Fig. 2).

#### 3.1. Oxidative damage

It is established that an increase in oxidative stress can lead to TL shortening in dividing cells by interfering with telomere DNA replication and telomerase activity (Barnes et al., 2019). The fact that isolated skeletal muscle fibers (Ludlow et al., 2014) and adipocytes (Monickaraj et al., 2013) display telomere shortening upon oxidative stress suggests the existence of a replication-independent, oxidative stress-dependent mechanism of TL shortening. Because the main DNA damage caused by oxidative stress is 8-oxoguanosine (8-oxoG), the G-richness of telomeres makes them a preferential target for oxidative lesions (Ahmed and Lingner, 2018; Fouquerel et al., 2019, 2016; Opresko, 2005). Several replication-independent events caused by the presence of 8-oxoG at telomeres could lead to TL shortening. First, oxidative damage can disrupt TRF2 binding to telomeric DNA (Opresko, 2005), which is expected to relax T-loop formation, rendering the telomere more sensitive to degradation (see below). Second, repair by the initiation of base excision repair (BER) by the OGG1 DNA glycosylase can introduce a nick, which could result in creation of a double-strand break (DSB) by a single-strand nuclease, thus fragmenting the distal part of telomeres. An alternative possibility is the formation of 8-oxoG on both strands in the immediate subtelomeric region as a result of the presence of degenerate telomeric DNA repeats leading to nearby nicks on both strands, resulting in DSB (Fig. 2A). Interestingly, it was recently shown that telomeric DSBs caused by oxidative damage are suppressed by increased formation of three-stranded structures containing a DNA:TERRA hybrid and a displaced DNA strand (telomeric R-loop) in a TRF2-dependent manner (Tan et al., 2019). It is possible that downregulation of TRF2 during LLPMC aging (Robin et al., 2020; Wagner et al., 2017) favors DSB formation upon increased oxidative damage via a decreased rate of telomeric R-loop formation.

To determine precisely how oxidative damage can shorten TL in LLPMC requires further studies (Trajano et al., 2018).

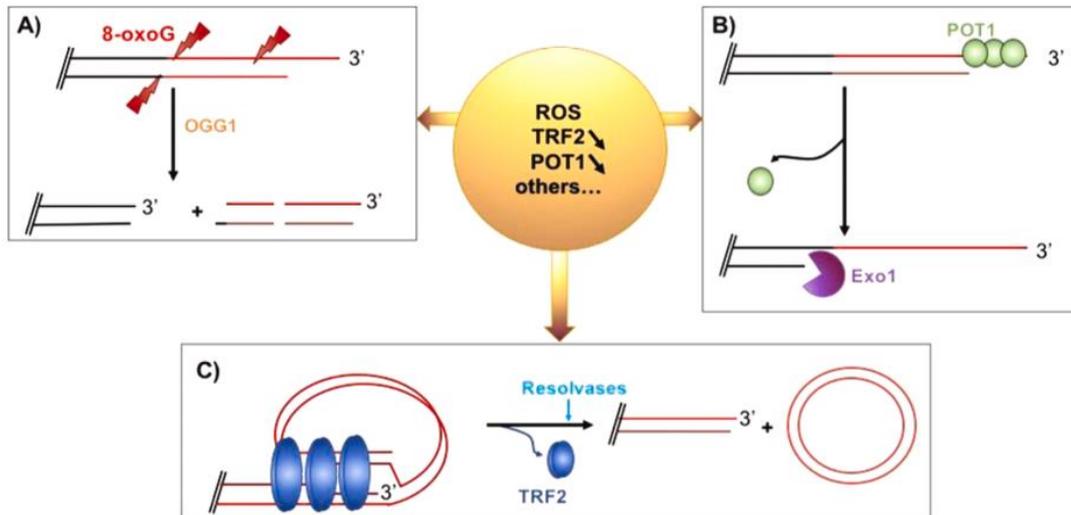
#### 3.2. Telomere degradation

One of the main functions of telomeres is to protect chromosomal

**Table 1**  
Telomere shortening during LLPMC aging.

Post-mitotic tissues/ cells	Organism	TL measurement	TL variations	References
Skeletal muscle	Zebrafish	TRF	Shortening	(Carneiro et al., 2016)
	Mouse	telo-qPCR	TL shortening in isolated muscle fibers exposed to oxidative stress TL shortening during chronological aging Decrease in short TL	(Ludlow et al., 2014) (Baek et al., 2019) (Decary et al., 1997)
	Human	TRF	TL shortening TL shortening in healthy adults	(Daniali et al., 2013) (Magi et al., 2018)
Brain	Mouse	Slot Blot telo-qPCR	TL shortening Hippocampus TL shortening during aging	(Robin et al., 2020) (Baek et al., 2019)
	Human	telo-Qpcr and qFISH	Neuron TL decreases in a cell cycle independent-manner during aging	(Ain et al., 2018)
Cardiomyocytes	Human	TRF	TL shortening in cerebral white matter in Japanese adults up to 70 years old	(Nakamura et al., 2007)
	Mouse	qFISH	TL decreases rapidly after birth triggering telomere dysfunction and cell cycle arrest Significant TL shortening in 30 vs 4 months old mice	(Aix et al., 2016) (Anderson et al., 2019)
Oocytes	Mouse	telo-qPCR and qFISH	TL shortening	(Yamada-Fukunaga et al., 2013); (Ge et al., 2019)
Retina	Mouse	telo-qPCR	Decrease in TL during mouse aging TL is shorter in subcutaneous and visceral fat of obese and type 2 diabetes patients	(Baek et al., 2019) (Monickaraj et al., 2012)
Adipocytes/ Fat	Human	telo-qPCR	TL decreases in adipocytes exposed to oxidative stress	(Monickaraj et al., 2013)
		TRF	TL shortening in subcutaneous fat	(Daniali et al., 2013)

Various methods are broadly used to measure TL: Terminal Restriction Fragment (TRF), Quantitative Fluorescence *in situ* Hybridization (qFISH), telomere quantitative Polymerase Chain Reaction (telo-qPCR) and Slot blotting.



**Fig. 2.** Possible mechanisms of TL shortening in LLPMC.

A) The OGG1 enzyme, in order to initiate the base excision repair, generates single-strand nicks at 8-oxoguanosine (8-oxoG) upon oxidative stress. These nicks can result in DSB by the action of a single-strand nuclease on the opposite strand or if they occur in close proximity in both strands. B) Telomeric factors, e.g. POT1, protect DNA extremities against nucleases. If over lifetime, the expression of these factors is downregulated, telomeric DNA can be degraded over a long distance by the action of exonucleases such as Exo1. C) TRF2 prevents resolvases to recombine the basis of T-loop leading to its excision. During aging, if TRF2 is downregulated, T-loop can be recombined leading to T-loop sized TL shortening.

DNA termini from unwanted nuclease action while controlling the formation of the limited 3'-overhang necessary for T-loop formation and telomerase action. This is based on a complex process during the cell cycle involving the shelterin subunits POT1 and TRF2, the TRF2-associated factor Apollo, as well as various types of DNA repair enzymes, nucleases and replication factors (Lam et al., 2010; Wu et al., 2010; Ye et al., 2010). In particular, the telomeres of yeast and mammalian cells are capable of preventing extensive 5' resection by the Exo1 exonuclease (Garvik et al., 1995; Ngo and Lydall, 2010; Wu et al., 2012). These mechanisms were studied in dividing cells. It is possible that some of the actors controlling telomere DNA resection become dysfunctional during LLPMC aging, promoting telomeric DNA degradation (Fig. 2B).

### 3.3. Telomere recombination

The T-loops could serve as intermediates for an intrachromatid deletion event leading to marked telomere shortening (Ancelin et al., 2002; Cesare and Reddel, 2008; Pickett et al., 2009; Wang et al., 2004). Indeed, the DNA strand-invasion formed at the basis of T-loops could promote the formation of Holiday junctions, which, if processed by a resolvase, can lead to T-loop excision and TL shortening. In normally protected telomeres, the access of resolvases to T-loop is restricted by TRF2 (Poulet et al., 2009; Saint-Léger et al., 2014; Schmutz et al., 2017). Therefore, the downregulation of TRF2 reported in LLPMCs (Robin et al., 2020; Wagner et al., 2017) or its displacement upon oxidative damage (see above) could favor T-loop excision and TL shortening (Fig. 2C).

### 4. Changes in shelterin expression during LLPMC aging

Not only the TL changes during aging but also the expression of the shelterin subunits that form the protective chromatin cap. Whether such telomeric chromatin changes are coordinated with TL shortening and their contribution to the aging phenotype are exciting questions for future studies.

The trend appears to be a general decrease in the expression of the shelterin subunits during aging in a tissue-specific manner. The first

indication of such an age-related decrease was the finding that, during replicative senescence, the expression of TRF2 is downregulated in a p53-dependant manner via degradation by siah1 (Fujita et al., 2010; Lototska et al., 2020). Whether this also occurs upon p53 activation in LLPMC is unknown. In fact, few studies have investigated shelterin expression during aging in post-mitotic organs. In zebrafish, the mRNA levels of the genes encoding the six shelterin subunits decreases with age in various tissues, including brain and muscle (Wagner et al., 2017). Whether this decrease occurs in LLPMCs is unclear because, unlike humans, zebrafish have a considerable regenerative capacity in post-mitotic organs (Chablais and Jazwinska, 2012; Wan et al., 2012). In skeletal muscle, the TRF1 protein level decreases with age in mouse (Derevyanko et al., 2017). By contrast, in human biopsies from individuals of different ages, of the six shelterin subunits, only the TRF2 protein level showed a marked decrease (Robin et al., 2020). These age-related TRF1 and TRF2 decreases appear to contribute to aging because: i) TRF1 overexpression in old mice rescued aging-related phenotypes such as cognitive and neuromuscular capacities (Derevyanko et al., 2017); ii) TRF2 inhibition in both mouse and human muscles leads to mitochondrial dysfunction and increased ROS production (Robin et al., 2020).

### 5. Telomere deprotection and senescence during LLPMC aging

A better indicator of telomere protection than TL and shelterin expression is the detection of DDR factors, such as gH2AX and 53BP1, at telomeres. This is usually assessed as the colocalization of a telomere marker (PNA recognizing telomeric DNA or antibodies directed against TRF1 or TRF2) by confocal microscopy and expressed as the number of telomere dysfunction-induced foci or TIF (Takai et al., 2003) (also named telomere-associated foci or TAF). There are reports of several examples of age-related TIF accumulation in LLPMCs and post-mitotic organs: cardiomyocytes during mouse and human aging (Anderson et al., 2019), mouse oocytes (Ge et al., 2019), human melanocytes (Victorelli et al., 2019), mouse retinal ganglion cells (Oubaha et al., 2016) and baboon hippocampus (Fumagalli et al., 2012).

The fact that deprotected telomeres can lead to LLPMC senescence is supported by several reports: i) the accumulation of TIFs in aging mouse

cardiomyocytes triggers senescence, as attested by cellular hypertrophy, p21<sup>WAF1</sup> and p16<sup>CDKN4a</sup> upregulation, heterochromatin opening, SASP and SA- $\beta$ -gal positivity (Anderson et al., 2019). ii) Mice deficient in telomerase, fourth-generation *terc*<sup>-/-</sup> mutants, displayed a p21<sup>WAF1</sup>-dependent senescence phenotype compared to the wild-type in cortical and Purkinje neurons (Jurk et al., 2012). iii) Short TL in neurons activates p53 (Ferrón et al., 2009). iv) Dysfunctional TRF2 in mature neurons activates p21<sup>WAF1</sup> expression (Zhang et al., 2007, 2006). v) Osteocytes from old mice have a larger number of TIFs and express p16<sup>INK4a</sup>, p21<sup>WAF1</sup>, and p53 and secrete SASP factors (Farr et al., 2016). vi) Aging adipocytes show hypertrophy correlating to telomere dysfunction (TL shortening and TIF) (Bouazzaoui et al., 2013; Monick-araj et al., 2013).

Interestingly, these age-related telomere deprotection and senescence events do not always correlate with TL and shelterin changes but to age-related oxidative damage, which can directly damage telomere structure (Anderson et al., 2019).

## 6. Protection-independent role of telomeres during LLPMC aging

Interestingly, TL shortening and shelterin downregulation in LLPMC do not always lead to telomere deprotection and senescence as in TRF2-compromised human myofiber (Robin et al., 2020) or in skeletal muscle of old baboons (Jeyapalan et al., 2007). Whether telomere changes directly affect aging-related processes other than chromosome end protection has important implications for the hierarchical relationships among age-related processes.

Much data indicate that telomeres directly regulate oxidative metabolism: i) Antioxidant proteins are specifically associated with telomeres (Aeby et al., 2016); ii) The catalytic subunit of telomerase TERT localizes in mitochondria, where it protects metabolism and DNA maintenance (Ahmed and Lingner, 2018; Miwa et al., 2016) in neurons upon oxidative stress (Spilsbury et al., 2015); iii) Telomerase RNA can be processed within the mitochondria, giving it a role in mitochondrial function (Cheng et al., 2018); iv) The shelterin subunits (with the exception of TRF1) have mitochondrial protective functions (Kim et al., 2017); v) The shelterin subunit TIN2 can be localized in mitochondria, where it induces mitochondrial dysfunction and ROS production (Chen et al., 2012); vi) Telomere dysfunction activates p53, which in turn binds and represses PGC-1 $\alpha$  and  $\beta$  promoters, leading to mitochondrial dysfunction (Chang et al., 2016; Sahin et al., 2011); vi) TRF2 regulates the expression of the subtelomeric mitochondrial Sirtuin SIRT3, leading to mitochondrial dysfunction in skeletal muscle (Robin et al., 2020). Some of the mechanisms connecting telomere structure to mitochondrial integrity in LLPMCs appear to be independent of telomere protection, e.g., a shuttle between telomere and mitochondria (Spilsbury et al., 2015) and telomere-dependent transcriptional regulation (Robin et al., 2020).

The transcriptional regulatory role of telomere factors in LLPMC concerns not only SIRT3 but also several genes involved in LLPMC differentiation (Ye et al., 2014): i) TRF2 inhibits the activity of repressor element-1-silencing transcription factor (REST), a key repressor of neuronal genes and, consequently, favors neurogenesis (Ovando-Roche et al., 2014; Zhang et al., 2011, 2008). ii) A non-telomeric isoform of TRF2 (TRF2-S) interacts directly with mRNAs to facilitate their axonal delivery (Zhang et al., 2015). iii) TRF2 binds at several telomeric interstitial sequences (ITS) throughout the genome, where it activates the expression of neuronal genes; for instance, several involved in heparan sulfate proteoglycan biosynthesis, vesicular transport, ion transport and axon growth (Biroccio et al., 2013; Cherfils-Vicini et al., 2019; Simonet et al., 2011; Ye et al., 2014). In skeletal muscle cells from patients with facioscapulohumeral muscular dystrophy, the expression of the subtelomeric *SORBS2* gene is upregulated as compared to control cells with longer telomeres (Robin et al., 2020, 2015).

Overall, the capacity of telomere to modulate gene transcription in subtelomeric regions by a process named Telomere Position Effect (TPE)

but also genome-wide by a still elusive mechanism is likely to play an important role during LLPMC aging (Ye et al., 2014).

## 7. A telomere aging clock for LLPMC?

The current model is that telomere dynamics, intrinsically linked to cell division, cannot act as a clock for LLPMC aging. In this review, we presented several recent findings that challenge this view by revealing that various types of telomere change occur during LLPMC aging. Moreover, there is evidence that these changes contribute to pathologic myocardium (Chang et al., 2016; Terai et al., 2013), skeletal muscle and fat aging (Carneiro et al., 2016; Daniali et al., 2013) and to neurodegeneration (Mamdani et al., 2016; Zglinicki, 2002).

Because the telomere changes programmed during LLPMC aging do not result from intrinsic mechanisms linked to successive cell division, what is the nature of the aging clock that orchestrates them? For instance, the increased oxidative stress during aging can have a direct impact on telomere structure (Anderson et al., 2019). A tempting hypothesis would link the telomere changes of aging LLPMC to the accumulation of senescent cells in the same or other organs, which could send extracellular signals triggering telomere changes in LLPMCs. This is supported by the recent finding that senescent melanocytes send paracrine signals to keratinocytes (Victorelli et al., 2019).

Importantly, the consequences of telomere changes in LLPMCs are not restricted to DDR activation and can impact cell function independently of senescence by regulating cell type transcription and by directly impacting mitochondrial function. For instance, the downregulation of TRF2 during skeletal muscle aging represses the expression of the subtelomeric gene SIRT3 required for mitochondrial integrity (Robin et al., 2020). These findings can serve as a paradigm of a new type of cellular aging, distinct from the classical senescence. In particular, it would be helpful to determine whether the other cellular processes that decline during LLPMC aging, such as autophagy, are also directly impacted by telomere changes.

Thus, there is a need to better understand in LLPMCs the mechanisms which can shorten TL, the cellular consequences of TL shortening, the regulation in space and time of the expression of the telomere-associated factors as well as their multiple functions. Altogether these understandings will greatly clarify the role played by telomeres in the sequence of events leading to LLPMC aging.

Another valuable source of knowledge should come from studies on the role of telomeres in the aging of organisms that are mostly composed of post-mitotic somatic cells such as the worm *Caenorhabditis elegans* or the fly *Drosophila melanogaster*. Strikingly, the molecular organization of telomeres in these organisms appears quite different from the one in vertebrates with, notably, an absence of TRF1 and TRF2 orthologs (Raffa et al., 2013; Raices et al., 2008) and, for the fly, an absence of telomerase as well as of telomeric DNA simple repeats (Casacuberta, 2017). Only a few and sometimes seemingly contradictory reports were published on the role of telomere changes on the aging of these organisms (see for instance Joeng et al., 2004 versus Raices et al., 2005). Interestingly, the fly telomeres are known to exert TPE (Cryderman, 1999), suggesting that telomere modifications during fly LLPMC aging could affect subtelomeric gene expression, as it was observed in humans and yeast (Ye et al., 2014).

We bet that more and more studies on telomeres will be focused on their role in non-dividing cells leading to unified models of the aging clock.

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Review

# The Power of Stress: The Telo-Hormesis Hypothesis

Maria Sol Jacome Burbano <sup>1</sup> and Eric Gilson <sup>1,2,\*</sup>

<sup>1</sup> Institut for Research on Cancer and Aging, Université Côte d'Azur, CNRS, Inserm, Nice (IRCAN), 06107 Nice, France; maria-sol.jacome-burbano@univ-cotedazur.fr

<sup>2</sup> Department of Medical Genetics, Archet 2 Hospital, FHU Oncoage, CHU of Nice, 06107 Nice, France

\* Correspondence: Eric.GILSON@univ-cotedazur.fr

**Abstract:** Adaptive response to stress is a strategy conserved across evolution to promote survival. In this context, the groundbreaking findings of Miroslav Radman on the adaptive value of changing mutation rates opened new avenues in our understanding of stress response. Inspired by this work, we explore here the putative beneficial effects of changing the ends of eukaryotic chromosomes, the telomeres, in response to stress. We first summarize basic principles in telomere biology and then describe how various types of stress can alter telomere structure and functions. Finally, we discuss the hypothesis of stress-induced telomere signaling with hormetic effects.

**Keywords:** telomeres; hormesis; stress response; adaptation



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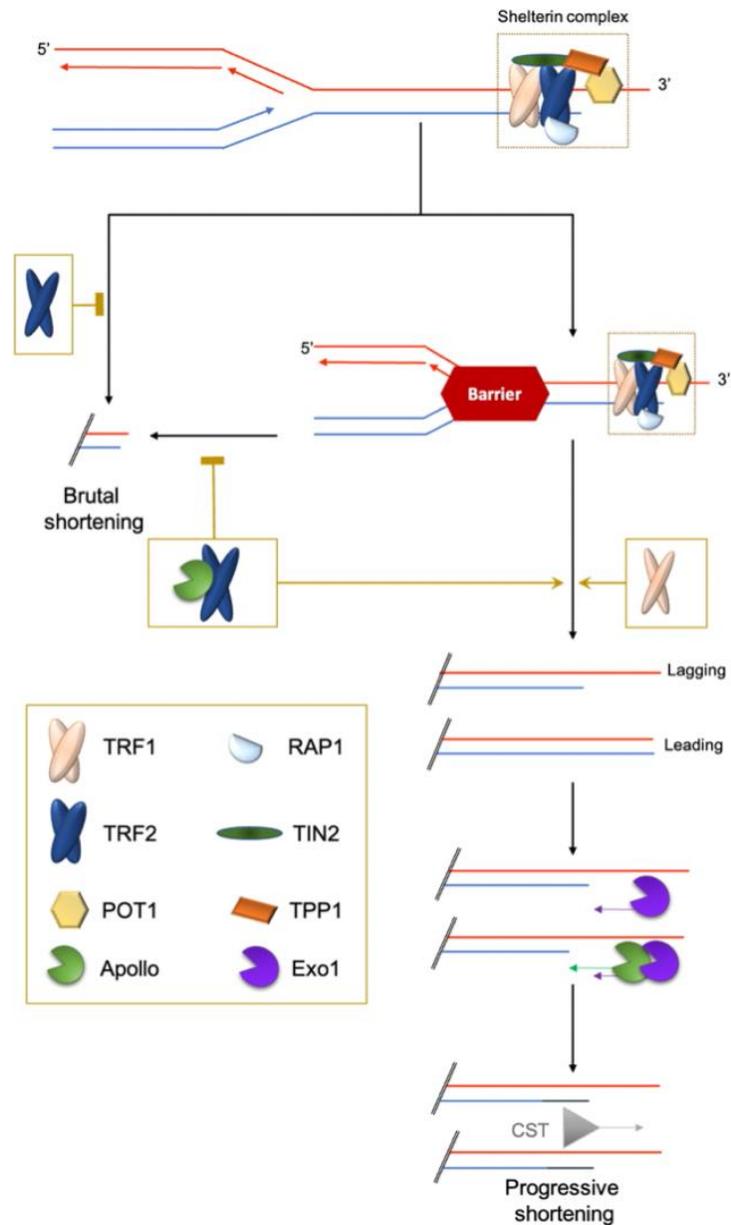
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## 1. Introduction

To survive and reproduce, living organisms must maintain homeostasis both in unchallenged (normal) and challenged (stressful) contexts. This requires the evolution of powerful stress response mechanisms adapted to a particular ecosystem and to regular environmental fluctuations. Thus, these mechanisms may be very diverse within the tree of life. The pioneering work of Miroslav Radman on the stress response in bacteria demonstrated the rapid and adaptive value of changing mutation rates for rapid evolution (the mutator effect) [1,2]. In other words, to facilitate the survival of a species, whether it be to respond to a replication blockade or to a stressful environment, it is better to rapidly evolve by generating more mutations, some being possibly lethal, than to die immediately. We believe that this principle applies to the complex dynamics of telomeres in eukaryotes, which become altered in response to stress. Contrary to the general view that these telomere changes are deleterious and inspired by Miroslav Radman's concepts, we discuss here the possibility that telomere modification in response to stress was selected for during evolution to respond to contextual fluctuations and physiological demands.

## 2. Telomeres: Basic Molecular Mechanisms in an Evolutionary Context

Telomeres are nucleoprotein structures at the ends of linear chromosomes that are required to maintain genome stability by providing a terminal cap [3]. Their function and replication require a complex interplay between specialized and general factors involved in replication and the DNA damage response. Strikingly, their structures change during an organism's lifetime, both as a result of developmentally regulated programs and external factors. This, together with significant variability among organisms in telomere structure and regulation, raises several fundamental questions regarding telomere evolution and their contribution to development, life history, aging, disease susceptibility and the stress response. To understand fully the "telomere logic" in the stress response, it is necessary to understand the molecular challenges that these structures must face and the evolutionary solutions adopted by different organisms.



**Figure 1.** Brutal and progressive telomere DNA shortening. Telomere replication at the leading or lagging strands is not similarly regulated. At the C'-rich lagging strand, due to the end-replication problem, the parental strand is not completely replicated, triggering a 3'-overhang. At the leading strand, there is a blunt end or 5' protruding that is eroded in a manner coupled to replication by the TRF2-dependent recruitment of the 5'-exonuclease Apollo. Next, at both strands, the Exo1 exonuclease prolongs the 3'-overhang. Finally, POT1 recruits the CST complex (CTC1-STN1-TEN1) to replenish the 5'-strands. The brutal appearance of critically short telomeres that cannot be explained by the progressive telomere shortening was first observed in yeast [11]. They are likely to stem from intra-telomeric recombination [12,13] or the collapse of DNA replication forks. Indeed, it is known that telomeric repeats can form recombinogenic t-loops [14,15] and are difficult to replicate [16–18].

## 2.2. Challenge 2: End Replication Problem

The full replication of the terminal DNA cannot be achieved by conventional replication machinery [19–22]. On the lagging telomeres, the G-tail formation may result simply from the removal of the last RNA primer. On the leading telomeres, the end product is either blunt or 5′ protruding. Since in many organisms a G-tail can be detected at both daughter telomeres, a 5′ resection activity must take place to convert it into a 3′ overhang. Indeed, coupled with replication, TRF2 targets the leading telomere with the 5′-exonuclease Apollo, which initiates the 5′ resection. At the lagging strand, POT1 inhibits Apollo resection [23]. At both ends, the Exo1 nuclease elongates the 3′ overhangs by pursuing a 5′ resection. It remains unknown how this resection is controlled. Finally, POT1 recruits the single-stranded DNA-binding trimeric complex CST (CTC1-STN1-TEN1) to refill the 5′-strands [23] (Figure 1).

There are several processes that compensate for this unavoidable shortening. One such mechanism relies on telomerase, a specialized reverse transcriptase with an RNA template that elongates the G-rich strand using the 3′ overhang as a substrate. Telomerase is composed of two subunits: TERT, the telomerase reverse transcriptase, and TERC, the telomerase RNA template. Using a single telomere length system and monitoring telomere DNA length (TL) evolution in budding yeast, it was shown that telomerase activity is reduced by over elongated telomeres: excess of Rap1p at the long yeast telomeres triggers a progressive *cis*-inhibition of telomerase [24]. This indicates that it is important to keep TL within a regulated range of size.

In mammalian species with large body mass, including humans and some birds, the level of telomerase expression is much higher in germ, stem and progenitor cells than in somatic differentiated cells. In some species, such as laboratory mice, telomerase expression is less repressed and more evenly distributed within cell types than in humans, but still higher in the germ and stem cell compartments [25–27]. Importantly, the amount of telomerase present in adult somatic stem cells and progenitors is generally not enough to replenish telomeric DNA fully when these cells divide to regenerate the corresponding tissue or to respond to external stimuli.

In many vertebrates, TL is subjected to a progressive erosion cadenced by cell divisions and the residual level of telomerase activity. There are also exceptions to this scheme; for instance, in the long-lived bat species *Myotis myotis*, mean TL does not shorten with age but changes with the environment [28]. In fact, the rate of telomere shortening in blood cells during development and aging is not strictly related to the level of telomerase activity [6,29]. This suggests that telomerase may have functions other than regulating telomere length and that TL might be regulated independently of telomerase. Indeed, the mean TL is the net result of shortening and elongation rates, rendering it difficult to interpret TL changes only in terms of telomerase-dependent elongation [24]. One interesting hypothesis is that the rate of telomere DNA shortening, but not the absolute length, controls the aging process [29]. It is not yet known whether there exist developmentally regulated mechanisms, independently of telomerase activity, that modulate the rate of TL shortening.

Interestingly, eukaryotic cells are able to use a telomerase-independent mechanism, ALT (alternative lengthening of telomeres), which is based on homologous recombination [30]. It is activated in a number of human tumors, in human cells immortalized in culture, and also in normal somatic tissues [31]. In plants, the ALT mechanism is activated upon telomerase dysfunction and possibly also during the earliest stages of normal plant development [32]. At the organismal level, ALT was suggested by Gomes and colleagues [6] for koala and wombat telomeres, based on the presence of long and interrupted telomere repeats and the absence of telomerase expression. However, it is not known whether these ALT-like telomeres use the same process of maintenance as the ALT + cancer cells. Along this line, a recent work that revealed the existence of an ALT-like mechanism in *Terc* knock-out (KO) mouse stem cells, with a unique non-telomeric sequence (mTALT) [33]. This process might be related to the one used in wombat. Hence, ALT or ALT-like mechanisms could give an advantage in the context of species with no telomerase. In order to avoid

cancer development, we can imagine ALT process is finely regulated in species using only this pathway to preserve their telomeres.

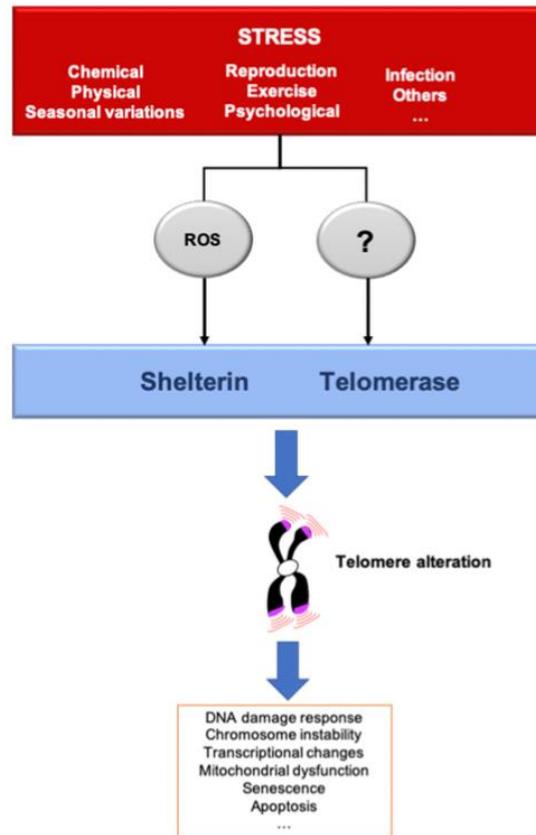
### 2.3. Challenge 3: The Hard-to-Replicate Problem

In addition to the end-replication problem, the progression of the replication fork through telomeric chromatin is difficult and may result in fork collapses leading to the loss of a large part of the telomeric DNA. This is prevented by specific mechanisms involving the shelterin subunits TRF1 and TRF2: TRF1 manages classical stalled forks leading to ATR activation and TRF2 manages topologically-constrained stalled forks [18,34] (Figure 1).

Interestingly, the function of TRF2 in this process requires the same factor, Apollo, as the one favoring the progressive shortening of the leading strand [18]. Inhibiting TRF2-Apollo interaction is associated with severe progeroid syndrome in humans [35]. The long-lived giant tortoises *Chelonoidis abingdonii* and *Aldabrachelys gigantea* carry an Apollo variation within the TRF2 binding domain that is not conserved in other reptiles, fishes or mammals and, thus, could contribute to the extreme longevity of these organisms [36]. Altogether, these findings suggest an evolutionary advantage, at least in some organisms, to control progressive shortening due to the end replication problem. This hypothesis seems at odds with the wealth of studies showing that TL is positively associated with improved healthspan and lifespan in vertebrates [37,38]. Usually, short telomeres are associated with chronological aging, cardiovascular and age-related diseases and poor prognosis [39]. However, long telomeres are not as beneficial as was initially thought: several studies have shown an association between long telomeres and some types of cancer. Actually, in a genome-wide association study, nine cancers out of 35 (including glioma, neuroblastoma and endometrial cancer) were associated with increased TL [40]. To understand the “raison d’être” of these different telomere trajectories between organisms and individuals, and their consequences for health, we need to understand more fully the complex interplay between genetic and environmental determinants regulating the response of telomeres to stress during development and aging.

### 3. Telomere Response to Stress

A stressor can be defined as a factor that leads to a rupture in homeostasis, whether it be at the cellular, systemic or organismal level. Thus, the environment can be defined as the combination of stressful situations to which individuals are exposed during their lifetime, including lifestyle, climate, pollutants, pathogens and social relationships. Organisms have evolved mechanisms to cope with their environments and predictable environmental fluctuations. Abnormal exposure to stressors is a well-known risk of age-related disease and premature aging [41]. Different types of stressors also lead to an acceleration of TL shortening, leading to critically short telomeres and cellular senescence, a mechanism that could explain the link between stress and aging. To give an example, the telomere shortening rate can increase by roughly five times in fibroblast cultured in hyperoxia compared to normoxia [42]. The rapidity by which these changes are signaled to the cells is not well understood. One can imagine two non-mutually exclusive modes of signaling. On one hand, shortened telomeres are phenotypically “silent” until they reach a critically short length that triggers senescence. On the other hand, even small changes in telomere length variation can have an impact, even before cells reach senescence. In the following paragraphs, we discuss how various stresses can modulate telomere structure and function (Figure 2).



**Figure 2.** Stressors affect telomere homeostasis. Several stresses such as changes in pH or temperature, exercise, irradiation, pathogens infection, reproduction/breeding efforts, exposure to chemicals or psychological pressure, among others, can affect telomere integrity. The most described mechanisms induced by several of these stressors include telomerase inhibition and an increase of ROS, that preferentially oxidizes the telomeric DNA, a process that we dubbed teloxidation [43]. Upon challenge, telomeric alterations induce the DNA damage response activation (DDR), senescence, transcriptional changes and mitochondrial dysfunction.

### 3.1. Inflammation

During aging there is an increase of circulating inflammatory markers and a decrease in immunological capacities, leading to a systemic chronic inflammatory state, also known as inflammaging [44]. Chronic inflammation is often associated with diseases such as rheumatoid arthritis or chronic obstructive pulmonary disease (COPD). Noteworthy, liver TL attrition is significantly exacerbated in cirrhosis or chronic hepatic livers [45]. In mouse, repeated *Salmonella* exposure decreases TL in leukocytes, but not in hepatocytes or splenocytes [46]. If these results indicate that inflammation can drive TL shortening, the reverse can also occur and a wealth of data present telomere dysfunctions as drivers of chronic inflammation (see e.g., [47].) In this review, we will focus on the processes of telomere shortening triggered by inflammation.

Inflammation-induced TL shortening may be due to the inhibition of telomerase (Figure 2). In support of this hypothesis, TNF $\alpha$  treatment shortens telomeres via ATF7-dependent telomerase disruption [48]. However, infection is capable to trigger accelerated TL attrition in telomerase-deficient mice [49], revealing the existence of telomerase-independent mechanisms linking inflammation to TL shortening. These mechanisms

might include the production of ROS during inflammation; for example, via secretion by immune cells such as neutrophils [50]. Indeed, the rate of telomere shortening increases under oxidative stress, and the G-richness of telomeric DNA renders it highly susceptible to oxidization, leading to the accumulation of 8-oxo-guanine. This disrupts the binding of telomere protective factors and can induce DNA breaks and prevent extension by telomerase (Figure 2) [51,52].

### 3.2. Life Factors

Under psychological stress, the Hypothalamic-Pituitary-Adrenal (HPA) axis secretes high levels of glucocorticoids (GC): cortisol and corticosterone. Their release allows an immediate response to stress, but chronic exposure may be detrimental [53]. GC block certain non-essential processes to store energy during periods of stress [54] but are also associated with behavioral response. There are several examples of telomere changes due to psychological stress. For instance, TL shortens in children who experience a violent childhood [55]. Acute psychological stress also increases telomerase activity for short periods, correlated with cortisol/corticosterone levels in humans and rats [56,57]. In fact, glucocorticoid accumulation seems to modify telomere homeostasis, including TL, although the mechanisms involved remain elusive [58]. One possible mechanism links psychological stress and oxidative stress, which is one of the consequences of GC activity [59]. Indeed, GC regulating genes and downstream pathways are involved in ROS production (Figure 2) [54] and/or mitochondrial metabolism [60]. Highly stressed women have shorter TL, decreased telomerase activity and increased oxidative stress signatures [61]. Thus, oxidative stress may be a mechanism for the effects of psychological stress and GC on telomeres, as it is for inflammation.

Other daily way of life decisions, such as diet, smoking and physical activity have been linked to telomeric changes. To give an example, the Mediterranean diet has been highlighted by its anti-aging capacities and cohort studies suggest that it decelerates telomere shortening in humans [62]. Indeed, individuals suggested to a diet rich in fibers, vitamins and unsaturated fatty acids tend to have longer telomeres and lower oxidative stress. Caffein consumption, another strong antioxidant agent, has also been correlated with longer leukocytes TL [63]. Food can modulate TL but also telomeric protein state. For example, Chinese obese children had shorter TL but higher TERT promoter methylation [64]. Additional to food intake, several studies have analyzed an association between exercise and telomere length. To our knowledge, most of the published results show a positive correlation between TL and training, as compared to sedentary people [65]. Nevertheless, physical exercise might also lead to a certain level of telomere dysfunction: occasional intense exercise would tend to produce an excessive amount of ROS leading to higher DNA damage, and thus, telomere damage while low intensity but regular activity would lead to lower oxidative stress and better adaptation [66]. Physical exercise can also modulate telomere structure and function by increasing TERRA transcription [67].

### 3.3. Chemical Stress

Exposure to chemical stressors can greatly modulate the health of organisms. The stressors can be toxic/genotoxic compounds or pesticides and airborne pollutants. Several studies report an association between chemical exposure (benzene, polycyclic aromatic hydrocarbons, ethanol, etc.) and telomere attrition in human cells [68,69]. Ethanol triggers TL shortening in human fibroblasts, whereas in budding yeast it has a TL lengthening effect [69,70]. In humans, the shortening effect of ethanol appears to be mediated by oxidative stress (Figure 2), mainly induced by alcohol dehydrogenase [69,71]. Nevertheless, zebra finches exposed to trace metal elements exhibit decreased TL with no change in oxidative stress [72]. Overall, the effect of chemical stress on TL varies depending upon the nature of the chemical, the mode and duration of exposure and the organism exposed. The mechanisms appear to be quite diverse, but in at least some cases are mediated by oxidative stress.

### 3.4. Physical Stress

Physical stress is another source of external tension for telomeres. It is well known that genotoxic insult triggers persistent DNA damage at telomeres [73,74]. A particular case is UV irradiation, whose main photoproduct is cyclobutane pyrimidine dimers formed between two consecutive pyrimidine bases, which are often present in telomeric repeat DNA sequences. Thus, telomeres are a preferential target for UV-induced DNA damage [73]. If the telomeric photoproducts at telomeres remain unrepaired they can interfere with shelterin DNA binding, telomeric DNA replication and transcription, and thereby lead to TL shortening and uncapping [75]. Indeed, UV exposure is associated with accelerated TL shortening [76–78] and prevents TRF1 binding [79]. However, another study did not observe increased TL shortening in human fibroblasts irradiated with UVB rays [73]. It is possible that the ability of telomeres to repair UV-induced photoproducts varies among cell types and organisms, which could be interpreted as an adaptive strategy of telomeres to repair UV lesions only when needed.

Recently it was shown in astronaut leukocytes that long-duration spaceflights (6–12 months) lengthened telomeres, but that telomeres were rapidly shortened to a pre-spaceflight length within a few days after the astronauts returned to Earth [80,81]. The authors posited that these effects were caused by space radiation. Puzzlingly, this telomere elongation might not be due to the canonical telomerase activity, which decreases during spaceflight, but to an increased rate of DNA damage and recombination that could favor ALT mechanisms. Indeed, astronaut cells exhibit elevated DNA damage and increased mitochondrial stress and ROS production. One interesting hypothesis is that oxidative stress activates the ALT pathway by triggering replication stress [82] and relocating telomerase to mitochondria (Figure 2) [83].

Exposure to elevated temperatures is another environmental stress that could affect telomere dynamics. Indeed, in *Saccharomyces cerevisiae*, incubation at high temperatures shortens telomeres [70]. Human cells that undergo heat shock accumulate TERRA and disrupt TRF2 from telomeres [84,85].

Finally, pH variation may be a cause of stress within an ecosystem. TL was observed to be reduced in HeLa cells cultured at low pH, but the heterogeneity of individual TL was narrow [86]. The authors proposed that telomerase elongates short telomeres under low pH, while long telomeres are more strenuously eroded.

### 4. Telomere and Life-History Trade-Off

Individual lifespan is influenced by life-history traits such as time of breeding, lifespan and the size of offspring. These life decisions are often highly energy-consuming and are known as life history tradeoffs. Among these trade-offs, the disposable soma theory states that the soma is just a vehicle to preserve the “immortal” germline. An important current debate on telomere biology is to determine whether developmentally programmed telomere change can contribute to life-history. For instance, in several species (e.g., human, rhesus monkey, zebra finch, dog) telomerase is inhibited in somatic cells after embryonic development but its expression is preserved in the germline [6,86,87], suggesting a trade-off between the cost of telomere maintenance in the soma and the need to allocate resource toward reproduction.

A high level of somatic telomerase downregulation appears to have been selected for in long-lived, large-body-mass animals, including mammals [6,88,89]. Accordingly, long-lived mammals have shorter telomeres (14 kb in African elephants or 5–15 kb in humans) than short-lived mammals (over 40 kb in the laboratory mouse). These observations can be interpreted with caution due to the extraordinary diversity in life-history trajectories in the tree of life. Thus, it is important to tackle this question by comparing related species but with different environment and life-history traits. For instance, mouse strains recently derived from the wild have shorter TL than those already established in laboratories. Overall, among these various mouse strains, no correlation exists between TL and lifespan [87]. This was also observed in rats [88]. Thus, to harbor shorter TL appears

more related to the wild conditions than to regulate lifespan. In agreement with this view, a recent analysis showed that wild mammals have shorter TL than those that domesticated one [89]. Overall, these studies suggest that possessing short telomeres is advantageous in a wild habitat with limited resources.

Another interesting link between telomere and life history is the correlation between the rate of TL somatic shortening and different strategies of growth and reproduction. Several organisms, including captive and wild metazoans, exhibit a negative correlation between reproduction and TL [90–96]. For instance, there is evidence that longer TLs triggered a reduced fertility but no effect on lifespan in *Drosophila melanogaster* [97]. In birds, earlier breeding is associated with more successful reproduction but shorter TL. One study found that in the common tern *Sterna hirundo*, brood size is inversely correlated with parental blood TL [92]. The authors reported that each fledgling in the brood shortened parental telomeres to the same extent as four years of normal aging. Telomere shortening appears to be one of the costs of reproduction and a negative correlation between reproduction and longevity has been reported in numerous species (e.g., humans, *Caenorhabditis elegans*) [98,99]. Overall, these findings suggest that TL shortening contributes to accelerated aging as a consequence of reproductive activity. However, there are exceptions to this telomere-mediated reproduction trade-off. For instance, in the eusocial insect *Bombis terrestris*, bumblebee queens live 4 to 6 times longer than workers and exhibit increased telomerase activity in fat bodies [100].

In many species, reproductive success does not rely solely on producing offspring but also on supporting their survival through early life care and breeding. In the Seychelles warbler (*Acrocephalus sechellensis*), a cooperatively breeding species, elderly dominant females that have subordinate helpers exhibit reduced telomere attrition and senescence markers, as well as improved probability of survival [101]. This may be because the dominant females expended less breeding effort due to helper incubation attendance and support for chick provisioning. In the common tern, males take care of feeding chicks and their telomere attrition associated with breeding is greater than that of females [92]. Finally, TL shortening is greater in human mothers with chronically ill children [61], showing that care-related stress is directly associated with telomere dynamics. These results suggest that not only reproduction must be considered in telomere attrition cost but also caregiving for offspring.

The mechanisms explaining these various relations between telomere variations and life-history traits remain largely unknown. For instance, how the energy saved by somatic telomere shortening could be reallocated to reproduction? One challenge in experimentally testing this role of telomeres in the germline/soma tradeoff is the difficulty of quantitatively comparing the energy cost to maintain telomere length and function with breeding function [102]. Telomere initial length and telomere attrition rate vary among species. For example, humans have short telomeres (5 to 15 kb) and their mean lifespan is estimated to be 72.6 years. Therefore, telomeres represent only 0.00016 to 0.0048% of the human genome. If we take into account the telomere shortening rates published in other reports, the base pair loss per year in leukocytes represents only 0.00000226% of human DNA. Laboratory mice have short lives (2 years) but long telomeres (50 kb); they lose just 0.000123% of their genomes every year despite their relatively high telomere attrition rate (7000 bp) [29]. Despite a relatively high level of somatic telomerase activity in mice, they have a rapid telomere erosion rate. Consequently, it is difficult to imagine that the energy saved by not preserving somatic TL could be enough to significantly offset the costs of growth and breeding. In support of this hypothesis, male Cory's shearwaters with larger offspring displayed shorter TL, whereas highly reproductive females had long telomeres [103]. Importantly, eggs were removed from nests to eliminate parental nesting/breeding effort, but TL values were compared with non-manipulated birds.

In summary, our knowledge of the role of telomeres in different life-history trajectories remains limited and puzzling. In particular, it remains difficult to ascertain whether somatic TL shortening is an evolutionary strategy to allocate resources to reproduction or merely a

consequence of breeding and care. It is also possible that the TL shortening cost linked to reproduction serves other functions in life history. If this is the case, it raises the question of whether TL shortening is a cost or a benefit.

### 5. The Telomere Hormesis Hypothesis

If the germ/soma trade-off cannot really explain the evolutionarily conserved process of telomere shortening during the ontogeny and in response to stress, what is its “raison d’être”? Is there any advantage to shorten telomeres upon exposure to stress or upon reproduction? Here, we address this question by using the concept of hormesis that can be defined as a dose response process in which low and high doses of stressors have opposite effects [104]. Applied to telomeres, a hormetic effect could be beneficial in, a priori, two types of situations: i) a low level of dysfunction (shortening or chromatin changes) leading to a beneficial effect, for instance by triggering a redox signaling or changing gene expression, while a higher dose (critical shortening or profound chromatin destruction) would trigger senescence, chromosomal instability and aging; ii) a low level of senescence, triggered by telomere dysfunction, being beneficial (for instance to limit the proliferation of pre-cancerous cells) while too many senescent cells would favor aging and even cancer. Pioneer data from Seiichi Yokoo and colleagues, suggested telo-hormesis effect on human keratinocytes exposed to low doses of hydrogen peroxide [105]. H<sub>2</sub>O<sub>2</sub> effects regarding the number of population doublings, ROS amounts and TL were better than those observed on keratinocytes exposed to antioxidant agents. Later, this concept was discussed in a study on *cdc13-1* dysfunctional budding yeast, which found that pre-exposure to a low dose of telomere stress led to more resilient yeast under acute stress [106]. A recent report showed that TL was shorter in mammals living in the wild than in domesticated species [89], suggesting that possessing short telomeres in a wild habitat confers a selective advantage (Figure 3). Since a wild habitat forces organisms to cope with both resource scarcity and fluctuating environments, we can postulate that short telomeres may improve survival and fitness during exposure to stress.

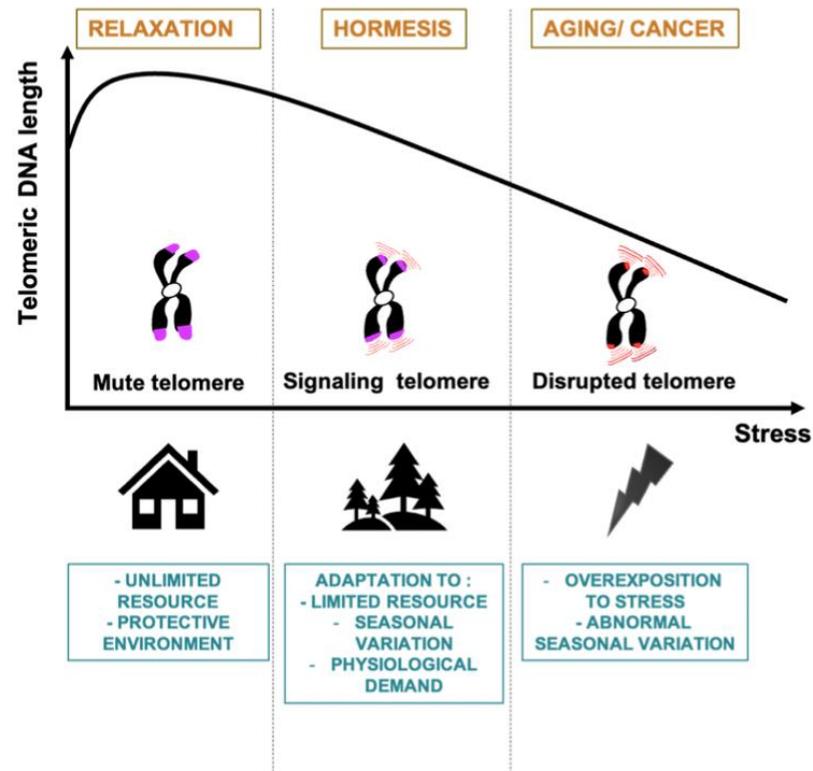
#### 5.1. Telomere Changes to Prevent Tumor Formation

An experimentally documented advantage of telomere shortening is preventing tumor progression by sending pre-cancerous cells with uncontrolled cell division to senescence. Indeed, oncogenic stress can lead to telomere dysfunction and senescence [107,108]. Moreover, short telomeres are frequently observed in long-lived, large-body-mass species, which are at relatively high risk of developing tumors [6,89,92,109]. Moreover, in Genome-Wide Association Studies (GWAS), mutations in genes favoring telomere elongation have been associated with increased risk of lung cancer and melanoma [110]. Accordingly, protection against melanoma development might be a driver of short TL in European populations compared to Sub-Saharan Africans [111]. These findings raise the question of whether UV damage may distinctly affect DNA from different populations. To our knowledge, some studies have compared the SNPs within DNA damage repair genes among different ethnic [112]. However, whether there is an ethnic difference between UV-induced DNA damage repair in European and Sub-Saharan populations, has not been studied yet.

Corroborating these observations, mice with dysfunctional telomerase are protected from chemical-induced skin cancer [109], while mice overexpressing telomerase develop large tumors relatively quickly [113]. Furthermore, crossing late generations of telomerase-deficient mice with strains prone to developing cancer reduces tumor frequency and increases survival [114–118]. For instance, in a lymphoma mouse model, short telomeres triggered a p53-mediated senescence process that limits tumorigenesis [116].

One can imagine that, if telomere attrition is an adaptive tumor-suppressor strategy, individuals with long telomeres should be more prone to cancer. However, Muñoz-Lorente et al. found that a mouse model carrying hyperlong telomeres, but with normal telomerase expression, exhibited lower cancer incidence and longer lifespan [119]. Thus, telomere-related changes other than TL shortening can interfere with cancer formation. For instance,

crossing telomerase-dysfunctional mice with colon carcinoma-prone mice (*Apc<sup>Min</sup>*) revealed that short telomeres can promote tumor initiation by inducing chromosome instability, but also prevent tumor progression [115]. In addition to promoting chromosome instability and tumor initiation, telomere dysfunction in stromal cells can favor carcinoma development by creating a pro-inflammatory environment [120,121].



**Figure 3.** The telo-hormesis hypothesis. Under exposure to predicted stresses (such as regular seasonal fluctuations and limited resource) or normal/physiological demands, telomere integrity might be affected in order to trigger a hormetic signal through transcriptional changes and mito-hormesis. To stop an early oncogenic event, this hormetic signaling can also result in cellular senescence. Under unexpected, overexposure and acute stresses, telomere integrity can be dramatically disrupted leading to detrimental effects with an excessive chromosome instability resulting in premature aging and cancer development. In contrast, in protective, unchallenged conditions, one can speculate that telomeres are in a “mute” state that just protect chromosome ends but without leading to any beneficial or detrimental signaling, resulting in the “relaxation” of the TL regulation processes, possibly leading to heterogenous and long telomeres, such as those present in laboratory mice.

Apart from telomere length variation, modulation in the expression of telomere factors can directly favor tumor formation and progression. For instance, the reactivation of telomerase observed in the large majority of human cancers might mediate pro-oncogenic effects through non-canonical functions [122]. Changes in shelterin proteins can also be drivers of tumorigenesis. Indeed, POT1 variants are correlated with several types of cancer [123–127]. In a large number of human cancers, high levels of TRF2 are associated with poor prognosis, favoring neoangiogenesis and immune-escape [128–131].

These opposite roles of telomeres in oncogenesis can explain the seemingly contradictory results obtained regarding the link between TL and cancer in the general population (Figure 3). Moreover, they invite us to revisit the view that telomere shortening was selected as an anti-cancer response. According to the pleiotropic antagonism theory of evolution, telomere shortening may limit tumorigenesis during youth but promote it later in life. In this case, the anti-cancer hormetic effects of telomere dysfunction would be restricted to the reproductive period of life. In contrast, in older adults, excessive telomere shortening and checkpoint failure might favor tumor formation both by cell-autonomous and cell-non autonomous mechanisms. Thus, before proposing a pro-telomerase therapy for older adults, it must be determined whether telomere hormetic effects can still be a factor later in life.

### 5.2. A Hormetic Effect of the Telomere Position Effect

Telomeres play a transcriptional regulation role known as the telomere position effect (TPE) [132]. In short, a consequence of telomere shortening due to stress might be to mount an adaptive transcriptional response (Figure 2). This mechanism has been thoroughly studied in budding yeast: Rap1 binds to double-stranded telomeric DNA, where it recruits the silencing factors Sir2, Sir3 and Sir4, initiating the spread of a silencing complex through subtelomeric chromatin [133]. Thus, TPE depends on both TL and telomere proteins. The extent of its spreading is modulated in a chromosome-end-specific manner. Insulator and proto-silencer sequences are present in the subtelomeres and, through a complex combination of chromatin looping, lead to a discontinuous spreading that can silence genes located far away from telomeres at the yeast chromosome scale [134]. A similar TPE is conserved in *Drosophila*, mammals and *Trypanosoma* [135–141]. As in yeast, human TPE is regulated by long-range chromatin loops whose formation depends upon TL and shelterin subunits [142–145]. The existence of long-range chromatin-loops has first been described in yeast [146–150], then in plant [151] and in mammalian cells [146,147,149,152,153]. Although the mechanisms allowing these long-range interactions remain elusive, it has been shown that human telomeres can interact with interstitial telomeric sequences, or ITS, in a Lamin A/C and TRF2 dependent manner [145,154]. This chromatin looping is expected to have a direct impact on the transcriptional regulation of the associated subtelomeric genes [132]. TL shortening can decrease the strength of TPE, thus upregulating subtelomeric genes, while releasing telomere factors that bind to internal sites, where they regulate the expression of another set of genes. This complex transcriptional regulation mediated by TL changes was first described in budding yeast [155,156] but also occurs in mammalian cells [157].

Remarkably, the genes whose expression is regulated by TL (such as those involved in carbon source and nutrient utilization [152], cell wall and stress responses [153,158,159]) can be adaptive in response to stress because they are in a subtelomeric position or targeted by released telomere factors. For instance, subtelomeric derepression occurs in response to glucose starvation via a global decrease in histone levels [160], and the derepression of subtelomeric genes encoding cell wall components contributes to the adaptation of yeasts to the membrane-deforming molecule chlorpromazine [158]. Upon rapamycin treatment, Sir3 is hyperphosphorylated, subtelomeric silencing is reduced and yeast acquired greater stress resistance. Besides, still in budding yeast, mitochondrial ROS are sensed by DNA damage kinases to extend chronological lifespan. This mechanism is mediated by enhanced subtelomeric gene silencing through the removal of the histone demethylase Rph1 favoring the Sir3 spreading in subtelomeric regions [161]. In several human cell lines, the ubiquitin-like protein ISG15 (interferon-stimulated gene 15) is overexpressed upon telomere shortening. *ISG15* is involved in the innate immune anti-viral response [162]. Altogether, these results substantiate the telo-hormesis concept, showing a direct telomere adaptation in response to stress via TPE modulation (Figure 3).

Beyond yeast and mammals, clustering stress response genes at subtelomeres appears to be a widely conserved strategy throughout evolution. For instance, in humans, a family

of olfactory receptor genes is located at subtelomeres [163]. However, it remains unknown whether their expression is regulated by telomeric changes. Remarkably, a similar strategy is used by *Candida glabrata* and *Trypanosoma brucei* to regulate the expression of subtelomeric variant surface glycoprotein (VSG) genes, to evade the host immune system [141,164]. The link between telomere changes and the precise regulation of VSG gene expression is unclear, but it may be related to stress signals emanating from the host.

In order to prevent activation of mobile DNA elements, subtelomeres, as other repetitive DNA sequences, are usually transcriptionally repressed. For subtelomeres, this repression is mediated by TPE. Regarding the telo-hormesis hypothesis, the concept of dose-response is well conserved in TPE maintenance. In fact, modest TL shortening triggers derepression of advantageous stress-response genes, while chronic TL changes can relieve TPE and activate mobile DNA elements leading to high-order disorganization and instability.

### 5.3. Telomeres and Mito-Hormesis

The most studied pathway of hormesis is mito-hormesis, defined as mitochondrial stress leading to better health and viability [165]. Mitochondrial dysfunction is signaled by the nucleus through a retrograde communication pathway triggering a differential gene expression response. Among the mito-hormesis signals of stressed mitochondria, the major factors are ROS, but there are also metabolites and the mitochondrial unfolded protein response (UPR).

There is a wealth of data showing a strong link between telomeres and mitochondria (Figure 3). Much data indicate that mitochondrial dysfunction leads to telomere stress and *vice versa*. Telomeric repeats are preferential targets of oxidative stress-induced DNA damage, due to its G-rich sequences [52], a process dubbed teloxidation [43]. Telomere shortening is exacerbated by oxidative stress [166] and enhanced antioxidant contexts tend to lengthen TL [167,168]. Some antioxidant proteins are associated with telomeres (PRDX1 and MTH1) [169,170]. Recently, it was shown that the ROS secreted by activated neutrophils leads to teloxidation of neighboring cells and senescence [50]. Besides, extra telomeric functions of the telomerase subunits TERT and TERC have been related to modulating mitochondrial metabolism. First, upon stress, TERT is exported from the nucleus to the mitochondria, where it protects from DNA damage, apoptosis and oxidative stress [171–175]. Second, the telomerase RNA TERC is processed within the mitochondria to be later released again to the cytoplasm [176]. It is likely that cleaved TERC inform the nucleus about mitochondrial functional state and activity [175]. In telomerase-deficient mice, short telomeres induce p53 activation, which in turn directly represses *PGC-1 $\alpha$*  and *PGC-1 $\beta$* , increasing ROS production. Since ROS signaling is the main mechanism of mito-hormesis and since dysfunctional telomeres could trigger mitochondrial instability and ROS production, telomere-induced oxidative metabolism changes could have a hormetic effect.

Moreover, KO of each shelterin subunit show that they are all, except from TRF1, involved in mitochondrial metabolism [177]. Specially, the glycolysis and the pentose phosphate pathways were modulated upon shelterin KO. It suggests that telomere dysfunction influences metabolism to better adapt the energy consumption upon stress. Interestingly, TIN2 can be localized within the mitochondria and affects its activity and ROS production [178]. TIN2 specific KO affects metabolites of the tricarboxylic acid cycle (TAC), supporting its role in mitochondrial function. Additionally, in human and mouse skeletal muscle, the expression of the mitochondrial sirtuin *SIRT3*, a subtelomeric gene, is activated by TRF2 fixation at its locus through a long-distance chromatin loop. *SIRT3* is down-regulated upon TRF2 inhibition, leading to mitochondrial dysfunction, characterized by higher mitochondrial DNA content and ROS levels [145]. TRF2 downregulation in skeletal muscle occurs naturally during young adulthood. Is the mitochondrial dysfunction due to TRF2 downregulation an adaptive state to respond to physiological demand and exercise? (Figure 3).

Overall, telomere and mitochondria are functionally interconnected. Telomeric changes are likely to be precursors, at least in part, of the mito-hormesis response. Further studies are required to reveal the mechanisms of this telomere/mitochondria cost/benefit balance and its implications in response to stress.

## 6. Conclusions

Telomere changes may be considered deleterious when they lead to telomere shortening and mitochondrial dysfunction but, in certain circumstances, they could also be part of hormetic tradeoff, improving stress resilience and adaptation to adverse situations. This process appears to be conserved across evolution. Clearly, further studies are needed to explore the mechanisms, the inducers and the evolutionary advantages of short TL to substantiate the telo-hormesis hypothesis. One interesting possibility may be that programmed telomere changes play a role during development via a hormetic effect.

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## **Intégrité télomérique dans des cellules musculaires striées squelettiques post-mitotiques : homéostasie tissulaire et rôle de FOXO3a dans la protection télomérique**

Parmi les processus conduisant au vieillissement, le raccourcissement des télomères est le seul clairement programmé au cours du développement dans de nombreux vertébrés. Ceci est dû au problème de réplication observé à chaque division cellulaire. Les télomères sont composés par des répétitions d'ADN (T2AG3), d'un complexe de protéines spécifique (shelterin), d'un ARN non-codant (TERRA) et d'un ensemble de facteurs ayant des fonctions dans les mécanismes de réparation et de réplication. Chez l'humain, le complexe shelterin est composé de six protéines dont TRF2 (telomere repeat-binding factor 2) qui permet la formation d'une boucle d'ADN (t-loop), qui cache l'extrémité des chromosomes d'une activation non voulue de la réponse à l'ADN endommagé. Étant donné que les cellules post-mitotiques différenciées ne se divisent pas, le rôle des télomères dans ces cellules a été longtemps négligé. Donc pendant ma thèse, je me suis intéressée au rôle des télomères dans les cellules musculaires squelettiques différenciées.

Dans la première partie de mon projet, j'ai contribué à un travail déterminant que l'expression de TRF2 diminue dans le muscle squelettique humain, cette diminution commençant chez le jeune adulte. Par la suite, j'ai étudié les conséquences d'une inhibition de l'expression du gène codant pour TRF2 (*TERF2*) dans les cellules musculaires squelettiques différenciées. Contrairement à ce que l'on aurait pu s'attendre, l'inhibition de TRF2 n'expose pas les télomères aux facteurs de reconnaissance et de réparation de l'ADN endommagé. Ceci est observé dans des cellules musculaires humaines en cultures (myotube) et dans les cellules musculaires d'un modèle de souris où le gène *TERF2* est invalidé spécifiquement dans les cellules musculaires squelettiques différenciées. Nonobstant, ces cellules musculaires privées de TRF2 développent un phénotype de dysfonctionnement mitochondrial avec une augmentation du contexte oxydatif qui s'explique, au moins en partie, par un rôle de TRF2 dans l'expression de la sirtuine mitochondriale SIRT3.

Dans la deuxième partie de mon travail de Thèse, j'ai étudié les mécanismes de protection des télomères dans les myotubes privés de TRF2. J'ai pu mettre en évidence un rôle non-canonique du facteur de longévité FOXO3a dans la protection de ces télomères dépourvus de TRF2.

Enfin, dans la troisième partie de mes études, j'ai caractérisé le modèle de souris d'inhibition de *Terf2* spécifiquement dans la fibre musculaire mature. Cette inhibition entraîne, chez les femelles, un retard dans le vieillissement des cellules musculaires squelettiques associé à une légère augmentation de la force musculaire et un rallongement de leur longévité.

En résumé, mes résultats ont révélé dans les cellules musculaires squelettiques différenciées un mécanisme inédit de protection des télomères impliquant un rôle non-canonique de FOXO3a, une connexion entre le facteur shelterin TRF2 et le fonctionnement des mitochondries ainsi qu'une fonction paradoxale de TRF2 dans le vieillissement musculaire et la longévité. Je discute ces résultats dans le contexte d'un continuum développement-vieillessement.