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AAV9 gene therapy with TRF1 telomere protective protein in adult and old mice prolongs mouse health span

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DOCTORAL THESIS

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SUMMARY

Telomeres are nucleoprotein structures at the end of linear chromosomes composed by TTAGGG DNA tandem repeats and a six-protein complex, called shelterin, which protects telomeres and prevents them from being recognized as dsDNA brakes and trigger DNA damage response, leading to cellular apoptosis or senescence. Telomeres are located above coding regions and protect chromosomes from degradation and fusion events. However, due to incomplete replication of the ends of eukaryotic linear chromosomes, telomeres shorten with each round of cell division, and if reaching a critically short length, the cell viability would be compromised.

TRF1 is an essential component of shelterin, with important roles in telomere protection and telomere replication. TRF1 deficiency in the context of different mouse tissues leads to loss of tissue homeostasis owing to impaired stem cell function. Recent reports suggest that TRF1 levels decrease during *in vitro* cellular aging induced via cell passaging. Furthermore, and subsequent overexpression of TRF1 in these “aged” cells at late passages reduced DNA damage at telomeres and decreased senescence, suggesting that age-related loss of TRF1 can contribute to aging phenotype *in vitro*.

Therefore, we first addressed whether TRF1 levels decrease is associated with aging *in vivo*. We measured TRF1 levels in tissues of different ages and found that TRF1 levels decrease during organismal aging both in mice and humans.

To test if natural TRF1 loss with age contributes to organismal aging impairments we overexpressed TRF1 via TRF1 gene therapy in both adult (1 year old) and old (2 years old) mice and studied its effects on aging process. To this end, we used the non-integrative adeno-associated serotype 9 vector (AAV9), which transduces the majority of mouse tissues allowing for moderate and transient TRF1 overexpression. AAV9-TRF1 gene therapy significantly prevented age-related decline in neuromuscular function, glucose tolerance, cognitive function, maintenance of subcutaneous fat, and chronic anemia. Interestingly, although AAV9-TRF1 treatment did not significantly affect median telomere length, we found a lower abundance of short telomeres and of telomere-associated DNA damage in some tissues. Together, these findings suggest that rescuing naturally decreased TRF1 levels during mouse aging by using AAV9-TRF1 gene therapy results in an improved mouse health span.

RESUMEN

Los telómeros son estrucruras nucleoprotéicas que se encuentran en los extremos de los cromosomas lineales, y que consisten en repeticiones en tándem de la secuencia de ADN, TTAGGG y un complejo de seis proteínas, llamado shelterina, que protege al ADN telomérico y evita que estos sean reconocidos como rupturas de doble cadena de ADN, lo que desencadenaría una respuesta de daño al ADN, que podría conducir a la apoptosis o senescencia celular. Los telómeros están ubicados encima de las regiones codificantes y protegen a los cromosomas de la degradación y fusiones cromosómicas. Sin embargo, debido a la replicación incompleta de los extremos de los cromosomas lineares eucarióticos, los telómeros se acortan por cada ciclo celular, y a una longitud críticamente corta se comprometería la viabilidad celular.

TRF1 es un componente esencial de complejo shelterina, con funciones importantes en la protección y la replicación de los telómeros. Se ha demostrado que la deficiencia de TRF1 en el contexto de diferentes tejidos de ratón conduce a la pérdida del homeostasis del tejido debido a la función deteriorada de las células madre. Publicaciones recientes sugieren que los niveles de TRF1 disminuyen durante el envejecimiento celular *in vitro* inducido a través de los pases de células. Además, la posterior sobreexpresión de TRF1 en estas células "envejecidas" en pasajes tardíos redujo el daño del ADN en los telómeros y disminuyó la senescencia, lo que sugiere que la pérdida de TRF1 relacionada con la edad puede contribuir al fenotipo de envejecimiento *in vitro*.

Por lo tanto, en nuestro trabajo primero abordamos si disminución de los niveles de TRF1 se asocia con el envejecimiento *in vivo* en ratones. Para ello, medimos los niveles de TRF1 en tejidos de diferentes edades y encontramos que los niveles de TRF1 disminuyen durante el envejecimiento del organismo tanto en ratones como en humanos.

Para evaluar si la pérdida natural de TRF1 durante el envejecimiento contribuye al empeoramiento relacionado con la edad, sobre-expresamos TRF1 a través de la terapia génica con el gen de TRF1 en ratones adultos (1 año de edad) y viejos (2 años de edad) y estudiamos sus efectos sobre el proceso de envejecimiento. Para tal fin, utilizamos el vector serotipo 9 del virus adeno-asociado no integrativo (AAV9), que transduce la mayoría de los tejidos del ratón, permitiendo así, una sobreexpresión moderada y transitoria de TRF1.

La terapia génica con AAV9-TRF1 evitó significativamente, la disminución en las funciones neuromusculares y cognitivas, la tolerancia a la glucosa, el mantenimiento de la grasa subcutánea y la anemia crónica, todos ellos factores relacionados con la edad. Adicionalmente, aunque el tratamiento con AAV9-TRF1 no afectó significativamente la longitud media de los telómeros, encontramos una menor abundancia de telómeros

cortos y daño del ADN asociado a los telómeros en algunos tejidos. En resumen, estos resultados sugieren que el rescate de los niveles de TRF1, que disminuyen de forma natural durante el envejecimiento del ratón, mediante el uso de la terapia génica con AAV9-TRF1 resultaría en una mejor salud del ratón.

ABBREVIATIONS

AAP – assembly-activating protein

AAV – adeno-associated virus

AAVR – AAV receptor

ALT – alternative lengthening of telomeres

alt-NHEJ – alternative NHEJ

APCs – antigen-presenting cells

ATM – ataxia telangiectasia mutated

ATR – ataxia telangiectasia and Rad3 related

a. u. – arbitrary units

AUC – area under the curve

53BP1 – p53-binding protein 1

BR – basic region

BMD – bone mineral density

CAG –CMV + chicken b-actin + rabbit b-globin

CBA – chicken β-actin

cDNA – complementary DNA

CLIC – clathrin-independent carrier

CMV – cytomegalovirus

CHK1 – checkpoint kinase 1

CHK2 – checkpoint kinase 2

c-NHEJ – classic NHEJ

DDR – DNA-damage response

DEXA – Dual Energy X-ray Absorptiometry

D-loop – displacement loop

DNA – deoxyribonucleic acid

dsDNA – double-stranded DNA

DSB – double-strand break

EF1a – elongation factor 1a

EM – electron microscopy

FISH – fluorescence *in situ* hybridisation

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GEECs – glycosylphosphotidylinositol- anchored protein enriched endosomal compartments

GTT – glucose tolerance test

Hb – hemoglobin

Hct – hematocrit

HDR – homology-directed repair

HOMA-IR – homeostatic model assessment of insulin resistance

HT Q-FISH – high-throughput Q-FISH

IP-GTT – intra-peritoneal glucose tolerance test

ITR – inverted terminal repeat

IV – intravenous

miRNA - microRNA

NHEJ – non-homologous end joining

NLS – nuclear localization signal

NPC – nuclear pore complex

Oct3/4 – octamer-binding transcription factor 3/*4*

ORC – origin recognition complex

ORF – open reading frame

PBMC – peripheral blood mononuclear cell

PCR – polymerase chain reaction

PI – post-injection

PLA1 – phospholipase A1

PLA2 – phospholipase A2

Pol II P – polymerase II promoter

Pol III P – polymerase III promoter

POT1 – protection of telomeres protein 1

Q-FISH – quantitative fluorescence *in situ* hybridisation

rAAV vector – recombinant adeno-associated viral vector

RAP1 – repressor / activator protein 1

RBC – red blood cell

RNA – ribonucleic acid

RT-qPCR – reverse transcription quantitative PCR

SEM – standard error of measurement

shRNA – short hairpin RNA

snRNA – small nuclear RNA

ssDNA – single-stranded DNA

SV40 – simian virus 40

TERC – telomerase RNA component

TERT – telomerase reverse transcriptase

TIF – telomere dysfunction-induced foci

TIN2 – TRF1- and TRF2-interacting nuclear protein 2

TGN – *trans*-Golgi network

T-loop – telomeric loop

TRF1 – telomeric repeat-binding factor 1

TRF2 – telomeric repeat-binding factor 2

TRFH – telomeric repeat-binding factor homology domain

TPP1/ACD – TINT1/PTOP/PIP1 / adrenocortical dysplasia homolog

TZAP – telomeric zinc finger-associated protein

XPF – Xeroderma Pigmentosum Group F

yH2AX – gamma phosphorylated histone H2AX

INTRODUCTION

I. TELOMERES

1. TELOMERES AND THEIR FUNCTION

Mammalian telomeres are heterochromatic structures at the end of linear chromosomes that consist of TTAGGG repeats bound by an array of associated proteins known as shelterin, which prevent chromosome ends from being recognised as double-strand DNA breaks and from chromosome end-to-end fusions (Blackburn 2000; de Lange 2005). Telomere length varies between the species, with an average length 15-20 kb in human germline and 25-150 kb in laboratory mice.

Telomeres evolved in eukaryotic cells as a solution to the "end-replication problem", which refers to the incomplete DNA replication of the ends of linear chromosomes by the DNA replication machinery (Watson 1972; Olovnikov 1973), thus leading to telomere shortening with each round of cell division (Harley et al. 1990). To start a DNA synthesis DNA polymerase requires a 3'-OH group, which in eukaryotic cells is provided by RNA-primers placed on unzipped ssDNA. Upon the end of replication, RNA primers are removed and the gaps are filled by deoxyribonucleotides. However, when removing primer from a 5'-very end of chromosome there is no 3'-OH group above to prime DNA synthesis, giving rise to the single strand 3' G-overhang (**Fig. 1**). In this way DNA getting shorter with each cell division.

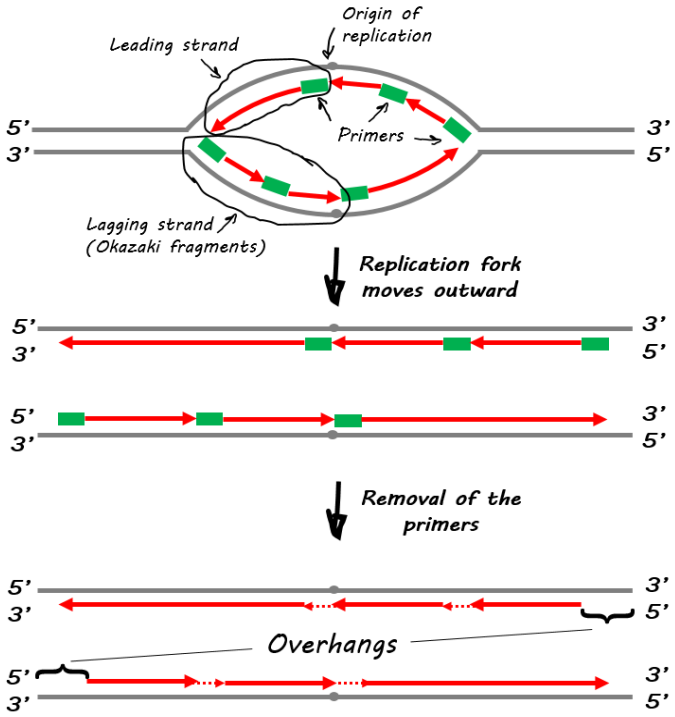

Figure 1. End-replication problem.
Incomplete DNA replication of the lagging strand leads to 3' overhangs formation.

2. MECHANISMS OF TELOMERE ELONGATION

TELOMERASE

There is an active form to maintain telomere length carried out by a ribonucleoprotein called telomerase. Telomerase is a reverse transcriptase (TERT). It elongates telomeres *de novo* by adding telomeric repeats on chromosome ends, using as template an RNA component (TERC), and thus preventing telomere erosion (Greider & Blackburn 1985). It hybridises with a single strand overhang of DNA and telomerase synthesizes the DNA strand complementary to its RNA subunit, translocates to the end of the synthesized strand and the process repeats. When a single stand overhang has been elongated, DNA primase synthesizes RNA primer near the 3 prime end and DNA polymerase fills the space (**Fig. 2**). Telomere elongation by telomerase is regulated by cell cycle and occurs at S phase, being coupled to DNA replication (Ten Hagen et al. 1990; Wright et al. 1999; Marcand et al. 2000; Zhao et al. 2009).

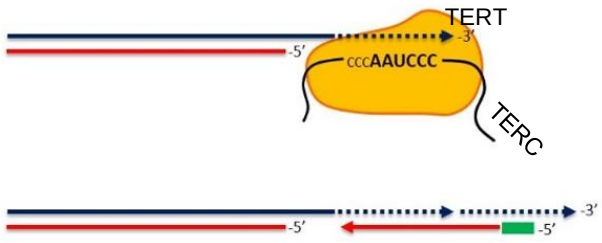

Figure 2. Telomere elongation by telomerase.
Telomerase (in yellow) (TERT) using its RNA component (TERC) as a template elongates 3' telomeric overhang. Then RNA primase synthetizes RNA primer (in green) and DNA polymerase completes *de novo* the synthesis of telomeric repeats on the complementary strand.

ALTERNATIVE LENGTHENING OF TELOMERES (ALT)

ALT is an alternative-to-telomerase telomere lengthening pathway, used by some cancer cell in the absence of telomerase activity (Bryan & Reddel 1997; Henson et al. 2002), which can also take place in normal somatic mouse tissues (Herrera et al. 2000; Neumann et al. 2013). ALT is based on recombination-mediated elongation and occurs via invasion of one telomere by a ssDNA of another telomere, which serves as a template to synthetize new repeats (Dunham et al. 2000; Henson et al. 2002).

3. T-LOOP AS A TELOMERIC SECONDARY STRUCTURE

Free DNA end at linear chromosome termini would be vulnerable to different cellular processes such as DNA repair. However, strand 3' G-overhang does not always exist as a free extension, but has been proposed to fold back invading the double-stranded telomeric region and forming so-called telomeric (T)-loop and displacement (D)-loop structures (**Fig. 3A**). Sequestration of the G-overhang in the T-loop helps to distinguish chromosome ends from sites of DNA damage, preventing a DNA damage response (DDR), inappropriate DNA repair and protecting chromosomes from degradation (Griffith et al. 1999).

It is important to note that replication of the telomere on the leading strand will end up in a blunt end. The absence of 3' G-overhang would make impossible the elongation telomeres by telomerase, due to the fact that telomerase is incapable of extending the blunt-end DNA duplex (Lingner & Cech 1996). Interestingly is was shown that most of eukaryotic chromosomes have 3' G-overhangs on both sides. Evidence suggests that the blunt-side end of the chromosome is processed by nucleases (Wellinger et al. 1996; Dionne & Wellinger 1996), such as Apollo (Wu et al. 2010), forming likewise a 3'-G-overhang and making it possible to form a protective T-loop structure at both ends of the chromosome.

T-loop structures for the first time have been shown *in vitro* as a lariat-like structures on electron microscopy (EM) images, obtained after incubation of DNA structures, which were mimicking telomeric the very end DNA, with baculovirus-derived human TRF2 (**Fig. 3B**) (Griffith et al. 1999). Existence of the T-loops was further confirmed *in vivo* in telomere-enriched fractions from psoralen cross-linked nuclei from different mouse and human cell types (**Fig. 3C-D**) (Griffith et al. 1999). In the same study it was also demonstrated that T-loop size in mammalian cells correlates with telomere length (Griffith et al. 1999).

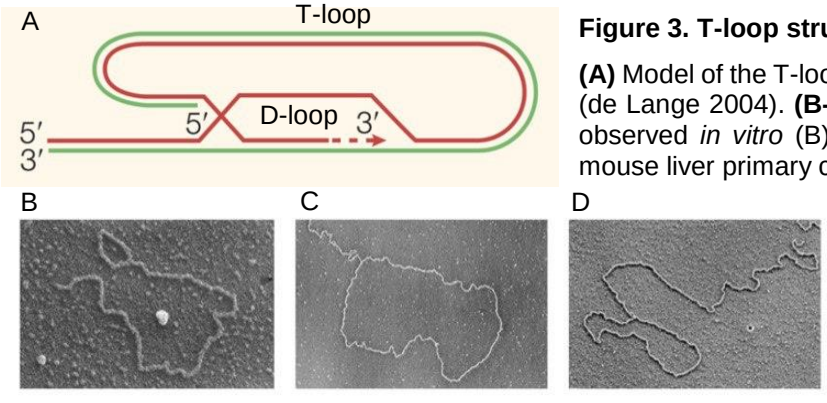

Figure 3. T-loop structure.

(A) Model of the T-loop DNA structure, adopted from (de Lange 2004). **(B-D)** EM visualisation of T-loops observed *in vitro* (B) and *in vivo* in HeLa (C) and mouse liver primary cells (D) (Griffith et al. 1999).

4. TELOMERIC NUCLEOPROTEIN ORGANIZATION. SHELTERIN COMPLEX

Telomere protection is achieved by a presence of the shelterin complex (**Fig. 4A-B**). Shelterin caps and compacts telomeres, inducing formation and maintaining a T-loop structure, thereby protecting telomeres from DNA-repair machinery. Blocking activation of ATM and ATR kinases, shelterin prevents nonhomologous end joining (NHEJ) and homology-directed (HDR) DNA repair pathway activation and a cell cycle arrest (de Lange 2002; d'Adda di Fagagna et al. 2003; d'Adda di Fagagna et al. 2004; de Lange 2005; Muñoz et al. 2006; Palm & de Lange 2008; Martínez et al. 2009).

Shelterin has been also proposed in the recruitment of telomerase to telomeres (Tejera et al. 2010). On the other hand, shelterin is behaving like a cis-acting inhibitor of telomere elongation. The amount of the shelterin on telomere is proportional to telomere length. Highly abundant shelterin on the very long telomeres blocks telomerase activity, preventing unnecessary telomere elongation (Smogorzewska & de Lange 2004; de Lange 2005). Interestingly, it was recently described a new player in telomere length regulation – Telomeric Zinc finger-Associated Protein (TZAP). TZAP is a specific telomere-associated protein, which binds to long telomeres and initiates telomere trimming (Li et al. 2017).

Shelterin consists of six proteins – TRF1 (telomeric repeat-binding factor 1) (Zhong et al. 1992; Chong et al. 1995), TRF2 (telomeric repeat-binding factor 2) (Bilaud et al. 1997; Broccoli et al. 1997), POT1 (protection of telomeres protein 1) (Baumann & Cech 2001), TIN2 (TRF1- and TRF2-interacting nuclear protein 2) (Kim et al. 1999), TPP1 (or ACD, adrenocortical dysplasia homolog) (Liu et al. 2004; Ye et al. 2004) and RAP1(repressor / activator protein 1) (Li et al. 2000) (**Fig. 4A-B**).

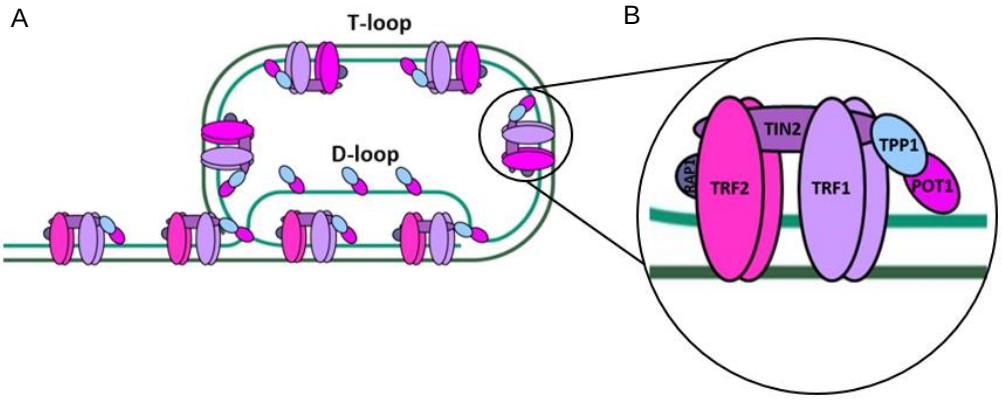

Figure 4. Shelterin complex.
(A) Shelterin complex on the T-loop structure, and **(B)** shelterins and their interconnection.

TRF1

TRF1 is one of the key components of the shelterin complex (Bianchi et al. 1999). TRF1 is the first of the shelterin proteins discovered (Zhong et al. 1992; Chong et al. 1995). Murine TRF1 is a 56 kD and 421 amino acids protein, (human: 50,2 kDa; 439 aa), which specifically binds to a dsDNA as a homodimer (Zhong et al. 1992; Bianchi et al. 1997; Court et al. 2005).

TRF1 contains four functional domains: an acidic N-domain, involved in protein-protein interactions, the TRF homology (TRFH) dimerization domain, which facilitates homodimerization and interaction with TIN2 protein, the nuclear localization signal (NLS) domain and one C-terminal SANT/MYB DNA binding domain, which recognizes 5'-YTAGGGTTR-3' sequence in a double-strand DNA with a high specificity (Chong et al. 1995) (**Fig. 5A-B**). TRF1 contains a single MYB domain. Interestingly, two MYB motifs are required for a stable TRF1-DNA complex formation, which means the formation of TRF1 homodimers fulfils this criteria (Bianchi et al. 1997). TRF1 does not bind to regions outside telomeric repeats, and that its binding is restricted to telomeric regions both in conditions of normal or critically short telomeres (Garrobo et al. 2014).

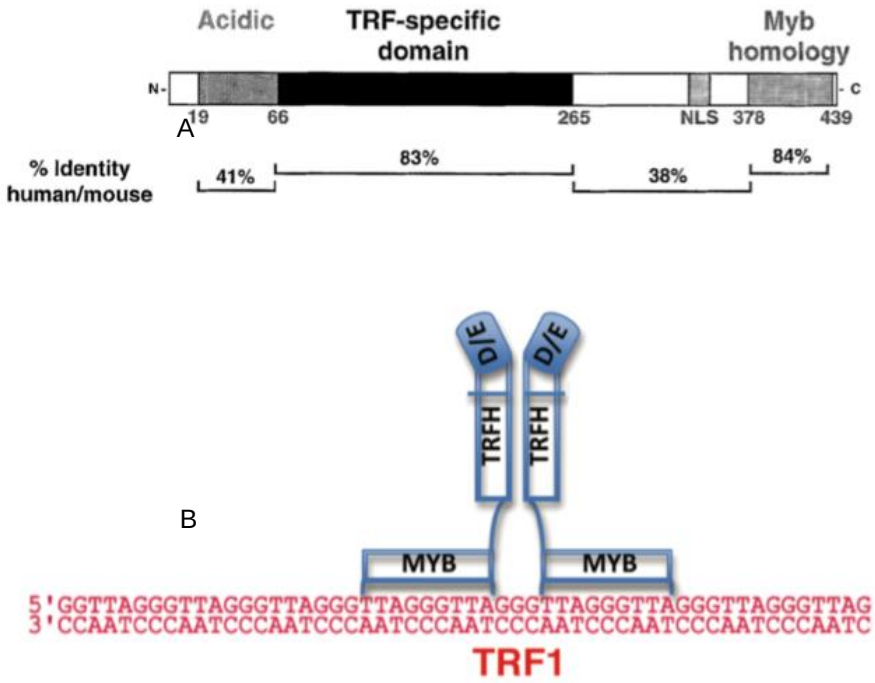

Figure 5. TRF1 domain organization
(A) Scheme of domains of human TRF1 (hTRF1) and it homology with mouse TRF1 (mTRF1) (Bianchi et al. 1997)
(B) TRF1 dimer formation through protein-protein interaction via TRFH homology dimerization domains. Binding to telomeric DNA occurs through MYB domains. D/E is a D/E-rich N-terminal acidic domain (Diotti & Loayza 2011).

Through its interaction with TIN2, TRF1 binds to TRF2, playing an important role in shelterin complex assembly (Xin et al. 2008; Diotti & Loayza 2011) (**Fig. 4A-B**). TRF1 is important both to prevent telomere fusions as well as for the replication of telomeric regions (Sfeir et al. 2009). Genetic manipulations with TRF1 overexpression demonstrated that TRF1, when systematically overexpressed, acts as a negative regulator of telomere length, mediating telomere cleavage by XPF nuclease (Muñoz et al. 2009). However, many *in vitro* and *in vivo* studies have assessed the importance of TRF1 in the healthy cellular and tissue homeostasis and showed that its insufficiency likewise leads to dramatic damages. In particular, deletion of TRF1 in mouse embryonic fibroblasts (MEFs) results in induction of senescence through activation of ATR/CHK1 and ATM/CHK2 checkpoint pathways, as well as in chromosome fusions and multitelomeric signals (aberrant number of telomeric signals per chromosome end) (Martínez et al. 2009; Sfeir et al. 2009). Homozygous deletion of TRF1 in mice leads to embryonic lethality, caused by severe telomere dysfunction (Karlseder et al. 2003). Importantly, these effects of TRF1 abrogation are independent of telomere length, as TRF1 deletion uncaps telomeres independently of telomerase and cell division (Karlseder et al. 2003; Martínez et al. 2009; Sfeir et al. 2009). Heterozygous deletion of TRF1 in mice leads to telomere and overall DNA damage and increased tumor incidence (Hartmann et al. 2016). In addition, conditional TRF1 abrogation in various mouse tissues has demonstrated the importance of TRF1 for tissue regeneration and tissue homeostasis (Beier et al. 2012; Povedano et al. 2015; Schneider et al. 2013; Martínez et al. 2009). Indeed, high TRF1 levels mark stem cell compartments as well as pluripotent stem cells, and are essential to induce and maintain pluripotency (Schneider et al. 2013). In this regard, it was previously shown that TRF1 is a direct transcriptional target of the pluripotency factor Oct3/4 (Schneider et al. 2013).

TRF2

TRF2 was identified by its homology with TRF1 in the database (Bilaud et al. 1996; Broccoli et al. 1997). TRF shares with TRF1 a similar architecture and a common domain structure in TRFH dimerization domain and MYB DNA binding domain (Bilaud et al. 1996; Broccoli et al. 1997; Court et al. 2005) (**Fig. 6**). As well as TRF1, TRF2 binds directly to a dsDNA as a homodimer (Court et al. 2005). However, despite these similarities, TFR2 plays a different role at telomeres.

TRF2 plays a major role in the T-loop formation and maintenance (de Lange 2002; Doksani et al. 2013; Griffith et al. 1999; Stansel et al. 2001). Mice deficient for TRF2 are early embryonic lethal (Celli & de Lange 2005). TRF2 inhibition results disruption of the

T-loop and in further loss of the 3' overhang, leading to ATM DNA damage signalling and activation non-homologous end-joining (NHEJ) DNA damage response (Celli & de Lange 2005; Sfeir et al. 2009). This provokes extensive chromosome end-to-end fusions, further leading to rupture of formed dicentric chromosomes and induction of apoptosis (Celli & de Lange 2005; Karlseder et al. 1999). TRF2 overexpression, likewise TRF1, leads to telomere degradation, mediated by XPF nuclease recruited to telomeres (Muñoz et al. 2005; Muñoz et al. 2006; Blanco et al. 2007).

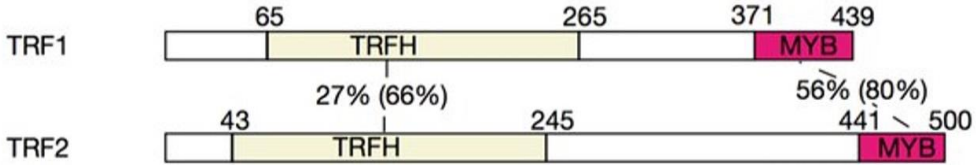

Figure 6. Scheme or TRF1 and TRF2 protein domain structure.
Homology between TRF homology domain (TRFH) and MYB DNA binding domain (MYB), shown as a percentage (Court et al. 2005).

POT1

POT1 is a shelterin, which possesses high specificity for the single-strand telomeric DNA and binds 3' G-overhang and displaced G-strand at the D-loop (Palm & de Lange 2008; Lei et al. 2004). POT1 is connected with the shelterin complex through binding to TPP1 (**Fig. 4A-B**). POT1 plays an important role in inhibition of DNA damage response and ATR signalling at telomeres, blocking the binding of RPA to telomeres (Denchi & de Lange 2007; Takai et al. 2011). POT1 protects 3' overhang and shapes telomere ends, determining the sequence of the 5' telomeric end (Hockemeyer et al. 2005), as well as playing a role in telomere length regulation (Loayza & De Lange 2003; Ye et al. 2004).

Unlike human POT1, mice contain two isoforms – POT1a and POT1b, where POT1a has been proposed to be required to repress DDR and activation of ATR at telomeres, whereas POT1b regulates 3' ssDNA overhangs (Hockemeyer et al. 2006; Wu et al. 2006). Lack of POT1a results in embryonic lethality, whereas mice lacking POT1b are viable and fertile (Hockemeyer et al. 2006).

TPP1

TPP1, encoded by the *Acd* gene, mediates POT1 binding to the rest of the sheltering complex (**Fig. 4A-B**) and to telomeres, contributing to a normal function of POT1/POT1a and POT1b and therefore playing a role in telomere protection and

preventing DDR at telomeres (Tejera et al. 2010; Kibe et al. 2010). It was also shown a role of TPP1 in recruitment of telomerase to telomeres (Tejera et al. 2010). Moreover human cells lacking TPP1 fail to recruit telomerase to telomeres, demonstrating the need of TPP1 for telomerase-dependent telomere elongation (Abreu et al. 2010). Deletion of TPP1 results in early embryonic lethality (Kibe et al. 2010).

TIN2

TIN2 binds to TRF1 and TRF2, acting as a bridge between two homodimers (**Fig. 4A-B**) (Kim et al. 2004). TIN2 also recruits TPP1/POT1 to the complex and loads it on telomeres (Ye et al. 2004; Takai et al. 2011). It was shown that the role of TIN2 in connecting various shelterin components is relevant to protection of telomeres from ATR and ATM signalling, ensured by TPP1/POT1 and TRF2 shelterins (Takai et al. 2011).

RAP1

RAP1 is proposed to be recruited to telomeres by TRF2 (**Fig. 4A-B**) (Li et al. 2000; Li & de Lange 2003). RAP1 is irrelevant for telomere capping, however it deficiency leads telomere recombination and frailty (Martinez et al. 2010; Sfeir et al. 2010). RAP1 binds both to telomeres and extra-telomeric TTAGGG sites, majority of which are found at intragenic positions or at the close proximity to gene-coding regions (Martinez et al. 2010). In this context RAP1 possesses several extra-telomeric functions such as silencing of subtelomeric genes and transcriptional regulation of genes involved in cancer, cell adhesion and metabolism (Martinez et al. 2010; Teo et al. 2010).

SHELTERIN ACCESSORY FACTORS

There are approximately 200 proteins involved in different aspects of telomere biology, contributing to their maintenance and to chromosome protection (Déjardin & Kingston 2009). A large number of them is recruited to telomeres through the shelterin complex, named as shelterin accessory factors. Most of accessory factors are transiently recruited to telomeres, whereas shelterin is constantly presented throughout the cell cycle. This factors are involved in DNA damage response (ATM kinase, MRE11-RAD50-NBS1 (MRN) complex) and DNA repair (Ku70/80 heterodimer, XPF/ERCC1, Apollo exonuclease), DNA replication (origin recognition complex (ORC), RecQ helicases) or chromatin structure (HP1 proteins), etc. Losses of shelterin accessory factors impair telomere maintenance and can lead to telomere shortening, loss or telomere fusion. Reviewed in (Palm & de Lange 2008; Burla et al. 2015).

5. CONSEQUENCES OF TELOMERE DYSFUNCTION

Although telomerase is aimed to maintain telomere length, mammalian cells stop expressing telomerase in the majority of tissues after birth (Blasco et al. 1995; Schaetzlein et al. 2004), leading to progressive telomere erosion throughout the lifespan of the organism. Telomerase expression in the adult organism remains restricted to a few cell types, such as germ and stem cells. Furthermore it was demonstrated that telomerase activity in stem cells is insufficient to prevent telomere shortening with continuous tissue renewal and proliferation (Broccoli et al. 1995; Chiu et al. 1996; Hiyama et al. 1996; Engelhardt et al. 1997; Wright et al. 2006; Brümmendorf & Balabanov 2006; Hiyama & Hiyama 2007).

When telomeres shorten below a threshold length or have lost shelterin protection, they are not able anymore to maintain the T-loop protective organization and become dysfunctional. Telomeric ends become indistinguishable from a double-strand DNA break (dsDB) and are exposed to endonucleases and DNA repair machineries, activating DNA damage response (DDR) at chromosome ends. DNA damage signalling cascade eventually leads to the activation of apoptotic and/or cellular senescence programs and thus to the deregulation of many functional processes (Martens et al. 2000; O'Sullivan & Karlseder 2010; Blackburn 2000). Two signalling pathways play a central role in the response to DNA breaks: ATM, which is activated by DSBs (Shiloh 2003), and ATR kinase pathway, activated by lesions after they have been proceeded to single strand intermediates (Zou & Elledge 2003). Activation of either pathway leads to H2AX histone phosphorylation on Ser139 (yH2AX). These activated sites of DNA damage at the chromosome ends can be visualized at telomeres as so-called Telomere Dysfunction Induced Foci (TIF). Once activated ATM and ATR pathways induce phosphorylation of checkpoint protein kinases CHK2 and CHK1. ATM and ATR pathways demonstrate an active cross talk in response to DNA damage (Cuadrado et al. 2006). Eventually they both lead to p53 phosphorylation (d'Adda di Fagagna et al. 2003; Gire et al. 2004; Guo et al. 2007; Denchi & de Lange 2007). Phosphorylation of p53 stimulates expression of the cyclin-depzendent kinase inhibitor p21, leading to senescence, or activates p53-dependent apoptosis, possessing a role in tumor suppression in cells with dysfunctional telomeres (Deng et al. 2008). Furthermore unprotected telomeric ends can be sensed by classical-NHEJ (c-NHEJ), alternative NHEJ (alt-NHEJ) and homology-directed repair (HDR) pathways (Sfeir & de Lange 2012). HDR provokes rapid changes in telomere length, telomere loss and in telomeric or non-telomeric sequence exchanges, resulting in genome instability and leading to deletions, inversions and translocations (de Lange 2005; de Lange 2009). C-NHEJ and alt-NHEJ repair lead to chromosome fusions and

formation of dicentric chromosomes, the rapture of which can lead to apoptosis or trigger genome instability and cancer (Martínez & Blasco 2010; O'Sullivan & Karlseder 2010) (**Fig. 7**).

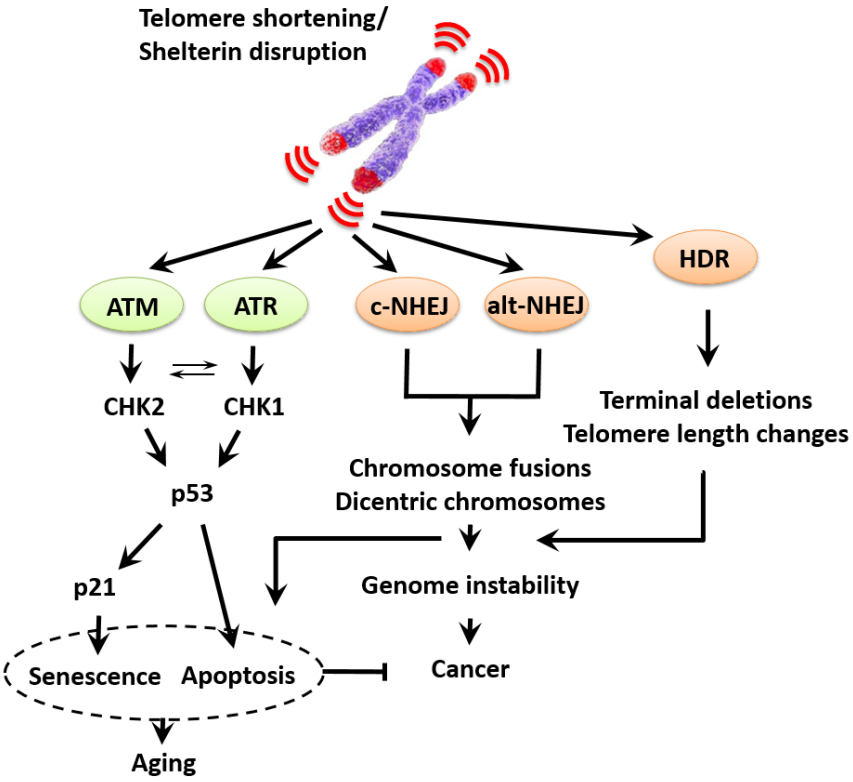

Figure 7. Schematic representation of the pathways activated upon telomere dysfunction

6. TELOMERES AND AGING

Aging is characterized by a time-dependent functional impairment of the organism in which several molecular pathways have been demonstrated to play a causal role (reviewed in Gladyshev 2016; López-Otín et al. 2013). Current efforts are directed at specifically targeting these fundamental aging events since intervening in these molecular pathways could delay or prevent age-related diseases and increase lifespan (reviewed in López-Otín et al. 2013). Telomere shortening has been identified as one of the primary hallmarks of aging (Blasco 2005; Martínez & Blasco 2017; López-Otín et al. 2013). Telomere shortening has been demonstrated to be sufficient to trigger age-related pathologies and shorten lifespan in mice (Blasco et al. 1997; Lee et al. 1998; Blasco 2005). Similarly, humans suffering from the so-called telomere syndromes, characterized by mutations in telomerase and other telomere maintenance genes, also show

premature age-related pathologies (Armanios & Blackburn 2012; Gilson & Londoño-Vallejo 2007).

Telomerase re-activation has been envisioned as a strategy to maintain telomeres, and therefore, to increase the proliferative potential of tissues, both in the telomere syndromes and in age-related conditions. Constitutive TERT expression in the context of cancer resistant mice was shown to be sufficient to maintain longer telomeres and less DNA damage with aging, as well as to delay age-related pathologies and increase mouse longevity by 40% (Tomás-Loba et al. 2008). More recently, telomerase over-expression in adult and old wild type mice by using non-integrative gene therapy vectors was sufficient to delay physiological aging and increase both median and maximum lifespan in wild-type mice in the absence of increased cancer (Bernardes de Jesus et al. 2012). Telomerase reactivation in mice with critically short telomeres owing to telomerase deficiency was also able to reverse tissue degeneration (Jaskelioff et al. 2011). Finally, small molecule telomerase activators such as TA-65 have been also described to delay some features of aging (Harley et al. 2011; Bernardes de Jesus et al. 2011).

Intriguingly, TRF1 levels have been recently shown to decrease with cell passaging, which simulates an aging process *in vitro*, for the first time showing that age intrinsic mechanisms lead to a reduction of TRF1 levels. Subsequent overexpression of TRF1 in “aged” cells at late passages reduced DNA damage at telomeres to the level observed in the “young” cells and decreased senescence, suggesting that decreased TRF1 levels with cell passaging can contribute to senescence and aging phenotype *in vitro* (Hohensinner et al. 2016).

II. rAAV GENE THERAPY

1. ADENO-ASSOCIATED VIRUS (AAV)

Adeno-associated virus (AAV) is a small (25 nm) replication-defective nonenveloped virus belonging to the family Parvoviridae. The AAV capsid is composed of 60 subunits compiled by three capsid proteins VP1, VP2 and VP3 in proportions of about 1:1:10, arranged with T = 1 icosahedral symmetry. VP1-3 share overlapping sequence, differing only at their N termini (Xie et al. 2002). AAV capsid packages a single-stranded (ssDNA) genome of approximately 4.7 kb size, with three open reading frames (ORFs), rep, cap, and assembly-activating protein (AAP), flanked by two inverted terminal repeats (ITRs). ITRs are sequences comprised by 145 bases each, which are palindromic and able to form a hairpin structure leading to self-priming and allowing primase-independent synthesis of the second DNA strand, thus are essential for efficient replication of viral genome (Bohenzky et al. 1988) as well as playing a role in viral packing (McLaughlin et al. 1988; Zhou & Muzyczka 1998). Rep gene encodes 4 regulatory proteins Rep40, Rep52, Rep68, and Rep72, involved in AAV genome replication. The cap gene, by means of alternative splicing and initiation of translation, gives rise to three structural proteins VP1-3 which form AAV capsid (Berns 1990). AAP is translated from the *aap* gene located in an alternate ORF overlapping the *cap* gene. It is required for AAV capsid assembly and is essential for virus reproduction (Sonntag et al. 2010; Naumer et al. 2012) (**Fig. 8**).

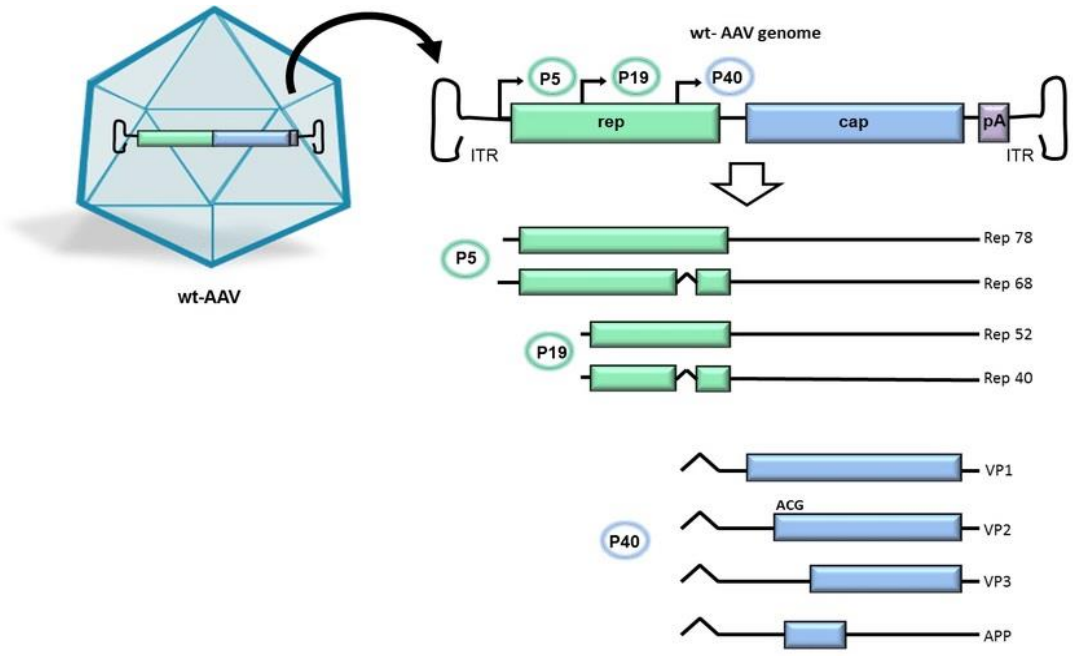

Figure 8. Representation of the wild-type adeno-associated virus genome (wt-AAV) (Saraiva et al. 2016).

2. rAAV AS A GENE THERAPY VECTOR

Interest in AAV virus was born in mid-1980s when it was first recognized that AAV can be used as a gene therapy vector (Tratschin et al. 1984; Hermonat & Muzyczka 1984) and to date they recognized as one of the most promising strategy for gene delivery.

In recombinant AAV vectors (rAAV) viral genome is substituted by a transgene cassette, <4,5 kb in length, cloned between 2 ITRs. Transgene cassette can correspond to a protein-coding complementary DNA (cDNA), microRNA (miRNA), or small nuclear RNA (snRNA) driven by a polymerase II promoter (Pol II P), or either to two inverted repeated sequences (sense and antisense), separated by a loop spacer, which upon transcription from a polymerase III promoter (Pol III P) form RNA which folds into a short hairpin RNA (shRNA) (**Fig. 9**) (Zacchigna et al. 2014).

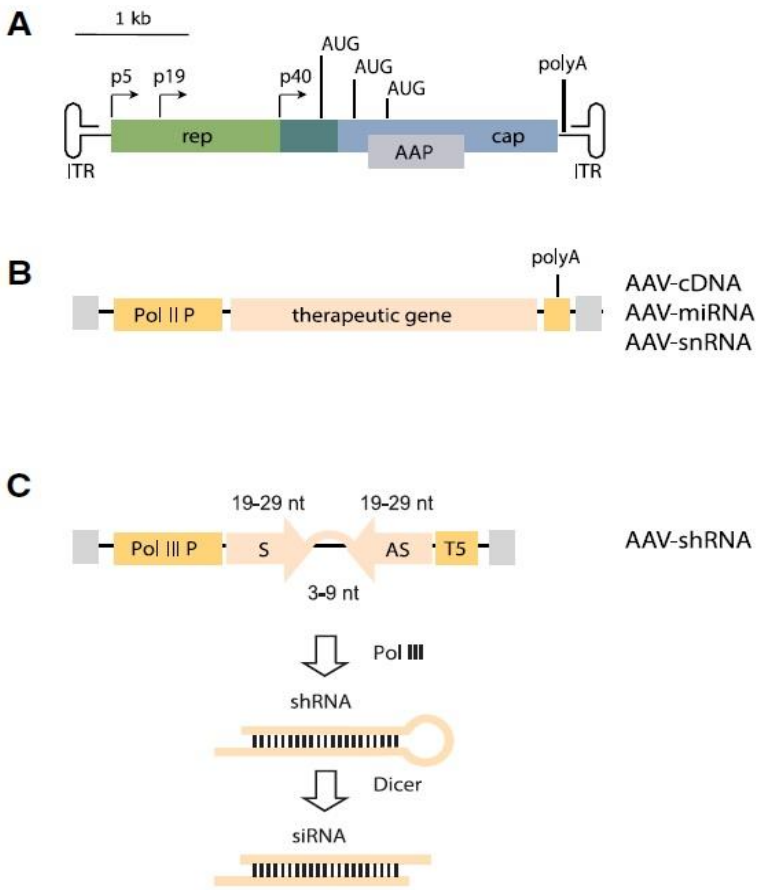

Figure 9. Schematic representation of AAV and recombinant AAV vectors.
(A) Genomic organization of wild-type AAV, which contains three ORFs – rep, cap, and assembly-activating protein (AAP), flanked by two inverted terminal repeats (ITRs). **(B)** AAV vector, expressing of therapeutic gene (protein-coding complementary DNA (cDNA), microRNA (miRNA), or small nuclear RNA (snRNA)), driven by a polymerase II promoter (Pol II P) and cloned into plasmid. **(C)** Expression of short hairpin RNAs (shRNAs). Inverted repeated sequences - antisense (AS) and sense (S), separated by a loop spacer, transcribed from a Pol III P. The resulting RNA folds back into a hairpin structure (shRNA), which is recognized and processed by the cellular endonuclease Dicer into a small interfering RNA molecule. (Zacchigna et al. 2014)

In many therapeutic strategies it is often desirable to achieve broad transgene expression in various tissues. Strong and constitutively active promoter assures high-level expression of the transgene. Commonly used promotors for this scenario are CMV (cytomegalovirus) promoter/enhancer, EF1a (elongation factor 1a), SV40 (simian virus 40), CBA (chicken β-actin) and CAG (derivative from CMV, chicken b-actin, rabbit b-globin). Among these constitutive promoters CAG is the strongest to achieve transgene expression in most cell types (Powell et al. 2015; Naso et al. 2017). However, in the scenario when it is important to achieve cell- or tissue-specific expression, the vector could be delivered locally, or a tissue-specific promoter can be used.

It is well known that AAV genome can integrate site-specifically into human genome via a non-homologous recombination pathway (Kotin et al. 1990; Kotin et al. 1991; Samulski et al. 1991). It is possible due to homology between Rep-binding element located on ITR of the virus and the AAVS1 sequence discovered on the human chromosome 19 (Kotin et al. 1992; Vance et al. 2015). However, to guarantee safety of the rAAV for using as a gene therapy vector, Rep gene as well as the element required for site-specific integration are removed from AAV genome. Therefore rAAV exists in an extrachromosomal state, preventing random mutagenesis much may be induced upon insertion (Vance et al. 2015).

3. rAAV CELLULAR TRANSDUCTION MECHANISM

Use of recombinant adeno-associated viral (rAAV) vectors for gene therapy applications have limitations related to their low transduction efficiency. For instance, in one study it was shown that only about 4%–5% of AAV2 particles attach to cell surface, and just 30% of them successfully reach the nucleus, meaning that eventually only 1%–2% of AAV virions will enter the nucleus and express (Xiao et al. 2012). Many studies were carried out to understand the cellular transduction mechanisms of rAAV. Using this knowledge it would be possible to improve transduction of AAV vectors and thereby to increase efficiency of the gene therapy, and also to avert untoward toxicity and/or an immune response (Weinberg et al. 2014).

First and the most critical step is entry and early trafficking of rAAV through the cell (**Fig. 10**). Cell-rAAV vector interaction starts from binding to glycan receptors (Huang et al. 2014), which leads to attachment of virions to the cell surface. Further cellular uptake is mediated by integrins and/or different transmembrane receptors (Berry & Asokan 2016), including clathrin-mediated endocytosis (Duan et al. 1999; Bartlett et al. 2000), micropinocytosis (Weinberg et al. 2014) and through CLIC/GEEC endocytic pathway (Nonnenmacher & Weber 2011). The entry pathway does also depend on rAAV vector

serotype as well as on the host cell environment (Weinberg et al. 2014). Moreover, some of these pathways lead to successful transduction, however another pathways in the same cell may be the 'dead end' for rAAV vector (Berry & Asokan 2016).

Once entering the cell AAV particles are conceivably trafficked to Rab5+ early endosome (Harbison et al. 2009). Further, rAAV vectors are trafficked to the Golgi apparatus in various ways: though Rab7+ late endosome, Rab11+ recycling endosome (Ding et al. 2006) or through syntaxin 5-dependent transport (Nonnenmacher et al. 2015), which as is a case with cell entry, differ in a serotype or/and host cell type dependant manner.

An important component which mediates rAAV transport through the endomembrane system to the *trans*-Golgi network (TGN) is so called AAV receptor (AAVR). This receptor largely localizes to Golgi apparatus and is essential for the efficient transduction (Pillay et al. 2016), however the mechanisms of AAVR mediated AAV transduction need to be further investigated.

After AAV particle passed through vesicular and Golgi compartments, rAAV escapes the endosome into the cytosol in phosphorilaze-dependent manner (phosphorilazes PLA1 and PLA2).

Nuclear import happens through the nuclear pore complex (NPC) mediated by importin-b1 (Nicolson & Samulski 2014). There are four basic regions (BR1-4) on rAAV conserved among serotypes 1–11, which are potentially playing function as nuclear localization signals (NLS). Upon nucleus entry to proceed with transgene expression rAAV particles need to be uncoated. Upon release, AAV ssDNA genome undergoes second-strand DNA synthesis (Berry & Asokan 2016), and then being circularized and concatemerized (Choi et al. 2005) by DNA recombination events. It was shown that several splicing factors, which belong to the U2 snRNP spliceosome complex, can bind to the exposed genome and the capsid, restricting processes starting from nuclear entry to second-strand DNA synthesis, eventually suppressing transcription (Schreiber et al. 2015).

The knowledge on rAAV transduction mechanisms will lead to development of various strategies to target the host factors that have been implicated in AAV trafficking and thereby to enhance the potency of rAAV vectors.

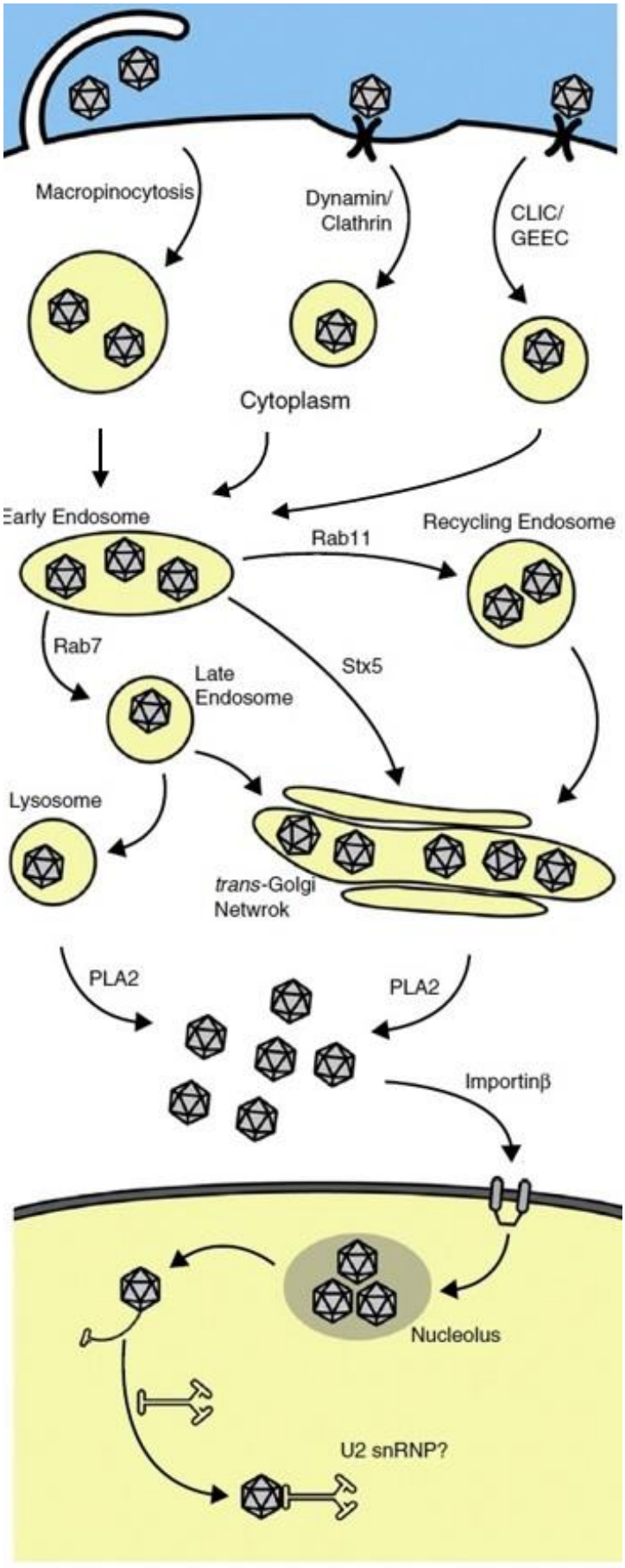

Figure 10. Summary of intracellular trafficking events leading to successful AAV transduction.
Adapted from (Berry & Asokan 2016)

4. rAAV VECTOR PRODUCTION

The most established method of production of rAAV particles is by co-infection of HEK293 cells by rAAV vector cloned into a plasmid and a plasmid carrying the AAV *rep* and *cap* genes (Grieger et al. 2006). Helper functions important for AAV successful replication could be supplied by co-transfecting cells with a helper virus, e.g., adenovirus, or either by co-transfecting with helper plasmid carrying genes from adenovirus (E4, E2a and VA). Notably, *rep* and *cap* genes can be supplied with helper genes within the same plasmid (Grimm et al. 1998). Double instead of triple- transduction has several advantages, including elimination of helper virus contamination in rAAV stocks as well as increased titres due to higher cell viability (Grimm et al. 1998; Ayuso et al. 2010). The replicating rAAV genomes are packed into pre-formed capsids within a nucleus of infected HEK293 cells. Cells are then harvested, homogenized and viral articles are purified from cell homogenate (**Fig. 11**).

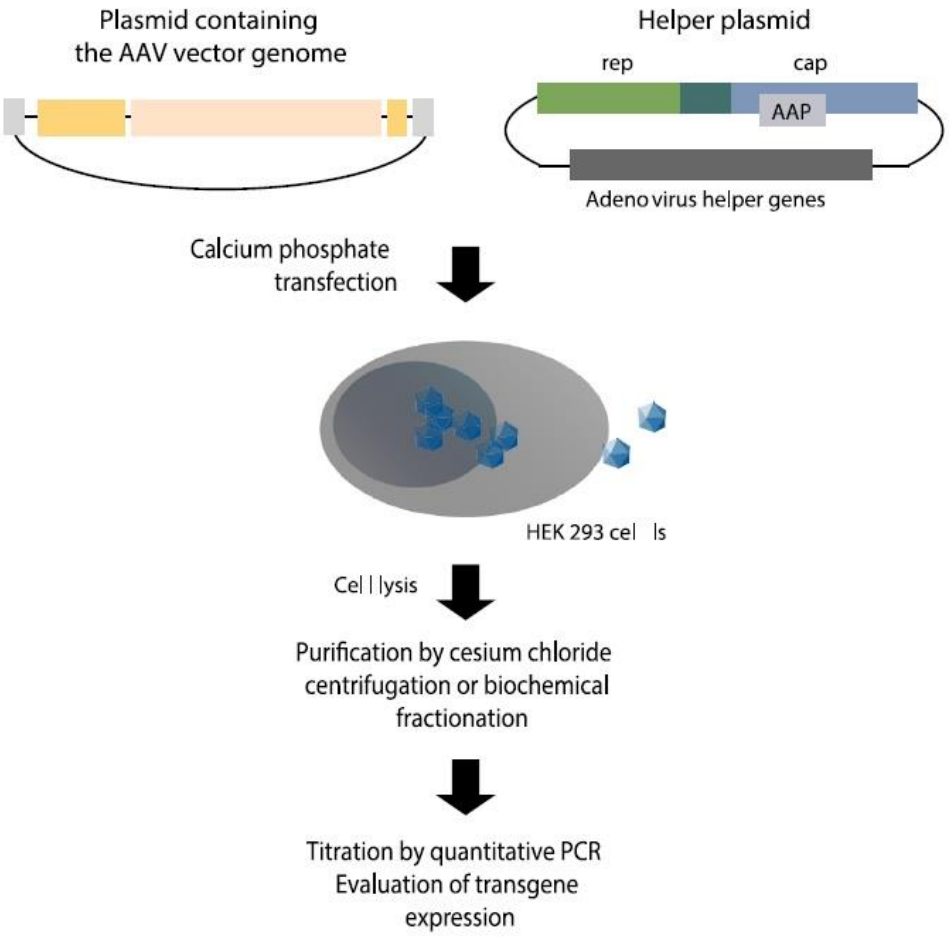

Figure 11. Production of recombinant AAV vector particles (Zacchigna et al. 2014)**.**

5. IMMUNE RESPONSE TO rAAV

Initially rAAVs were considered as non-immunogenic vectors due to their inefficiency in transducing antigen-presenting cells (APCs) in mice (Jooss et al. 1998). However when the first clinical trials were conducted, they showed that rAAV gene therapy in humans could actually activate innate and adaptive immune response to a viral capsid, a DNA genome and the therapeutic product of this transgene (Mingozzi & High 2013). Immune response can lead to clearance of transduced cells, therefore limiting the therapeutic efficacy of the gene therapy (Mingozzi et al. 2007). Different factors such a dose, the choice of AAV serotype and the administration route can influence the rAAV vector immunogenicity. More studies need to be done to understand how to influence the initiation of an expression limiting immune response, however the main obstacle is that so far there is no animal model that could recapitulate the human immune response to the viral vector, and the majority of the questions answered to date are based on information from clinical studies.

Many efforts has been done to prevent immune response to rAAV vector. At the moment the most successful results are given by glucocorticoid steroid immunosuppressant drugs (Flanigan et al. 2013).

It was shown that organism, in contrast with immune response, can also develop tolerance to rAAV vectors through induction of T regulatory response and silencing of T lymphocytes, however the mechanism of tolerance is still lacking precise characterization (Lin et al. 2007; Velazquez et al. 2009; Flotte et al. 2011; Mueller et al. 2013).

Summing up, even taking into account rAAV immune activating properties, they remain much reduced compared to other viral and non-viral vector.

6. SAFETY CONCERNS OF rAAV GENE THERAPY

AAV gene therapy possess a great potential for treating human diseases and is actively undergoing clinical trials, however the safety of AAV vectors still needs more detailed assessment. Even when designed to prevent site-specific integration, rAAV vectors at low frequencies but can integrate at non-homologous sites in the host genome. Integration happens into so-called “hot spots”, which represent sites of genomic instability and are prone to double-strand breaks or other forms of DNA damage, activating non-homologous recombination (Nakai et al. 2005; Inagaki et al. 2007; Deyle & Russell 2009). rAAV vector integration into genome may lead to insertional mutagenesis, altering the expression of chromosomal genes and even causing

malignant transformation (Nakai et al. 2005; Miller et al. 2005; Donsante et al. 2007; Deyle & Russell 2009), and therefore should be taken into account. However, the frequency of AAV vector genome integration is too low, which is one of the main advantages of AAV vectors, and therefore they are actively being tested in clinical trials (Anon n.d.; Vance et al. 2015).

7. AAV SEROTYPES AND THEIR BIODISTRIBUTION

Chronologically, a majority of the first discoveries and applications were associated with AAV serotype 2 (Srivastava et al. 1983), however over the last years, thirteen human serotypes of AAV (AAV serotype 1 [AAV-1] to AAV-13) (Srivastava 2016) and more than 100 serotypes from nonhuman primates have been discovered and isolated (Daya & Berns 2008). Knowledge on different serotypes opens many opportunities, because serotypes were found to differ in infectivity rates and tissue specificity. Variability of serotypes is distributed within looped out domains, displayed on the surface of the capsid structure (Gao et al. 2003), which are primarily involved in cellular interactions. Therefore, these differences are more likely define differences between AAV serotypes in tropism and transduction efficiency. The efficacy ad tissue specificity of some of the AAV serotype vectors has been evaluated in different animal models (**Fig. 12**) (Srivastava 2016).

AAV vectors share a high homology among different human serotypes (Vance et al. 2015), as well as have high similarity in sequences with non-human serotypes, which efficiently distribute and are able to cross various species barriers, making them also suitable for treating human disease (Vance et al. 2015).

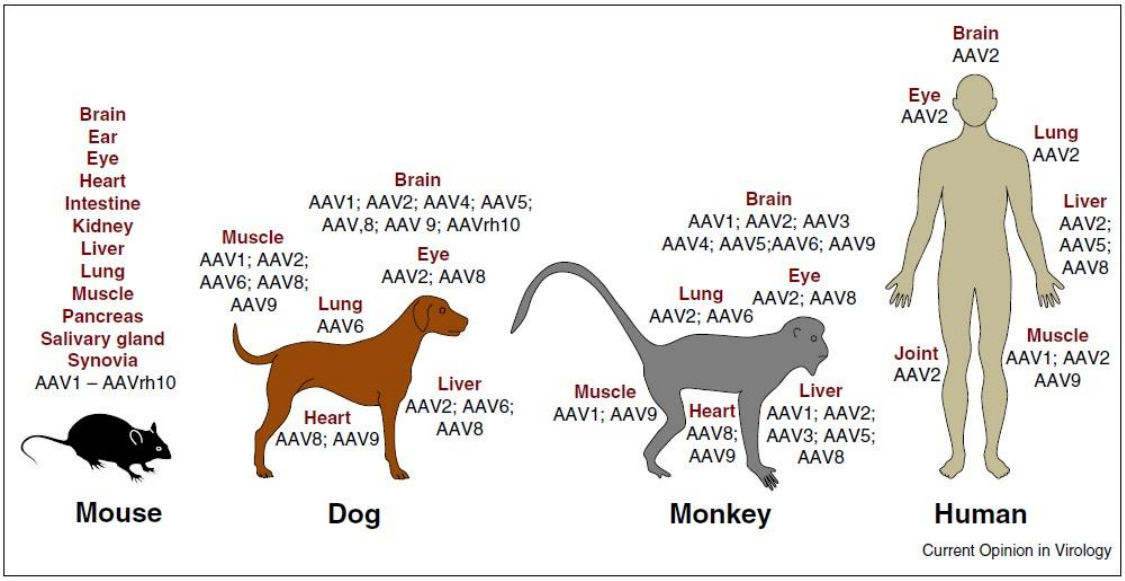

Figure 12. Schematic representation of various studies carried out with AAV vectors of different serotypes in animal models and in humans (Srivastava 2016)**.**

8. AAV9 SEROTYPE

Recombinant AAV9 vector owns a list of beneficial properties, which makes it one of the most popular vectors used for therapeutic applications.

First, AAV9 demonstrates one of the fastest onset and the highest expression of transgene as well as the best viral genome distribution among other serotypes upon systemic administration (Zincarelli et al. 2008). Superior to other serotypes it efficiently targets heart, liver and skeletal muscle (Bish et al. 2008; Inagaki et al. 2006; Pacak et al. 2006; Zincarelli et al. 2008; Vandendriessche et al. 2007). AAV9 tissue binding was shown to be primarily mediated by N-linked glycans with terminal galactosyl residues, which are abundant in various animal tissues, explaining the broad tropism of AAV9 serotype (Shen et al. 2011).

Moreover, AAV9 is one of the few serotypes (together with AAV8 to the lesser extent), which possess the ability to cross blood-brain barrier following intravascular administration (Foust et al. 2009), opening up new opportunities for basic and clinical neurology studies.

9. GENE THERAPY OF DISEASES AND AGING

Five decades after the discovery of adeno-associated virus (AAV) and more than 30 years since the first gene transfer experiment, to date over 183 clinical trials worldwide are in process what makes up 7.3 % of virus-vectored gene-therapy trials (Anon n.d.) (**Fig. 13**).

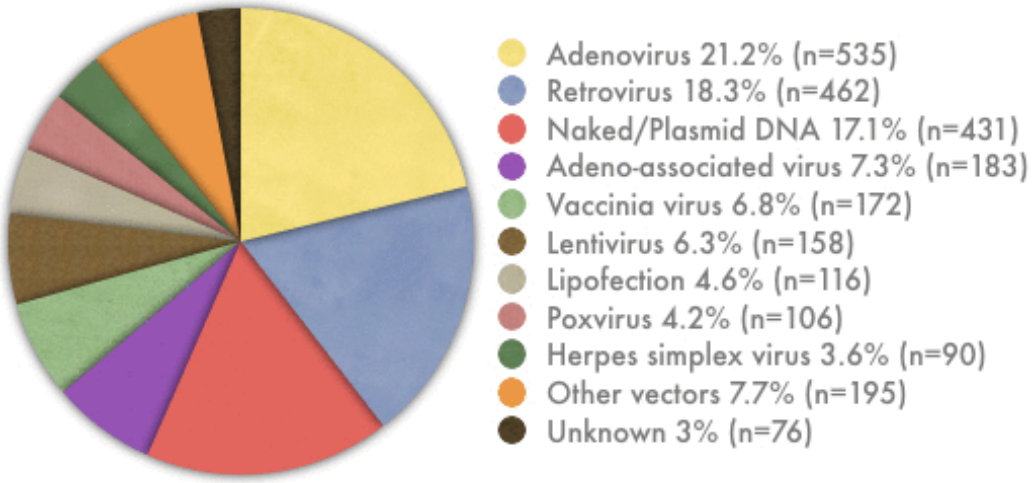

Figure 13. Vectors Used in Gene Therapy Clinical Trials, 2017 (Anon n.d.) .

Extensive preclinical studies led to many clinical trials and to date one AAV vector is already approved for use in Europe (Anon n.d.; Ylä-Herttuala 2012; Scott 2015). Most of clinical trials, which are running today, are aimed to target broad types of diseases such as monogenic, inherited diseases, replacing defective gene (hemophilia A and B), neurological (Parkinson's disease, Canavan disease, Alzheimer's disease), cardiovascular (congenital heart failure, cardiomyopathies), ocular (Leber congenital amaurosis) and infectious (HIV) diseases, muscular dystrophies as well as arthritis and cancer (scuamos cell head and neck cancer), etc (Vance et al. 2015). Recent pre-clinical studies have also demonstrated efficiency of using AAV vectors to target aging. Delivering TERT gene via rAAV gene therapy affected one of the hallmark of aging, telomere shortening, leading to telomere lengthening and decreased abundance of short telomeres, improving the life and health span in aging mice (Bernardes de Jesus et al. 2012; Boccardi & Herbig 2012). Growing knowledge and application is showing a promising success of AAV gene therapy for different needs.

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OBJECTIVES

I. **To study the dynamics of TRF1 expression in aging in vivo in mouse and human tissues**

II. **To study potential effects of AAV9-TRF1 gene therapy in adult and old mice on aging**

1. To produce AAV9 vectors carrying m*Trf1* transgene and confirm their transduction efficiency and TRF1 overexpression in various tissues

2. To perform AAV9-TRF1 gene therapy in adult and old wild type mice and to study it effects on life span and health

3. To study the molecular consequences of AAV9 mediated TRF1 overexpression

MATERIALS AND METHODS

1. MICE AND ANIMAL PROCEDURES

Experiments were performed with wild type mice of a >95% C57BL/6 background. All of the data was collected in a quantitative way and subjective evaluations were not made. Mice were produced and housed in the specific-pathogen-free animal house of CNIO, Madrid. All procedures performed on mice were approved by the CNIO-ISCIII Ethics Committee for Research and Animal Welfare (CEIyBA). Mice were treated according to Spanish laws and the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

Mice were tail-vein injected with a dose of $2*10^{12}$ viral genomes per mouse– AAV9-TRF1 in the experimental group and AAV9-empty in the control group. Injections were performed at different ages: adult mice of ~1 year of age (64-65 weeks old) and old mice ~2 years of age (109-110 weeks old). Between different groups within the same age, an equal ratio males/females was maintained.

2. RECOMBINANT AAV9 VECTORS

Mouse TRF1 was cloned into *pBABE puro* plasmid vector at CNIO (Madrid), which was used for AAV9-TRF1 production. AAV9-TRF1 and control vectors were produced and purified as described previously (Matsushita et al. 1998; Ayuso et al. 2010). Briefly, recombinant AAV9 vectors were produced through triple transfection of HEK293 cells by plasmid carrying a TRF1 expression cassette, plasmid carrying the AAV *rep* and *cap* genes, and an adenovirus helper plasmid. AAV9 vector purification was achieved by CsCl gradient centrifugation, following CsCl removal by dialysis against PBS and filtration. Titres of viral particles were determined by quantitative RT-PCR.

3. RNA ISOLATION AND RT-QPCR

Analysed tissues were homogenized and total RNA was isolated using the RNeasy Mini Kit (QIAGEN). The concentration and purity of the RNA was determined with a NanoDrop ND-1000 spectrophotometer. Total RNA was retrotranscribed using the iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's guidelines. Expression levels of Trf1 mRNA were determined by real-time PCR, performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7900HT Fast Real-Time PRC System (Applied Biosystems). Each reaction was performed in triplicate and normalized to GAPDH mRNA levels as an endogenous control. Sequences of the mouse primers used for quantitative real-time PCR in this work are listed: *TRF1*-Fw: 5'-TCT AAG GAT AGG CCA GAT GCC A-3', *TRF1*-Rv: 5'-CTG AAA TCT GAT GGA GCA CGT

C-3', *GAPDH*-Fw: 5'-TTC ACC ACC ATG GAG AAG GC-3', *GAPDH*-Rv: 5'-CCC TTT TGG CTC CAC CCT-3'.

4. IMMUNOFLUORESCENCE ANALYSIS

Immunofluorescence analysis was performed on paraffin-embedded tissue sections as described previously (Tejera et al. 2010; Martínez et al. 2009). To target TRF1, a homemade rat monoclonal anti-TRF1 antibody and polyclonal goat anti-rat Alexa 555 as a secondary antibody were used. Images were captured on a confocal ultraspectral microscope Leica TCS-SP5-WLL. Fluorescence intensities were analyzed using Definiens Developer Cell software.

5. BLOOD SAMPLES AND CELL COUNTS

Blood samples were taken at different time points over the lifespan by facial vein puncture (~50 µL) and collected in EDTA anti-coagulant tubes. Blood cell counts, hematocrit (Hct) and hemoglobin (Hb) concentrations were measured on an *Abacus Junior* Vet veterinary hematology analyzer.

6. HIGH-THROUGHPUT Q-FISH (HT Q-FISH)

To perform a longitudinal study on telomere length dynamics in mouse peripheral white blood cells, blood samples were collected as described in the section above at the indicated time points after AAV9 injection. Blood samples were processed with erythrocyte lysis using Buffer EL (QIAGEN) and frozen in 10% DMSO/FBS. Prior to proceeding with the protocol, blood samples were de-frozen and plated in poly-L-lysine pre-coated clear bottom black-walled 96-well plates (Greiner). Samples were analyzed in duplicate. The HT Q-FISH protocol was performed as described in (Canela et al. 2007). In order to convert telomeres fluorescence values into kb, we used standard cell lines with stable telomere length: L5178Y-R (79.7 kb), HeLa1211 (23.8 kb) and CCRF-*CEM (7.5 kb)*. Images were acquired on an Opera High Content Screening System (PerkinElmer, Inc.) and analyzed with Acapella Image analysis software (PerkinElmer, Inc.).

7. TELOMERE Q-FISH ANALYSIS

Telomere analysis was performed on paraffin-embedded tissue sections, which were de-parafinized, hybridized with a PNA-telomeric probe and treated as described in (Zijlmans et al. 1997). Images were captured on the confocal ultraspectral microscope

Leica TCS-SP5-WLL. Analysis of images was performed using the Definiens Developer Cell software.

8. IMMUNO-FISH ANALYSIS

To assess the level of DNA damage at telomeres, we performed Immuno-FISH analysis. First we performed a Q-FISH protocol excluding a pepsin digestion step, followed by an immunofluorescence protocol, using rabbit monoclonal anti-53PB1 antibodies for the primary antibody and polyclonal goat anti-rabbit Alexa 488 antibodies for the secondary antibody. Images were acquired on a confocal ultraspectral microscope Leica TCS-SP5-WLL. Analysis of images was performed using the Definiens Developer Cell software.

9. NEUROMUSCULAR COORDINATION

Neuromuscular coordination and balance were evaluated in tightrope and rotarod tests. In the tightrope test, mice were placed onto a bar (100 cm long and 1.5 cm diameter). If the mouse was able to remain on the bar for 1 minute, it was considered as a success. Percentage of success out of five trials was determined. In the rotarod test, mice were placed onto a rod which was rotating with accelerating speed from 4 to 40 rpm during one minute. The mean time of the latency before falling was measured in 3 trials.

10. MEMORY ANALYSIS

Recognition memory was studied in the object recognition test as described in (Bevins & Besheer 2006). Each mouse was placed in a box to investigate two equal objects for 5 min. In a 2 h time gap one of the two objects was replaced with an object of different form and structure, and the mouse was placed back into the box for 5 more min. The memory score was quantified as the time spent investigating a novel object divided by the total time of investigation of both objects.

11. SUBCUTANEOUS FAT THICKNESS

Thickness of the subcutaneous fat layer was measured as described previously (Moynihan et al. 2005). Briefly, a total of 20 measurements were performed on 3 back sections of the skin for each mouse sacrificed at the humane end point. For these measurements, 17 AAV9-empty and 15 AAV9-TRF1 mice were used. Measurements were made using Panoramic Viewer software.

12. BONE MINERAL DENSITY

Bone mineral density (BMD) was measured on anaesthetized living mice using a Dual Energy X-ray Absorptiometry (DEXA) scan device.

13. INTRAPERITONEAL GLUCOSE TOLERANCE TEST (IP-GTT)

To measure the clearance of injected glucose into peripheral tissues, an IP-GTT test was performed as described (Moynihan et al. 2005; Tomás-Loba et al. 2008). Briefly, mice were fasted for 14 h and then injected intraperitoneally with 50% D-(+)-glucose solution (2 g/kg body weight). Blood was collected from the tail vein at 0, 15, 30, 60, 90 and 120 min after injection and blood glucose levels were measured with a glucometer and Glucocard Memory Strips (Arkray Factory, Inc., Japan). Glucose curves on changing of glucose levels with time were represented, and area under the curve (AUC) was calculated.

14. FASTING INSULIN LEVELS AND HOMEOSTATIC MODEL ASSESSMENT OF INSULIN RESISTANCE (HOMA-IR)

Serum was taken from mice after 14 h of fasting. Insulin levels were measured in blood serum with an Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc.), following the manufacturer's protocol.

HOMA-IR was performed as described (Heikkinen et al. 2007a). HOMA-IR index was calculated according to the formula:

$$\mathrm{HOMA-IR} = \frac{\mathrm{FPI} \times \mathrm{FPG}}{22.5 \times 18 \times 45.5}$$

where FPI is the fasting serum insulin (ng/mL) determined by ELISA and FPG is the fasting blood glucose concentration (mg/dL).

RESULTS

1. TRF1 LEVELS DECREASE WITH INCREASING AGE BOTH IN MICE AND HUMANS

To address whether the decrease of TRF1 is associated to physiological ageing *in vivo*, here we determined both mRNA and protein TRF1 levels in the epidermis of wild-type mice at different ages including young (6-to-8 weeks old), adult (52-to-57 weeks old), and old mice (89-to-104 weeks old). We found significantly decreased *Trf1* mRNA levels in the epidermis of adult and old wild-type mice compared to young mice (**Fig. 1A**). As an independent molecular marker of aging, we also measured p16 (a mediator of cellular senescence) mRNA levels, which are known to increase with age in almost all tissues in rodents and humans (Krishnamurthy et al. 2004; Satyanarayana & Rudolph 2004). As expected, p16 mRNA levels were higher in the epidermis of middle-aged mice compared to young mice, and this was further increased in old mice (**Fig. 1B**).

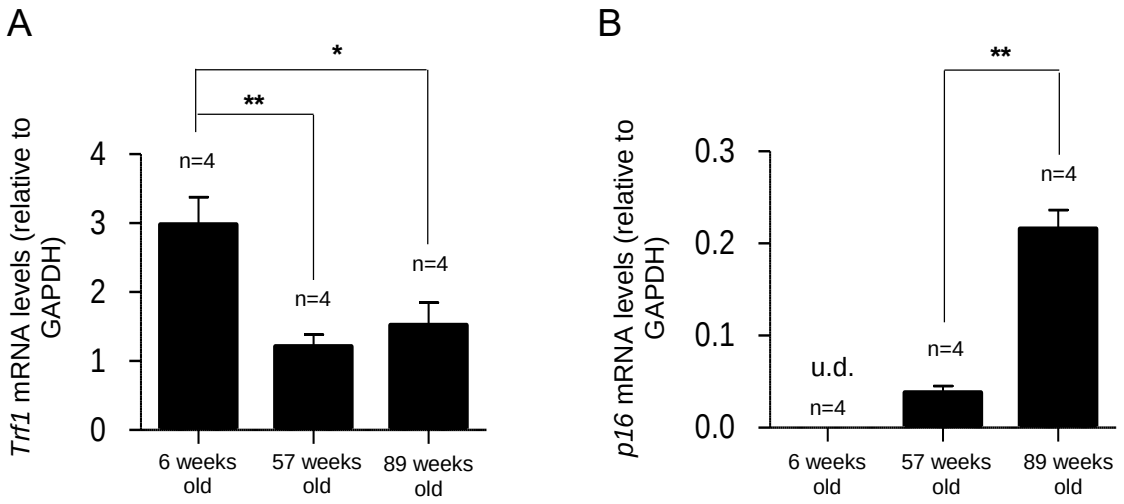


Figure 1. Decrease of TRF1 mRNA levels with aging in mouse skin.
(A-B) Trf1 (A) and p16 (B) mRNA levels determined by RT-qPCR in tail skin epidermis from 6, 57 and 89 weeks old mice. Trf1 and p16 mRNA values are normalized to GAPDH. Student's t-test was used for statistical analysis. Error bars represent SEM. U.d.- undetermined value *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice/individuals.

Earlier Dr. Ralph Schneider from our Group has shown decreased TRF1 protein expression levels in the mouse and human epidermis at different ages. Using immunofluorescence with antibodies against the TRF1 protein, he demonstrated that TRF1 fluorescence was significantly decreased in old mice compared to both adult and young mice (**Fig. 2A**). Importantly, TRF1 levels with aging were also decreased in the human epidermis. In particular, performing TRF1 immunofluorescence on human skin samples from young (2-to-12 years old), adult (31-to-40 years old), and old (75-to-85

years old) individuals, he observed significantly decreased TRF1 levels with age. In particular, adult human skin showed significantly lower TRF1 levels compared to young skin, and TRF1 levels were further decreased in old skin samples (**Fig. 1B**).

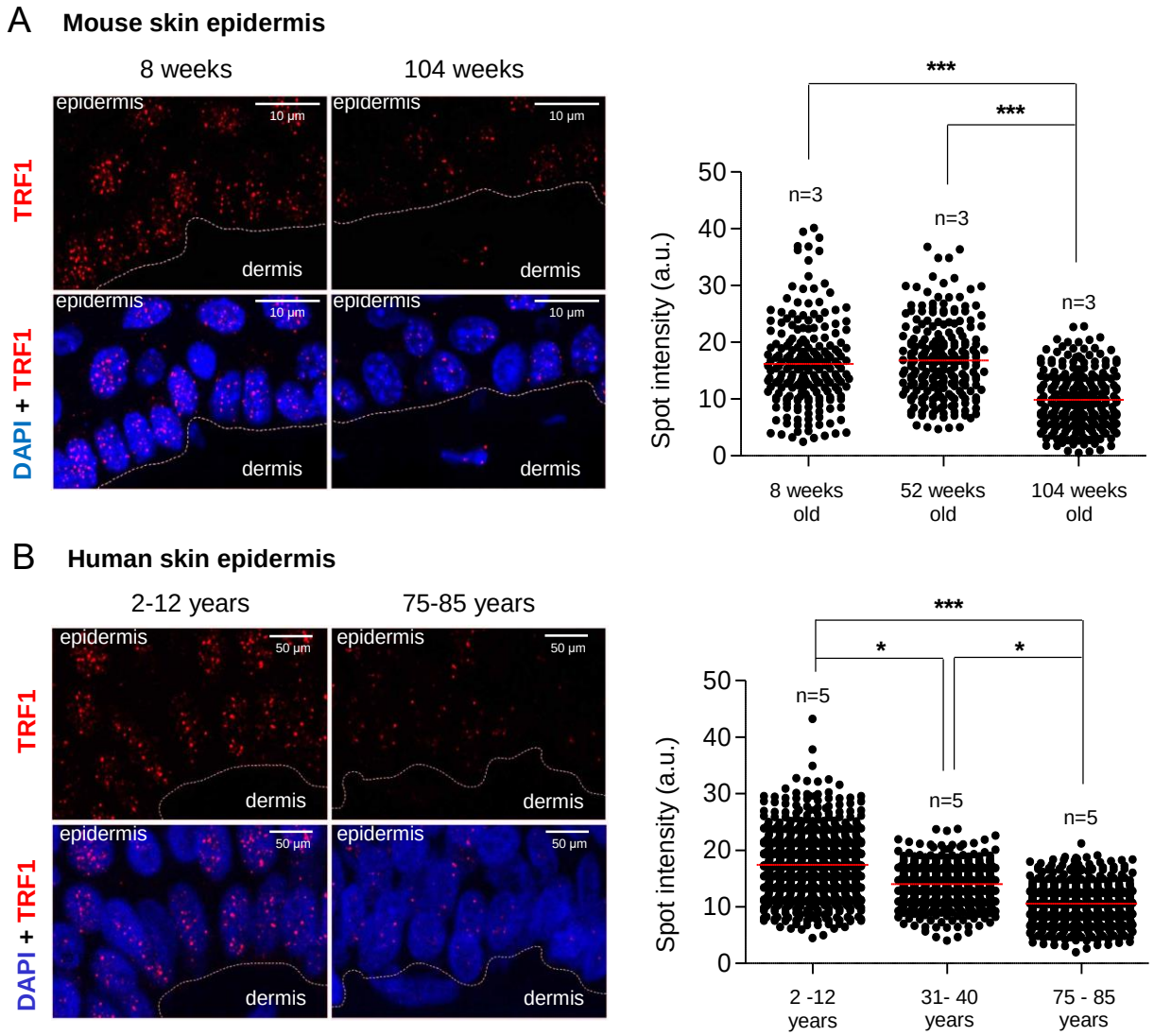


Figure 2. Decrease of TRF1 protein levels with aging in mouse and human skin.
(A) Representative images of TRF1 (in red) and DAPI (in blue) and quantification of TRF1 protein levels measured by immunofluorescence analysis in mice of 8, 52 and 104 weeks old in back skin epidermis. **(B)** Representative images of TRF1 (in red) and DAPI (in blue) and quantification of TRF1 protein levels measured by immunofluorescence analysis in skin epidermis of young (2-12 years), middle-age (31-40 years), and old (75-85) humans. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice/individuals. For each experiment, images were acquired with the same resolution and exposure parameters.

We extended these findings to the intestine, where we also observed a significant decrease in TRF1 protein levels in the old mice group compared to young mice group (**Fig. 3A**). In the case of post-mitotic tissues, we saw decreased TRF1 expression with aging in the muscle tissue in mice (**Fig. 3B**), but not in the liver (**Fig. 3C)**.

In summary, these findings indicate that TRF1 levels decrease with age in mice and humans, at least in the majority of tissues that have been studied here.

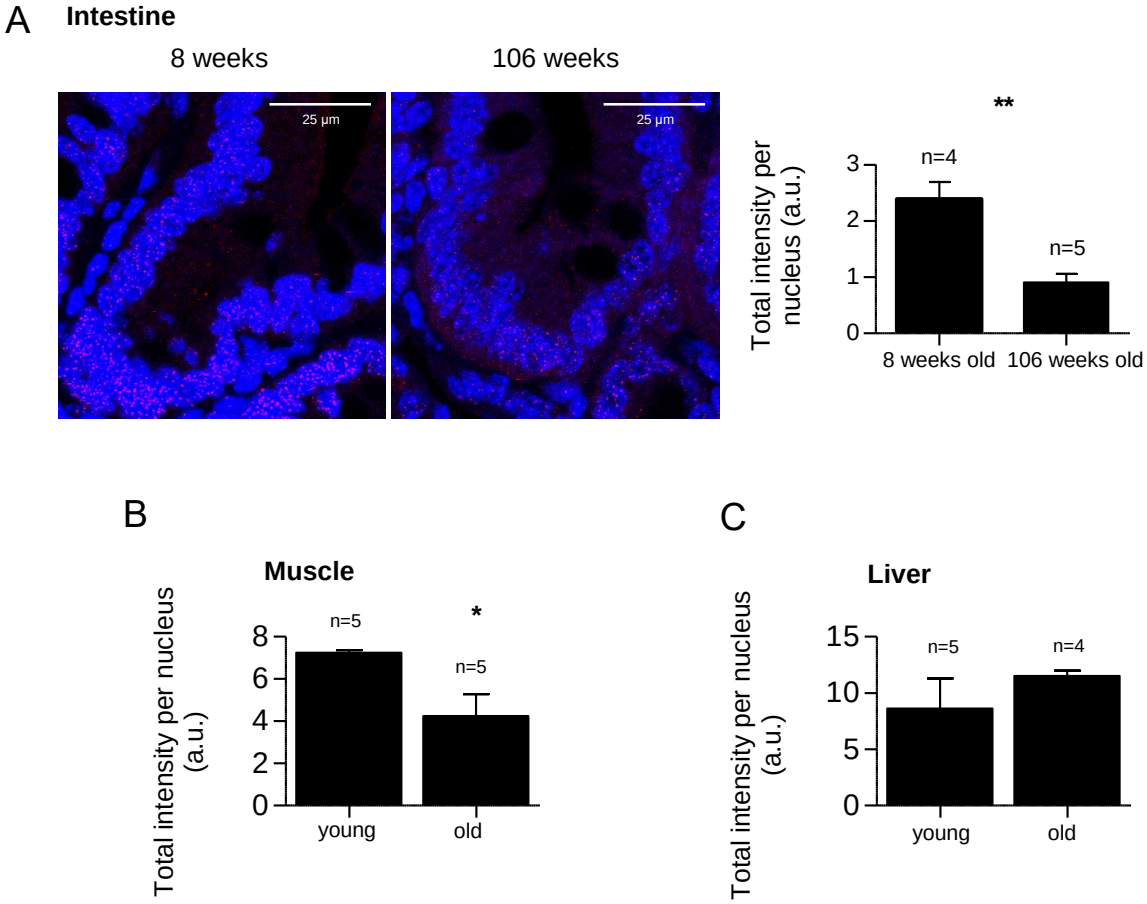


Figure 3. Decrease of TRF1 protein levels with aging in mouse intestine and TRF1 protein levels in post-mitotic tissues. (A) Representative images of TRF1 (in red) and DAPI (in blue) and quantification for TRF1 immunofluorescence analysis in mice of 8 weeks and 106 weeks old in intestine. **(B-C)** Mean TRF1 protein levels in mice at young (8 weeks) and old (> 2 years) ages in muscle (B) and liver (C). Expression levels are presented as total intensity per nucleus. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice. For each experiment, images were acquired with the same resolution and exposure parameters.

2. AAV9-TRF1 VECTORS DEMONSTRATE HIGH TRANSDUCTION EFFICIENCY AND LEAD TO INCREASED TRF1 LEVELS IN MULTIPLE MOUSE TISSUES

As TRF1 levels decrease with aging in mice and humans, we next set to study whether we could rescue phenotypes associated with aging *in vivo* by increasing TRF1 expression in adult and old mice. As we previously described that constitutive TRF1 over-expression in transgenic mice leads to XPF-dependent telomere shortening (Muñoz et al. 2009), here we set to over-express TRF1 in a moderate and transient manner. To this

end, we used recombinant AAV vectors, which are non-integrative (Ayuso et al. 2010), thus leading to a transient TRF1 overexpression. In particular, we used the AAV9 serotype, also previously used by us to deliver the TERT telomerase gene to many different adult tissues in mice (Bär et al. 2014; Bar et al. 2016; Bernardes de Jesus et al. 2012). AAV9 has a number of attractive properties such as poor immunogenicity, high transduction efficiency in a wide range of tissues, and the ability to cross the blood-brain barrier (Ayuso et al. 2010).

First, to assess the efficiency of transduction and expression of the AAV9-TRF1 vector, we intravenously injected 8.5 month-old mice with AAV9-TRF1 and 3 weeks post-injection determined TRF1 mRNA and protein levels in various tissues (**Fig. 4, 5A, 7**).

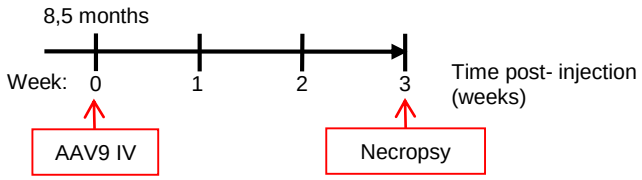


Figure 4. Scheme of mice injection for the transduction efficiency study.

Upon AAV9-TRF1 transduction we found significantly upregulated TRF1 mRNA levels in the liver, heart, muscle, and brain (**Fig. 5A)**. However, we did not see a significant increase of the rest of the shelterin components (**Fig. 5B**). We also found increased TRF1 mRNA levels in kidney, bone marrow, lung and intestine, although in these cases the differences did not reach significance (**Fig. 5A)**.

By using immunofluorescence analysis with anti-TRF1 antibodies, we confirmed TRF1 protein overexpression both when measuring mean TRF1 fluorescence levels and the percentage of nuclei with “high TRF1” levels. In particular, cells were designated as “high TRF1” if the TRF1 levels were above the 99th percentile determined from the control group (**Fig. 6**).

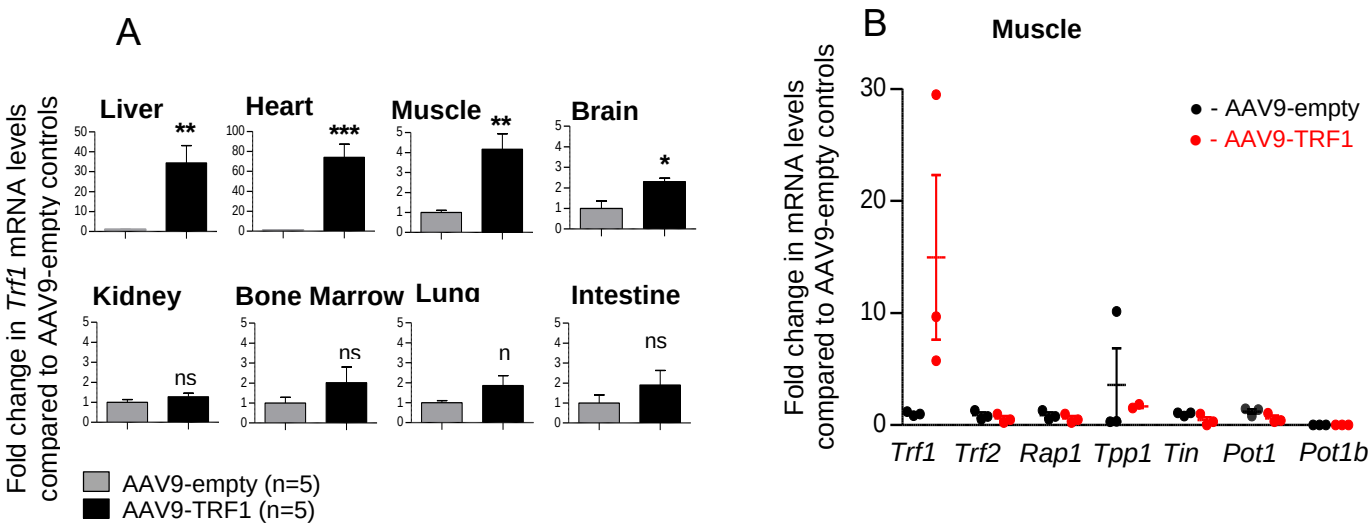


Figure 5. AAV9-TRF1 transduction efficiency, determined by RT-qPCR, and mRNA levels of other shelterins upon TRF1 overexpression.
(A) *Trf1* mRNA levels determined by RT-qPCR in several murine tissues injected with AAV9-TRF1 vector compared to mice injected with AAV9-empty vector (set to 1). 5 mice per group were used. *Trf1* mRNA values are normalized to GAPDH. **(B)** Trf1, Trf2, Rap1, Tpp1, Tin2, Pot1a and Pot1b mRNA levels determined by RT-qPCR in muscle from mice injected at 1 year of age with AAV9-TRF1 compared to AAV9-empty determined at humane time point. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates a number of mice.

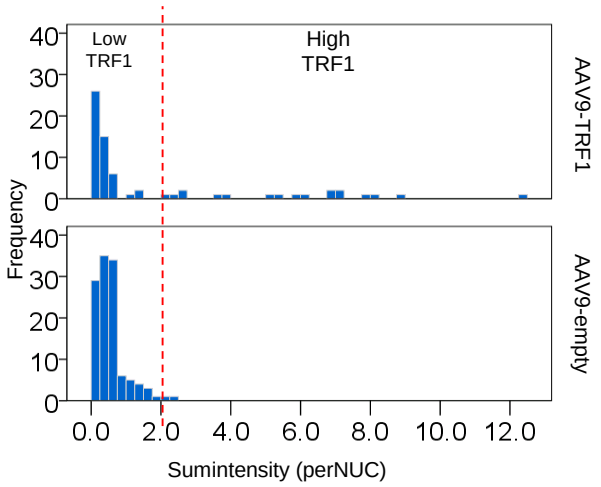


Figure 6. "High TRF1" nuclei designation. Histogram of the distribution of nuclei by their sum intensities calculated in muscle tissue of mice injected with AAV9-TRF1 and AAV9-empty. Nuclei with "High TRF1" levels are determined as nuclei with intensities above the 99th percentile of the control group.

We found increased TRF1 protein levels in liver, heart, muscle, and lung, when using both parameters (**Fig. 7**). This pattern of overexpression of TRF1 using AAV9 is consistent with previous reports (Schuster et al. 2014; Zincarelli et al. 2008; Bernardes de Jesus et al. 2012). Thus, by using AAV9-TRF1 vectors we can efficiently deliver TRF1 to different adult tissue types in the mouse.

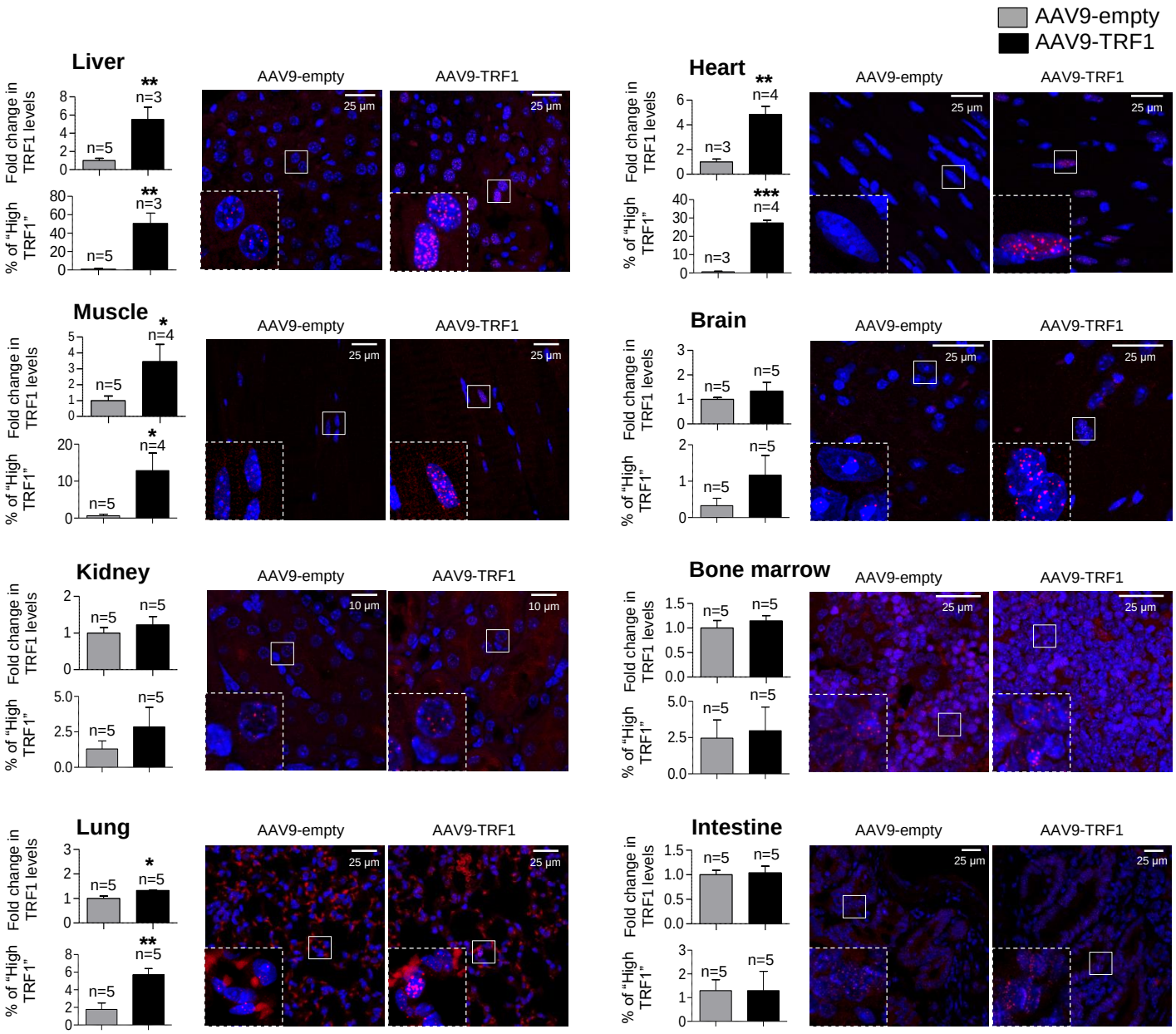


Figure 7. TRF1 expression efficiency upon transduction via AAV9 vector.
Representative images of TRF1 (in red) and DAPI (in blue) and quantification for TRF1 protein level and percentage of nuclei expressing "high TRF1" levels, measured by immunofluorescence analysis in various murine tissues in mice injected with AAV9-TRF1 vector compared to AAV9-empty vector (set to 1). Amplified images are shown in the insets. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates a number of mice. Within each tissue images were acquired with the same resolution and exposure parameters.

3. AAV9-TRF1 GENE THERAPY DELAYS PHYSIOLOGICAL MOUSE AGING

To study whether AAV9-TRF1 treatment was able to delay aging and age-related phenotypes in mice, middle aged (1 year old) and old (2 years old) mice were intravenously injected with a single dose of either AAV9-TRF1 or AAV9-empty vectors. Upon treatment with the vectors, the mouse cohorts were longitudinally followed to determine different parameters of aging, cancer, as well as overall survival. Telomere length in blood samples was also determined longitudinally as a molecular biomarker of aging. At the humane end point mice were sacrificed and a full histopathological analysis was performed (**Fig. 8**).

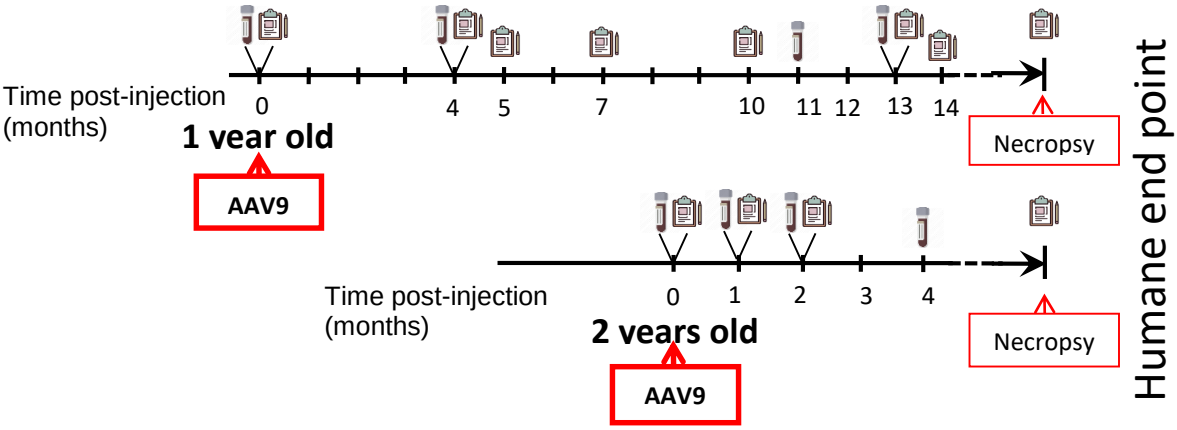


Figure 8. Scheme of mice injection for the study of effect of AAV9-TRF1 gene therapy on mouse aging.
Two groups of mice were intravenously injected with AAV9-TRF1 or a control AAV9-empty vector (AAV9 VI) at two different ages: middle age (1 year old) and old (2 years old). Aging studies ( ) and blood sampling ( ) were longitudinally performed at different time points, indicated as months post-injection. Mice were sacrificed when they reached a humane end point and the full necropsy was performed.

Neuromuscular coordination

Progressive loss of neuromuscular function is a characteristic of aging (Ingram & Reynolds 1986). Thus, we first evaluated neuromuscular function in AAV9-TRF1 treated mice compared to AAV9-empty treated controls in 2 sets of experiments: the tightrope test and rotarod test. Mice treated with AAV9-TRF1 at 1 year of age showed a statistically significant improvement of neuromuscular coordination in the tightrope test at 7 months post-injection compared to controls treated with the empty vector, and this difference was maintained at later time-points although the results did not reach statistical significance (**Fig. 9A**). A similar trend was also observed in the 2-year old group although it did not reach significance (**Fig. 9B**). In the rotarod test, we also found a trend for

improved performance in the 1-year old group treated with AAV9-TRF1 compared to the empty-vector treated group, although the differences did not reach significance (**Fig. 9C**). Thus, AAV9-TRF1 treatment improved neuromuscular coordination in the 1-year old group, and these differences were lower in the 2 year old group.

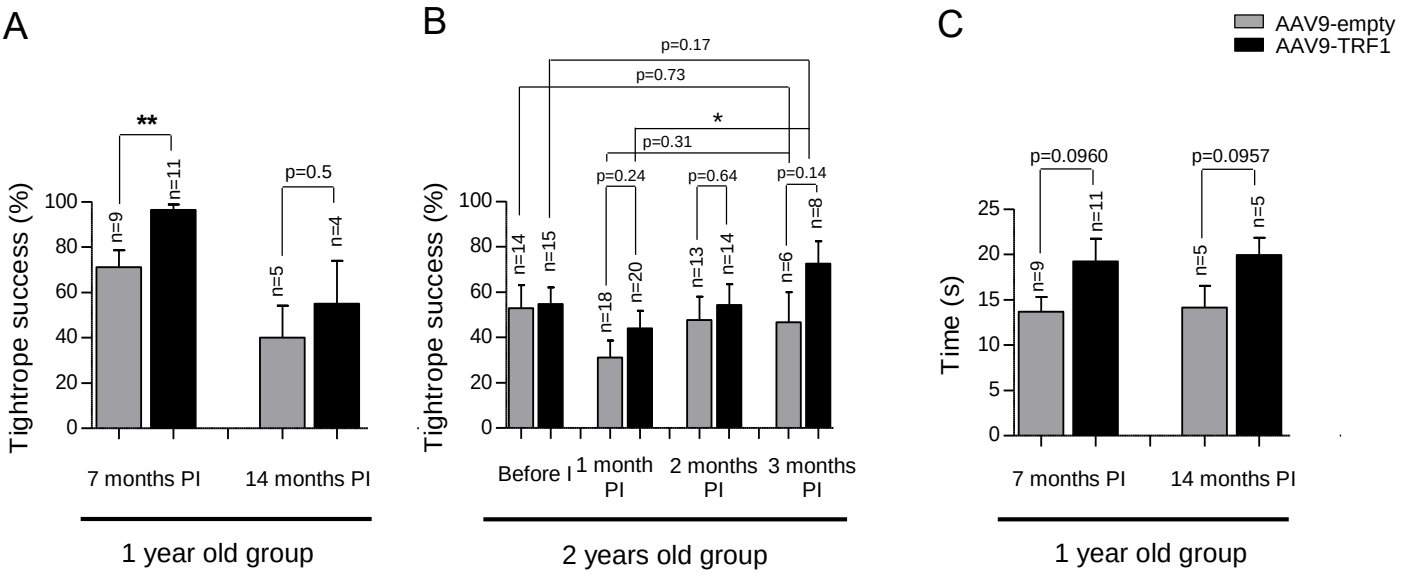
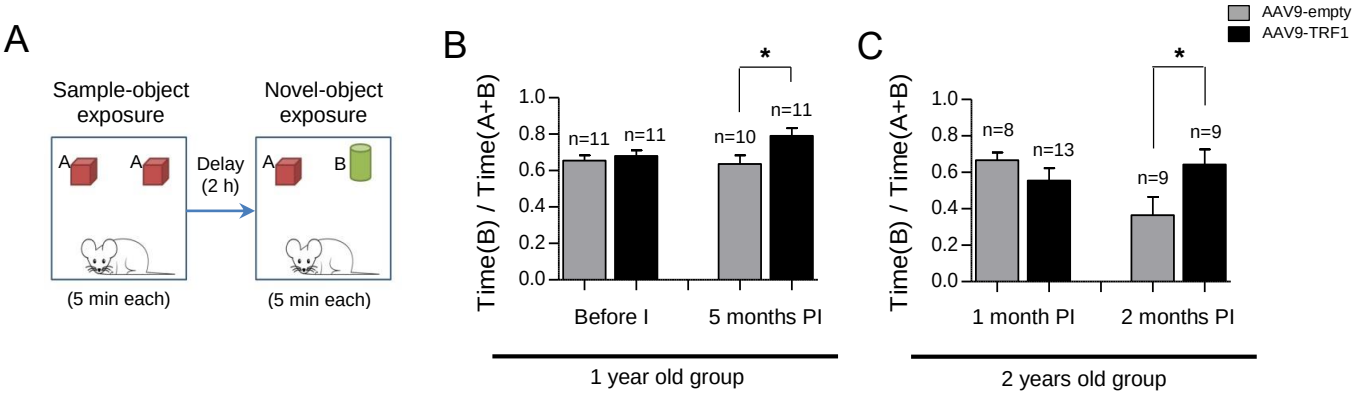


Figure 9. AAV9-TRF1 gene therapy improves neuromuscular coordination.
(A-B) Neuromuscular coordination using tightrope test in 1 year old (A) and 2 years old (B) mice injected with AAV9-TRF1 compared to AAV9-empty vector. **(C)** Neuromuscular coordination using rota-rod test in 1 year old mice. Time-points at time post-injection (PI) are indicated. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice.

Cognitive impairment

As AAV9 vectors can cross the blood-brain barrier and we indeed found TRF1 mRNA levels increased in the brain (**Fig. 5A**), we next investigated the effects of AAV9-TRF1 gene therapy on cognitive function. To this end we used the so-called object recognition test, which is widely used to assess cognitive decline associated with aging (**Fig. 10A**) (Scali et al. 1997). At 5 months post treatment with AAV9-TRF1, 1-year old mice showed improvement in recognition memory scores compared to mice treated with the empty vector (**Fig. 10B**). We also observed improved cognitive function in the 2-year old mice treated with AAV9-TRF1 vectors at 2 month post-injection compared to mice treated with the empty vector (**Fig. 10C**). Thus, AAV9-TRF1 gene therapy significantly ameliorates memory scores in both the 1 year old and 2 year old groups.

Figure 10. AAV9-TRF1 gene therapy improves memory scores.
(A) Design of the object recognition test. **(B-C)** Object recognition test in 1 year old (B) and 2 years old (C) mice. Results show the ratios of time spent investigating the new object *vs.* the total time spent with both objects. Time-points at time post-injection (PI) are indicated. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice.



Skin aging

Another characteristic of aging is the loss of the subcutaneous fat layer, which increases the risk of skin injury, reduces the ability to maintain body temperature, and raises the probability of infection (Shimokata et al. 1989). Thus, we measured the thickness of the subcutaneous fat layer at the time of death of mice in the 2 year-old group. Interestingly, mice from the 2 year-old group treated with AAV9-TRF1 showed a significantly thicker fat layer than the empty vector treated controls at their time of death (**Fig. 11A-B**), again showing a beneficial effect of AAV9-TRF1 therapy.

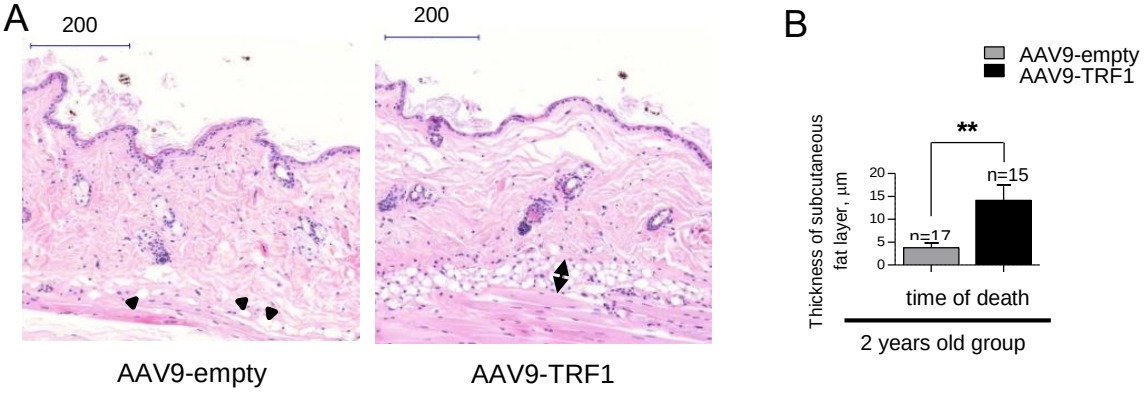


Figure 11. AAV9-TRF1 gene therapy delays age-associated subcutaneous fat loss.
(A) Representative hematoxylin-eosin images of a back skin section of AAV9-empty and AAV9-TRF1 injected mice. In the image of the AAV9-empty injected mouse, black arrows indicate residual adipocytes. In the image of the AAV9-TRF1 injected mouse, a black arrow indicates a subcutaneous fat layer. **(B)** Thickness of the subcutaneous fat layer of mice injected with AAV9-empty and AAV9-TRF1 at 2 years old at the time of death. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice.

Age-related osteoporosis

Bone deterioration is a well-characterized sign of the aging process both in mice and humans (Ferguson et al. 2003). It results from age related shift of the bone homeostasis to the direction of the bone resorption by osteoclasts, leading to osteoporosis and a high risk to bone fractures (Demontiero et al. 2012). As expected, we observed a significant decline of bone mineral density (BMD) with age, measured in femur (**Fig. 12A**), when monitoring mice injected with the empty vector at 1 year of age over a long period of time (**Fig. 12B**). However, we did not observe any changes when treating mice with AAV9-TRF1 vector at both aging cohorts (**Fig. 12C**), meaning that TRF1 gene therapy does not affect bone homeostasis under these experimental conditions.

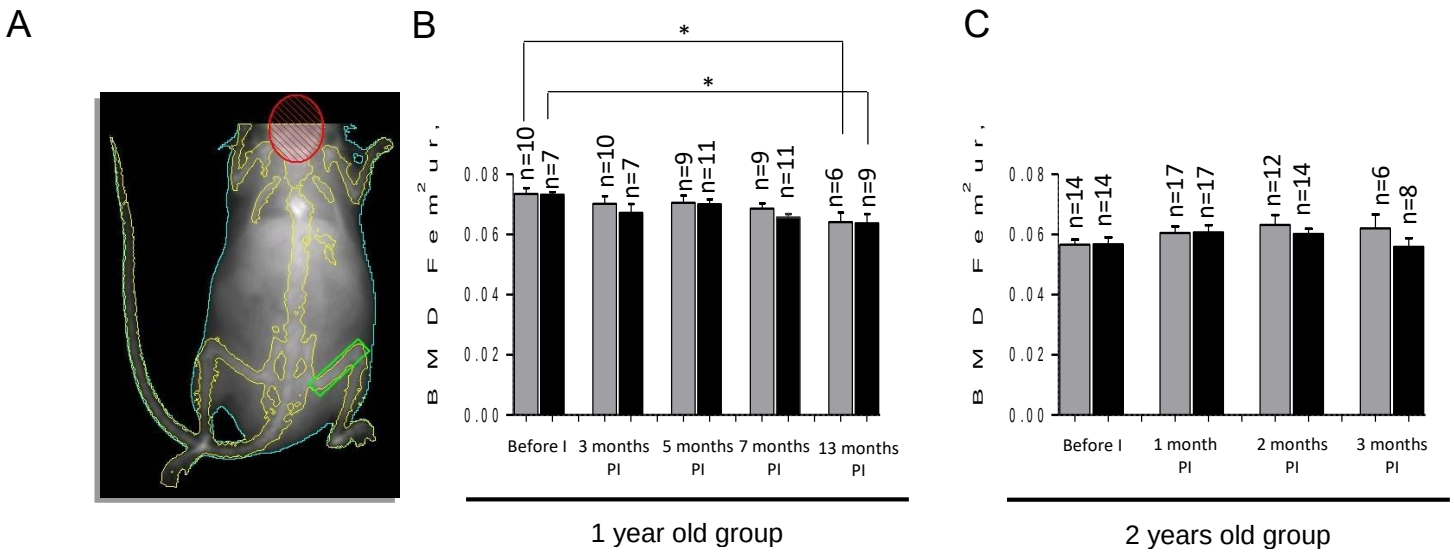


Figure 12. AAV9-TRF1 gene therapy does not prevent age-related osteoporosis.
(A) Representative image of a DEXA scan for measurements of bone mineral density in femur (framed in green) **(B-C)** Femur bone mineral density (BMD femur) measured in 1 year old (B) and 2 years old (C) mice. Time-points at time post-injection (PI) are indicated. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice.

Age related anemia

Blood chemistry and composition vary with the aging process. Anemia is a common chronic condition appearing with age (Ferrucci & Balducci 2008; Berliner 2013; Patel 2008). Anemia can be caused by various age-related events occurring in the organism, such as telomere shortening (Herrera et al. 1999; Beier et al. 2012). Thus, to

study the effects of AAV9-TRF1 treatment on blood changes with age, we followed longitudinally red blood cell (RBC) counts in both the AAV9-TRF1 and AAV9-empty treated cohorts. To this end, we collected blood at different time points in both groups of mice. As expected, we found a decline in RBCs with age in both 1 year and 2 year-old groups treated with the empty vector (**Fig. 13A, 13D**). We also found a decrease in hemoglobin (Hb) and hematocrit (Hct) levels in both age groups (**Fig. 13B-C, 13E-F**). Interestingly, mice treated with AAV9-TRF1 at 1 year of age showed significantly higher RBC counts compared to the control at late time points (**Fig. 13A**). Analogous improvement was also noticed in the hemoglobin (**Fig. 13B**) and hematocrit (**Fig. 13C**) levels. A similar trend was also observed in mice injected at 2 years of age, although differences did not reach statistical significance in this old group (**Fig. 13D-F**). These observations suggest that AAV9-TRF1 treatment improves the chronic anemia condition acquired during mouse physiological aging.

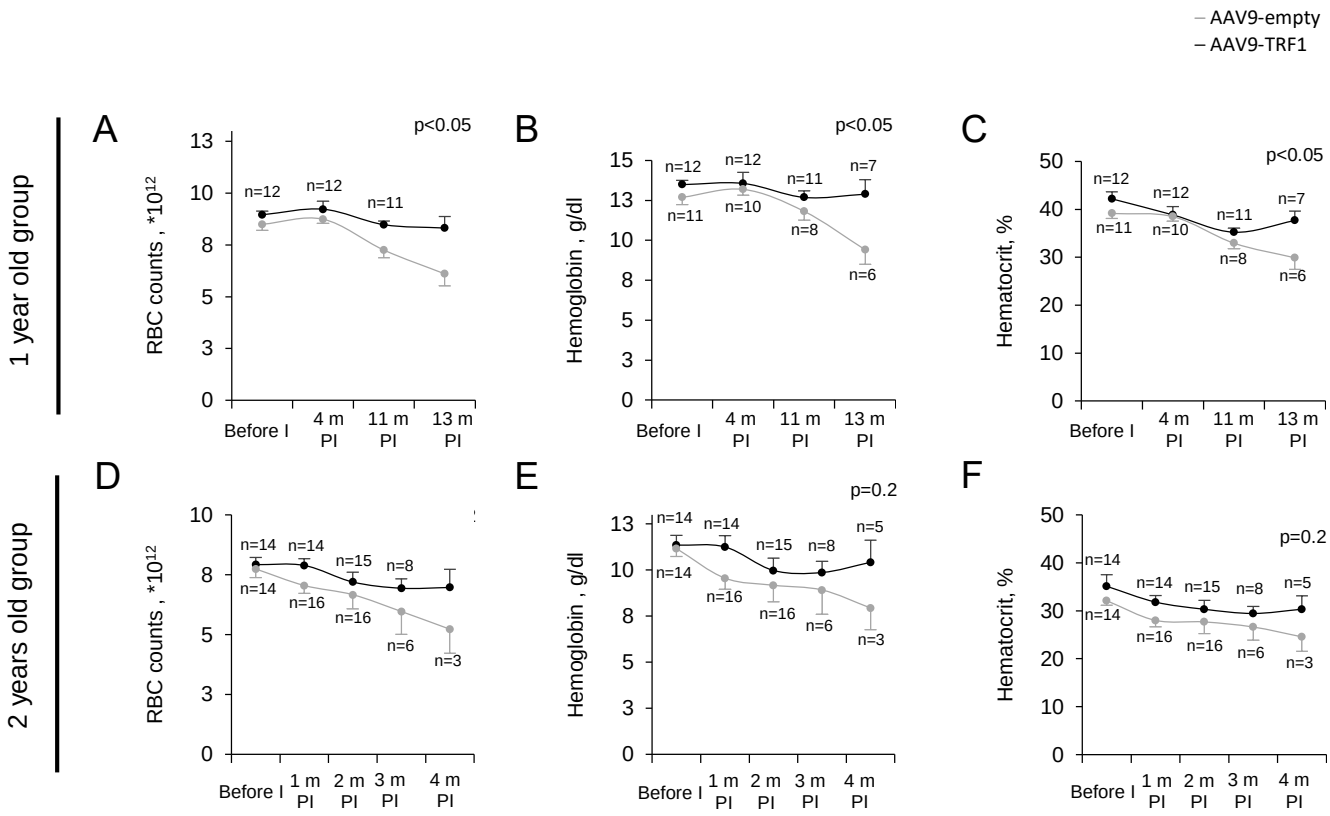


Figure 13. AAV9-TRF1 gene therapy rescues age-related anemia.
(A-F) Blood analysis for mice injected with AAV9 vectors at 1 year old (A-C) and at 2 years old (D-F). Red blood cell count (RBC) changes (A, D), hemoglobin levels (g/dl) (B,E) and hematocrit percentage (C, F). Time-points at time post-injection (PI) are indicated. Student's t-test was used for statistical analysis. Error bars represent SEM. n indicates the number of mice.

Glucose Intolerance

Glucose homeostasis likewise becomes dysregulated with age (Bailey & Flatt 1982). An association of impaired insulin secretion and glucose intolerance with short uncapped telomeres has been established (Kuhlow et al. 2010). In particular, short or dysfunctional telomeres trigger a DNA damage response, which can lead to cellular senescence of adult islet beta-cells, thus resulting in dysregulation of insulin secretion. Here, we studied whether AAV9-TRF1 treatment can provide better telomere protection and decrease glucose homeostasis dysregulation associated with physiological aging. To assess glucose tolerance, we performed an intraperitoneal glucose tolerance test (IP-GTT). We observed a significant improvement in glucose tolerance at 10 months post-injection in the 1-year old AAV9-TRF1 treated mice compared to control mice treated with the empty vector (**Fig. 14A-F**).

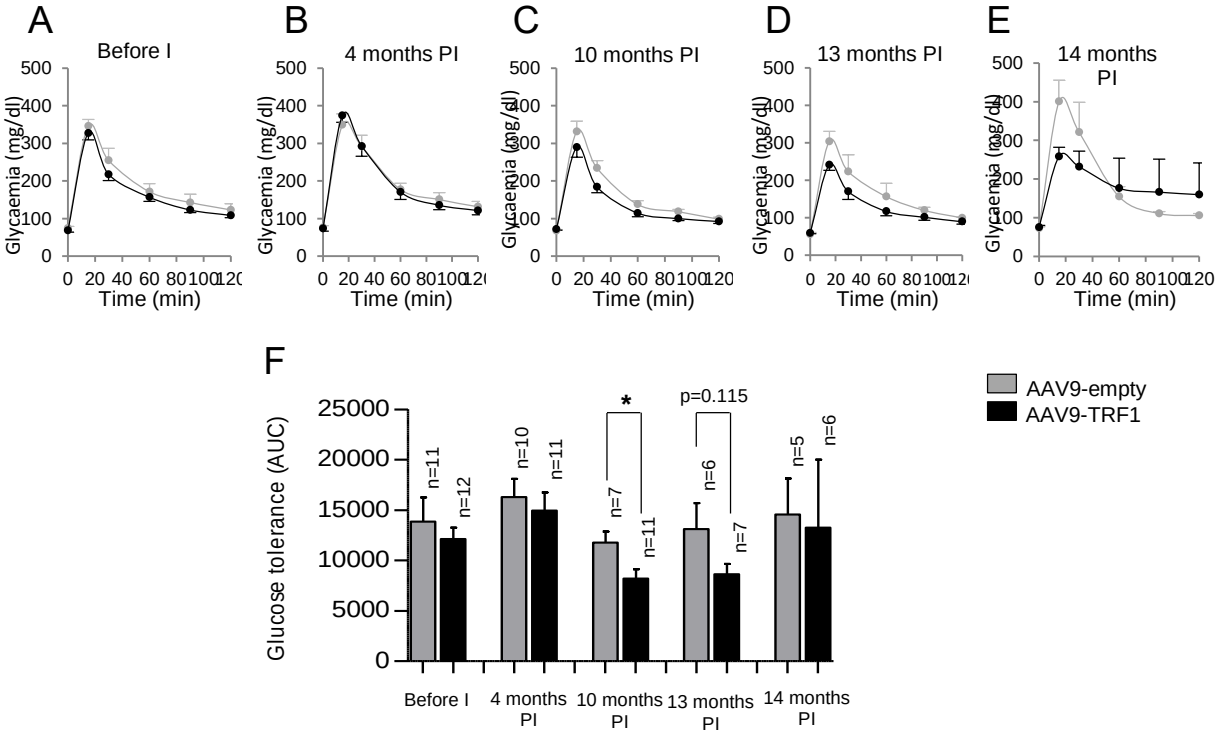


Figure 14. AAV9-TRF1 gene therapy delays glucose homeostasis dysregulation.
(A-F) Intraperitoneal glucose tolerance test (IP-GTT) performed on mice injected with AAV9-TRF1 and AAV9-empry at 1 year of age. Glucose curves before injection (A), at 4 months post-injection (B), 10 months post-injection (C), 13 months post-injection (D), 14 months post-injection (E) and AUC (area under the curve) values at these time-points (F) are represented. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice.

The same tendency was seen in the fasting insulin level, which is considered to be an indicator of insulin resistance and this level increases with age (Lindberg et al. 1997).

Levels of fasting insulin showed a lower trend in AAV9-TRF1 injected mice compared to controls at late time points post-injection (**Fig. 15A**). Finally, AAV9-TRF1 injected mice also showed a trend of improvement in homeostatic model assessment (HOMA-IR) compared to control mice treated with the AAV9-empty vector (**Fig. 15B**), again suggesting improved insulin sensitivity in the AAV9-TRF1 treated cohorts (Heikkinen et al. 2007b). Together, these findings suggest that AAV9-TRF1 gene therapy can improve glucose intolerance associated with age

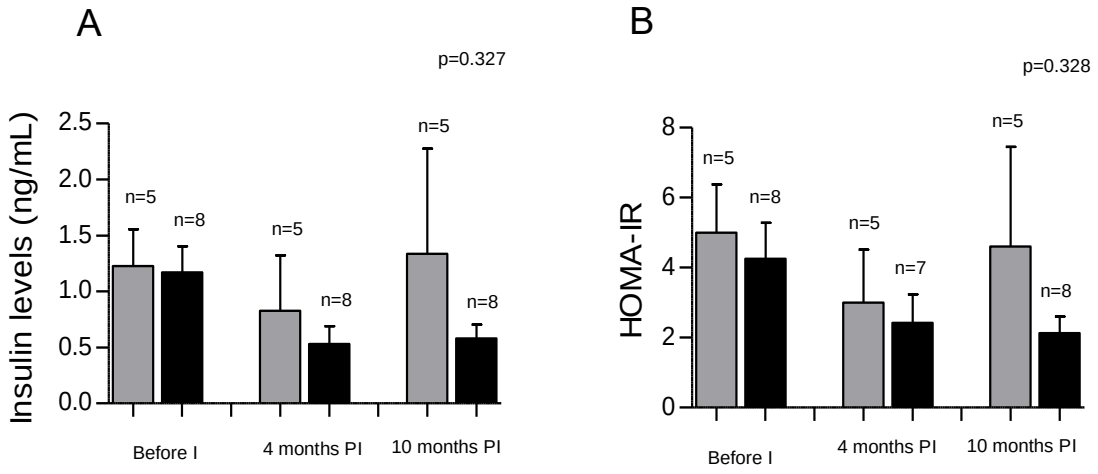


Figure 15. Effects of AAV9-TRF1 gene therapy on insulin sensitivity.
(A) Fasting insulin levels measured in AAV9-TRF1 mice compared to controls, injected at 1 year of age before injection, at 4 and 10 months post-injection. **(B)** HOMA-IR assessment using previous data on IP-GTT and on fasting insulin levels. Student's t-test was used for statistical analysis. Error bars represent SEM. n indicates the number of mice.

Correlation of TRF1 overexpression levels in mice with their performance in aging studies

To study whether the mice expressing high levels of TRF1 were also the ones showing less age-related pathologies, we determined the percentage of cells showing high TRF1 expression in muscle. We found that the percentage of cells with high TRF1 expression moderately correlated with improvement in the tightrope test (R=0.59; **Fig. 16A**) as well as in the object recognition test (R=0.51; **Fig. 16B**). There was also a strong correlation between the % of cells with high TRF1 expression and red blood cell counts (R=0.98; **Fig. 16C**) but not with the AUC in the glucose tolerance test (**Fig. 16D**).

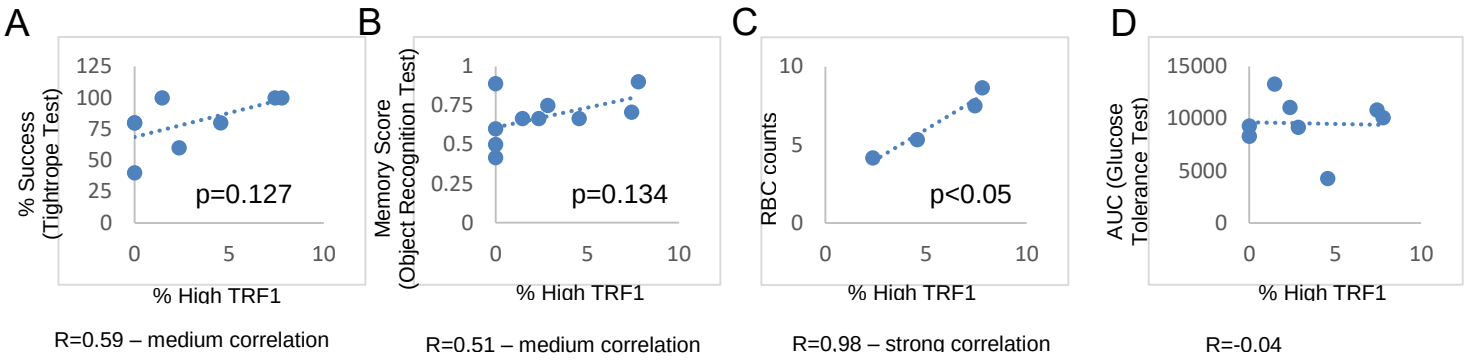


Figure 16. Correlation between percentage of nuclei overexpressing high TRF1 levels and readouts of aging.
(A-D) Correlation analysis between percentage of nuclei overexpressing high TRF1 levels and readouts of aging: success in tightrope test (A), memory function (B), RBC counts (C) and glucose tolerance (D). R indicates correlation coefficient.

4. AAV9-TRF1 TREATMENT IS NOT LEADING MICE TO BE TUMOR PRONE

Increasing age is the highest risk factor for cancer development both in humans and mice (White et al. 2014; Pawelec 2017). Furthermore, evidence suggests that TRF1 overexpression may favour tumorigenesis. TRF1 is upregulated in some human cancers (Matsutani et al. 2001; Oh et al. 2005). Moreover constitutive TRF1 over-expression in epithelial tissue on transgenic mice (K5TRF1 mice) led to moderately increased levels of chromosomal aberrations and chemically induced skin tumorigenesis (Muñoz et al. 2009). Therefore, we addressed effects of TRF1 increased expression in our experimental settings on tumor formation with aging. To assess the effects of AAV9-TRF1 gene therapy on cancer incidence, we performed a full pathological analysis at the time of death in both AAV9-TRF1 and AAV9-empty treated mouse cohorts. Mice treated with AAV9-TRF1 at 1 and at 2 years of age showed a tendency to show lower cancer incidence compared to the empty-vector treated mice, although the differences did not reach significance (**Fig. 17A-B**). This illustrates that increased TRF1 expression is not rendering these mice to be tumor prone.

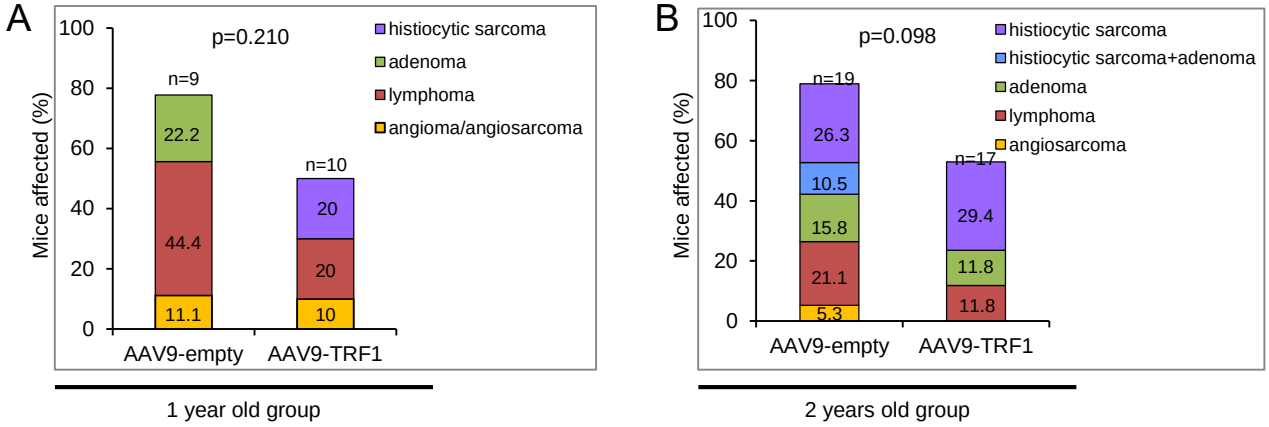


Figure 17. AAV9-TRF1 treatment does not affect cancer incidence in aging mice.
(A-B) Percentage of mice which developed tumors, for mice injected at 1 year (A) and at 2 years (B) of age.

5. AAV9-TRF1 TREATMENT DOES NOT AFFECT MEDIAN SURVIVAL

Another indication of absence of deleterious secondary effects associated to increased TRF1 expression is provided by the fact that median survival did not differ between control and AAV9-TRF1 treated mice in any of the age groups (**Fig. 18A-B**). Interestingly, mice injected with AAV9-TRF1 at 2 years of age showed increased maximum lifespan compared to the controls when comparing the longest-lived mice in each cohort (**Fig. 18B**).

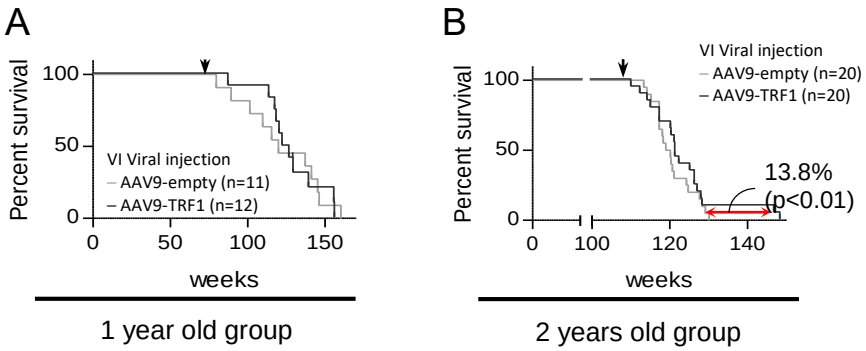


Figure 18. AAV9-TRF1 treatment does not affect median life span in aging mice.
(A-B) Survival curves for mice injected with AAV9-TRF1 compared to AAV9-empty vectors at 1 year (A) and 2 years (B) old. The determined difference in maximum survival for the 10% longest lived mice for mice injected at 2 years of age. Student's t-test was used for statistical analysis. n indicates the number of mice.

6. AAV9-MEDIATED TRF1 OVEREXPRESSION IS MAINTAINED WITH AGE

Previous reports have shown that AAV9 vectors allow for a long-term expression of the genes that they carry (Nizzardo et al. 2015; Bernardes de Jesus et al. 2012). To address whether AAV9-TRF1 treatment allowed for long term TRF1 expression, we compared TRF1 mRNA and protein levels at the humane endpoint in our 2-year old mouse cohort in which all mice had been already sacrificed. We found significantly higher TRF1 mRNA levels in the pancreas of AAV9-TRF1 treated mice compared to the controls (**Fig. 19A**). We also found significantly higher TRF1 protein levels as determined by immunofluorescence in several mouse tissues, such as liver and muscle (**Fig. 19B-C**). Interestingly, in agreement with the fact that AAV9 vectors are non-integrative and dilute as the cells divide, we noticed a decreased concentration of AAV9-TRF1 targeted cells with aging in all tissues (**Fig. 6, 19E-F**), suggesting that even though TRF1 overexpressing cells are present, they are present in lower numbers as the tissues age. For this reason, we set to calcule the percentage of nuclei expressing high TRF1 levels,

which reflects the cells maintaining high TRF1 expression at different time points. In most of the tissues analyzed, we identified significantly higher abundance of nuclei with “high TRF1” levels in AAV9-TRF1 injected mice compared to the controls (**Fig. 19B-F**). Thus, in muscle tissue of mice injected at 1 year of age where we could not detect any difference comparing mean TRF1 levels between the AAV9-TRF1 treated mice and the empty-vector controls. We readily detected a highly significant increase in “high TRF1” nuclei in the AAV9-TRF1 treated mice compared to the controls, confirming that at late time points TRF1 is still overexpressed (**Fig. 19E**), however in fewer cells than at earlier time points (**Fig. 7**).

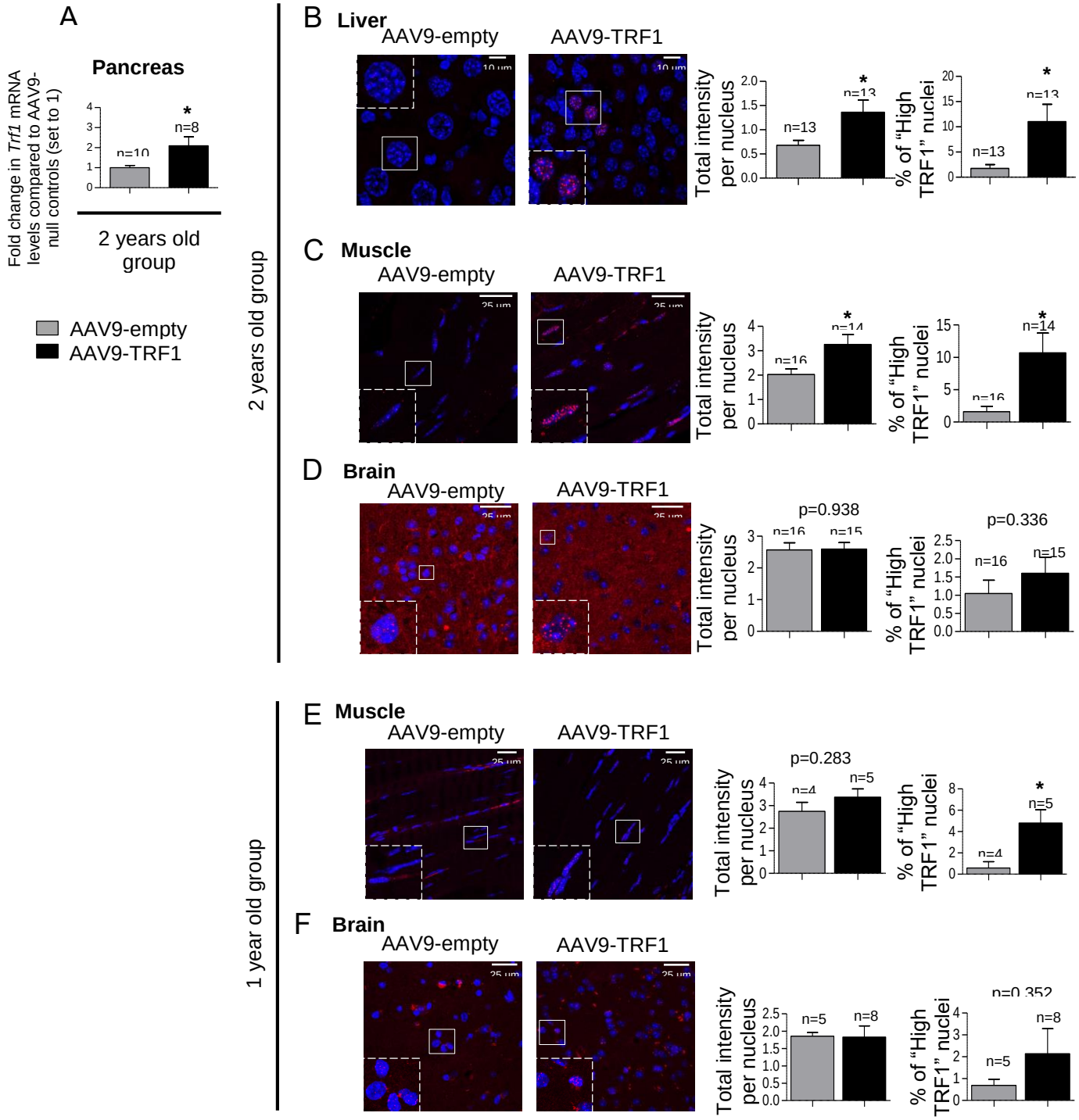


Figure 19. Sustained TRF1 overexpression upon AAV9-TRF1 treatment in mice.
(A) Trf1 mRNA levels determined by RT-qPCR in pancreatic tissue in mice injected at 2 years of age with AAV9-TRF1 vector compared to mice injected with AAV9-empty vector (set to 1). Trf1 mRNA values are normalized to GAPDH. (B-D) Representative images of TRF1 (in red) and DAPI (in blue), mean TRF1 expression levels and percentage of "high TRF1" nuclei in mice injected with AAV9-empty and AAV9-TRF1 vectors at 2 years of age measured at the humane end-point in liver **(B)**, muscle **(C)** and brain **(D)**. **(E-F)** Representative images of TRF1 (in red) and DAPI (in blue), mean TRF1 expression levels and percentage of "high TRF1" nuclei in mice injected with AAV9-empty and AAV9-TRF1 vectors at 1 year of age, measured at the humane end-point in muscle (E) and brain (F). Expression levels are presented as total intensity per nucleus. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice.

7. *AAV9-TRF1 GENE THERAPY DOES NOT HAVE NEGATIVE EFFECTS ON TELOMERE LENGTH AND PROVIDES PROTECTION FROM THE ACCUMULATION OF SHORT TELOMERES*

Next, we set to address the long-term effects of AAV9-TRF1 gene therapy on telomere length. We first performed a longitudinal study on telomere length dynamics in blood peripheral leukocytes using the high-throughput Q-FISH (HT Q-FISH) technique. We did not observe any significant difference in the mean telomere length or percentage of short telomeres between 1-year-old injected experimental mice and the control groups not in PMBC (**Fig. 20A-B**) neither in other tissues (**Fig. 21B-C**).

Q-FISH performed on muscle tissue of mice injected at 2 years of age also didn't show any difference in the mean telomere length (**Fig. 21A**). Interestingly, the percentage of short telomeres, determined here within a control group as telomeres with intensities lower than the 25th percentile, was lower in muscle tissue of mice injected with AAV9-TRF1 compared to controls (**Fig. 21A**). These results highlight that TRF1 overexpression through AAV9-TRF1 gene therapy does not trigger XPF-mediated telomere shortening, as it was observed in the case of constitutive TRF1 overexpression in a transgenic mouse model (Muñoz et al. 2009). Moreover, TRF1 overexpression in muscle tissue led to better protection of telomeres, delaying the accumulation of the short telomeres.

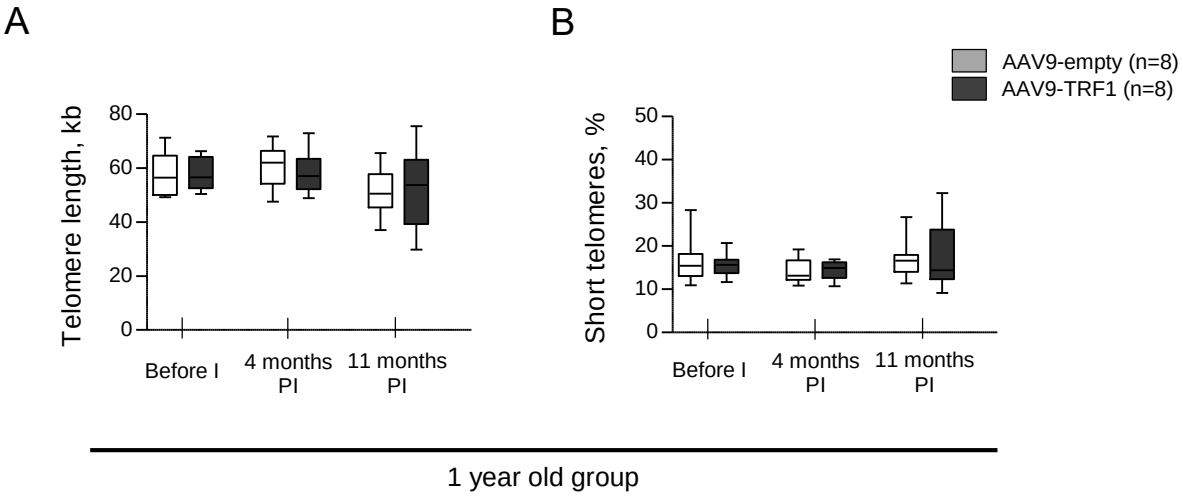


Figure 20. AAV9-TRF1 gene therapy has no effect on mean telomere length and number of short telomeres in peripheral white blood cells.
(A-B) Characterization of telomere length measured by high-throughput Q-FISH (HT Q-FISH). Comparison of mean telomere length (A) and percentage of short telomeres (B) measured in peripheral white blood cells in mice injected at 1 year of age with AAV9-TRF1 vector compared to the control. Time-points at time post-injection (PI) are indicated Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice.

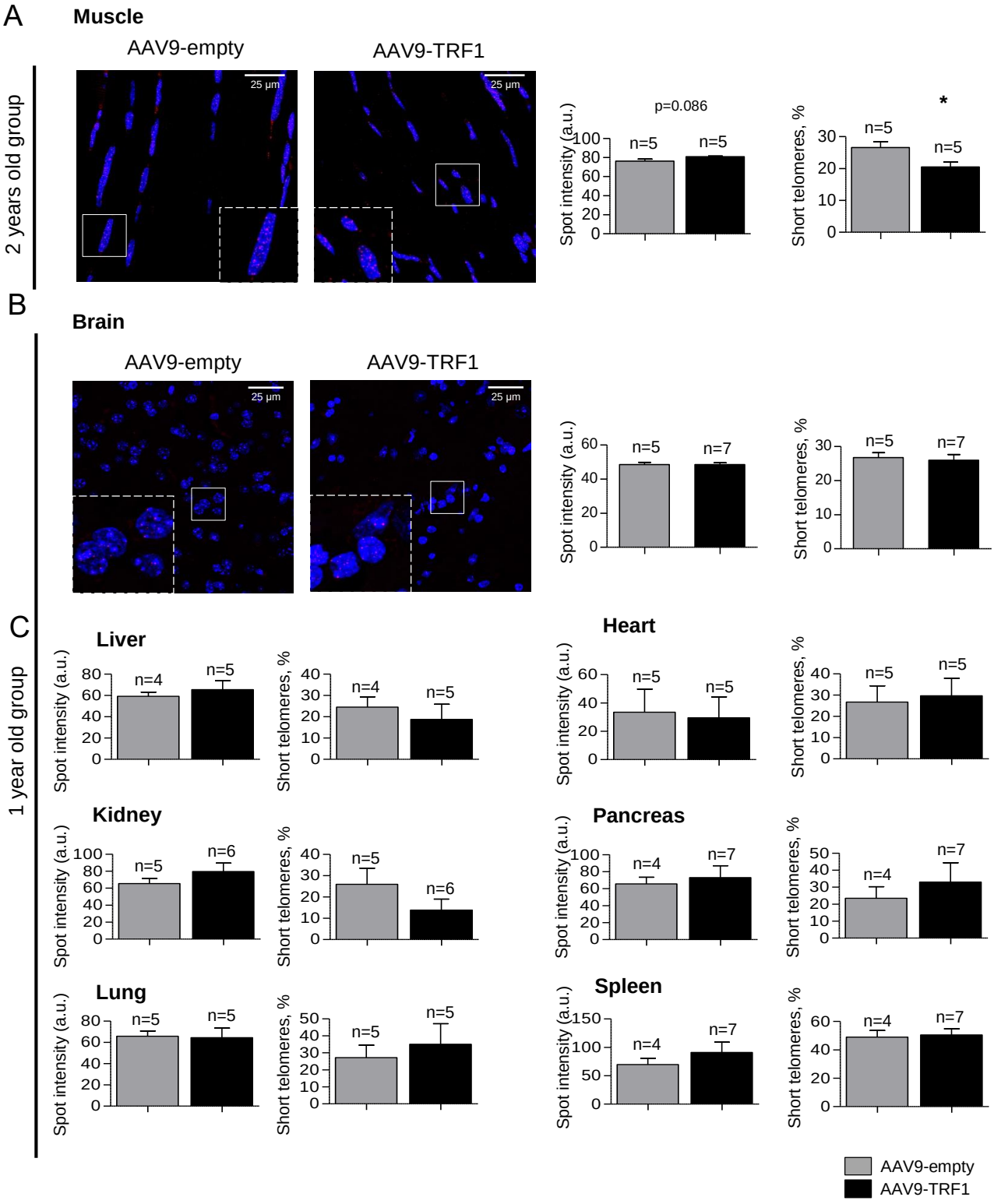


Figure 21. AAV9-TRF1 gene therapy has no effect on mean telomere length and rescues accumulation of short telomeres in mouse tissues.

(A-B) Representative images of telomere probe Cy3 (in red) and DAPI (in blue) and quantification of telomere length and percentage of short telomeres measured in muscle in mice injected with virus vectors at 2 years of age (A) and in brain of mice injected at 1 year of age (B). **(C)** Quantification of telomere length and percentage of short telomeres measured in mice injected with virus vectors at 1 year of age determined in liver, kidney, lung, heart, pancreas, spleen. Analysis performed at the time of death. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice.

8. AAV9-TRF1 INCREASES TELOMERE PROTECTION IN THE HIPPOCAMPUS AREA OF THE BRAIN OF MICE

Based on the finding that AAV9-TRF1 gene therapy was able to delay the accumulation of short telomeres, we further addressed the question of whether it also prevents DNA damage at dysfunctional telomeres. Since an improvement in recognition memory was observed, the hippocampus was further investigated. The hippocampus is a brain structure that plays a central role in the formation of new memories (Cohen & Eichenbaum 1993). The hippocampus exhibits neurogenesis, which continues through adulthood in a substructure named the dentate gyrus (Gross 2000). Aging and other conditions affect processes of neurogenesis, and this leads to impairment of cognitive function (Drapeau et al. 2003; Jin et al. 2003). As TRF1 is important for telomere protection, and moreover plays a role in stem cell function, we proposed that its deficiency may have molecular consequences and affect the stem cell pool of the dentate gyrus, thus leading to impaired neurogenesis and memory with age. In this case, AAV9-TRF1 gene therapy may rescue this impairment to some extent.

We first compared the DNA damage status in the brain of mice injected with AAV9-TRF1 and control vector at the adult age. Total DNA damage was determined as area covered by 53BP1 foci. We did not observe any difference in total DNA damage in the whole brain, neither in the dentate gyrus area or the hippocampus (**Fig. 22A-D**). Next, DNA damage at telomeres was determined by immuno-FISH analysis as the number of TIFs (Telomere Dysfunction Induced Foci) – spots of co-localization of 53BP1 foci and telomere probe Cy3 foci. Although we did not see differences in whole brain sections (**Fig. 22A, 22E**), we observed significantly less TIFs in the dentate gyrus of mice treated with AAV9-TRF1 gene therapy compared to the empty-vector group (**Fig. 22B, 22F**). Thus, AAV9-TRF1 gene therapy seems to prevent DNA damage at telomeres in the dentate gyrus, which may have led to the improvement of the memory function observed in the object recognition test (**Fig. 10B-C**).

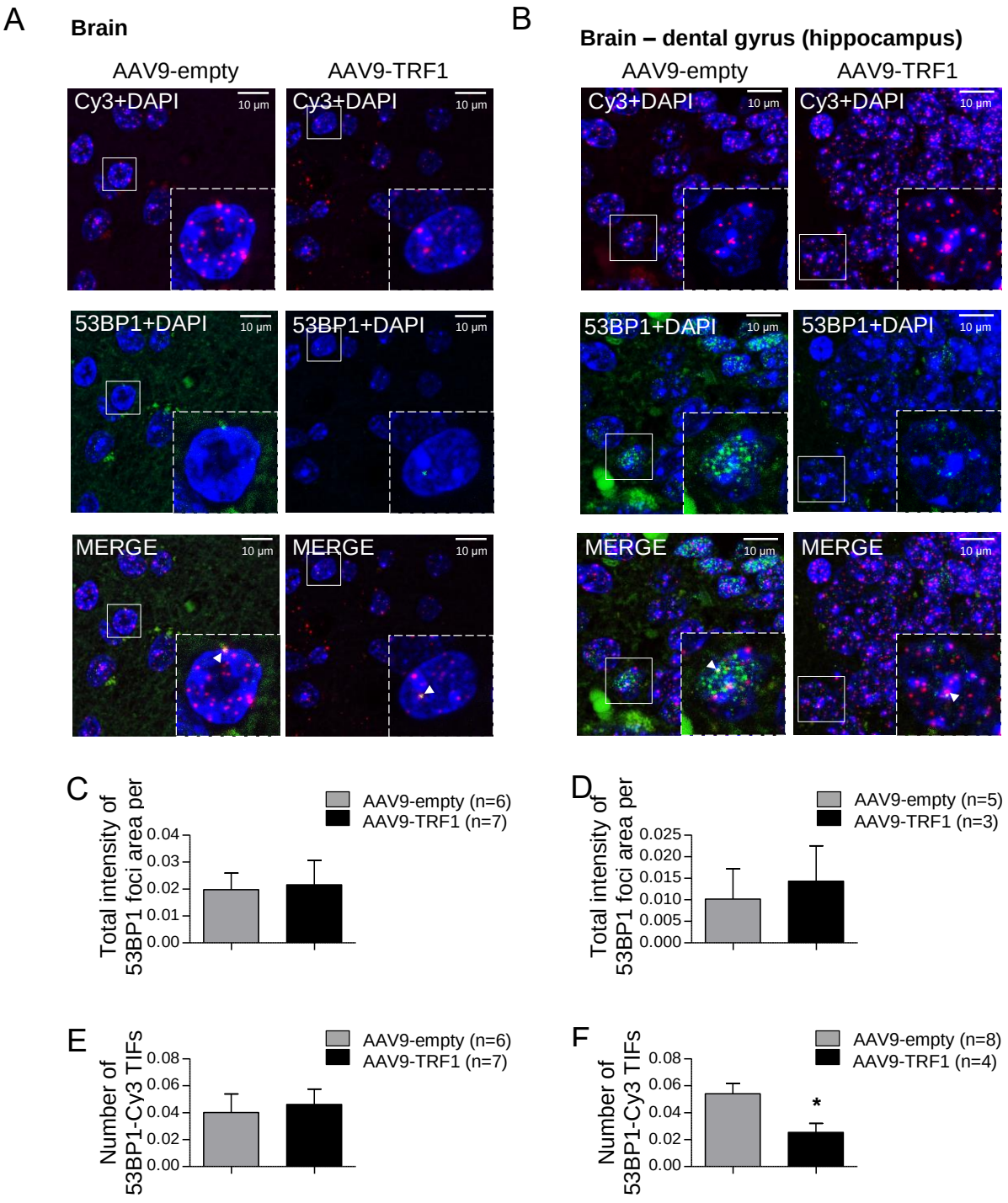


Figure 22. AAV9-TRF1 gene therapy reduces DNA damage levels at telomeres in the brain. **(A-B)** Representative images for immuno-FISH analysis on DNA damage and telomere-associated DNA damage in the whole brain (A) and specifically in the dentate gyrus of the hippocampus (B). Cy3 probe is presented in red, 53BP1 in green, and DAPI in blue. The representative images are merged from the Z-stack of images. Co-localization events (white arrows) were only counted as positive when detected in the individual confocal images. **(C-D)** Analysis of total DNA damage determined as the total intensity of 53BP1 foci per nucleus in the whole brain (C) and in the dentate gyrus area (D). **(E-F)** Analysis of telomere-induced DNA damages determined as the number of Cy3-53BP1 co-localization events per cells in the whole brain (E) and the dentate gyrus zone (F). Analysis performed at the time of death. Student's t-test was used for statistical analysis. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001. n indicates the number of mice.

9. CONTRIBUTING AUTORS

Ralph P. Schneider

Decreased TRF1 protein levels in the mouse and human epidermis (**Fig. 2A-B**).

Kurt Whittemore

TRF1 immunofluorescence in post-mitotic tissues (muscle and liver) in mice at young (8 weeks) and old (> 2 years) ages (**Fig. 3B-C**).

RT-qPCR to measure Trf1, Trf2, Rap1, Tpp1, Tin2, Pot1a and Pot1b mRNA levels in order to study effects of AAV9-TRF1 gene therapy on other shelterins (**Fig. 5B**).

Immunofluorescence analysis in order to measure telomere length and percentage of short telomeres in liver, kidney, lung, heart, pancreas and spleen of mice injected with virus vectors at 1 year of age. (**Fig. 21C**).

DISCUSSION

TRF1 is a central component of the shelterin complex, with an important role in telomere protection (Smith & de Lange 1997; Palm & de Lange 2008; Diotti & Loayza 2011). TRF1 also marks and is essential for both adult stem cells and pluripotent cells (Schneider et al. 2013). Interestingly, TRF1 levels decrease with increasing cell passages *in vitro* (Hohensinner et al. 2016), suggesting that TRF1 levels may also decrease with organismal aging.

Here we for the first time show that TRF1 levels decrease during mouse and human aging *in vivo*. This poses the interesting hypothesis that reduced TRF1 levels with aging may lead to telomere uncapping and telomere-induced DNA damage (Martínez et al. 2009; Sfeir et al. 2009), as well as to loss of tissue homeostasis (Schneider et al. 2013), eventually leading to tissue dysfunction and organismal aging. To test this hypothesis, here we set to rescue TRF1 levels in aging by overexpressing TRF1 in adult and old mice. Our Group previously described that constitutive transgenic overexpression of TRF1 results in telomere shortening in vivo (Muñoz et al. 2009). To overcome this problem, and to achieve a transient over-expression of TRF1, we used adeno-associated gene therapy vectors. As recombinant AAV9 vectors are non-integrative and can transduce the majority of mouse tissues, they provide transient overexpression of the target protein in several mouse tissues (Ayuso et al. 2010).

We describe here that mice treated with AAV9-TRF1 gene therapy showed significant improvements in health span, such as delayed anemia, and improvements in neuromuscular coordination, memory, epithelial barrier fitness, and glucose metabolism. Importantly, overexpression of TRF1 by using AAV9 vectors did not trigger telomere shortening.

The AAV9-TRF1 therapy affected TRF1 levels in post-mitotic tissues, and this may contribute to the effect of TRF1 gene therapy on aging. Although many of the cells in post-mitotic tissues do not proliferate, these tissues also contain stem cell compartments, which are responsible for maintaining tissue homeostasis throughout life. This is the case in the brain, the heart, and the muscle. For example, the brain exhibits neurogenesis, which continues throughout adulthood (Gross 2000). Muscle tissue in turn contain multipotent satellite cells, which are present in a quiescent state, but can be are activated and re-enter the cell cycle. They are indispensable for tissue growth and regeneration. Impaired function of satellite cells affects regenerative capacity of muscle tissue. Importantly, it was previously demonstrated that stem cell compartments exhibit increased TRF1 expression and that TRF1 is necessary for tissue homeostasis even in the presence of a normal telomere length (Martínez et al. 2009; Schneider et al. 2013).

Thus, TRF1 loss even in the presence of long telomeres can trigger severe defects in tissue homeostasis. Note that in our group it was previously demonstrated through whole genome ChIP-sequencing that TRF1 binding is restricted to telomeres and this is not influenced by shorter telomeres owing to telomerase deficiency (Garrobo et al. 2014). Therefore, it is unlikely that transient increased TRF1 expression owing to AAV9-TRF1 gene therapy may have major effects on gene expression elsewhere in the genome. This is in contrast to other shelterin components like RAP1, which can affect gene expression and this effect is influenced by telomere length (Ye et al. 2014; Martinez et al. 2010). We show here that TRF1 levels decrease with mouse aging in both highly proliferative tissues such as the skin and intestine, as well as in some post-mitotic tissues such as muscle. Since the deficiency of TRF1 leads to impaired telomere protection, this loss may play a role in age-associated impairments in the stem cell compartments as well. In addition, other processes associated with aging such defective DNA repair mechanisms could also result in de-protected telomeres (Grach 2013; Goytisolo et al. 2001; Samper et al. 2000; Espejel, Martín, et al. 2004; Espejel, Klatt, et al. 2004; Tarsounas et al. 2004).

In more details, we observed that AAV9 mediated TRF1 overexpression delayed accumulation of the short telomeres in muscle tissue. It is known that intracellular damage accumulated with age impairs the function of satellite cells, affecting the regenerative capacity of muscle tissue and contributing to development of sarcopenia, or age-related muscle wasting, which affects stability and balance (Ryall et al. 2008). This is consistent with our observations in neuromuscular coordination tests, where performance of the control group in tightrope test was impaired with age. Therefore evidence suggests that delayed accumulation of short telomeres upon AAV9-TRF1 gene therapy plays a role in improvement in neuromuscular coordination tests.

Interestingly, we observed less telomere-associated DNA damage foci upon gene therapy in the hippocampus area of the brain. Previous reports showed that age-associated accumulation of DNA damage in the hippocampus leads to impaired neurogenesis (Lemaire et al. 2000) and causes age-related cognitive alterations such as impaired memory function (Drapeau et al. 2003). In this regard, the improved memory scores observed here upon AAV9-TRF1 treatment, may suggest that AAV9-TRF1 gene therapy can delay age-related decline in hippocampal neurogenesis (Nacher et al. 2003; Drapeau et al. 2003; Lemaire et al. 2000), thus improving memory performance.

The effect of TRF1 on telomeres appears to be influenced by the level of TRF1 expression. In a publication of our Group (Muñoz et al. 2009), TRF1 was highly over-

expressed in a constitutive manner over the lifetime of mice. This persistent TRF1 over-expression lead to telomere shortening mediated by the XPF nuclease. However, in this work, TRF1 is transiently expressed through AAV9-TRF1 gene therapy. After a single injection of these non-integrative vectors, the TRF1 signal becomes diluted with time in replicative tissues. Therefore, the levels of TRF1 overexpression achieved are much lower. As demonstrated here, the levels of TRF1 overexpression needed to negatively regulate telomere length are not reached after the TRF1 gene therapy as telomere shortening was not observed. Indeed, we observed fewer short telomeres and telomere-associated DNA damage in some tissues, which is consistent with *in vitro* data showing beneficial effects of TRF1 overexpression in aged cells (Hohensinner et al. 2016). Regarding, how TRF1 stabilizes telomeres, our Group have previously demonstrated that persistent TRF1 downregulation in bone marrow stem cells can lead to cellular senescence of stem cells and to a compensatory hyperproliferation of the remaining stem cells to maintain tissue homeostasis. This compensatory hyperproliferation also leads to telomere shortening (Beier et al., Blood, 2012). Thus, decreased TRF1 levels with aging may also lead to loss of stem cells and the extra proliferation of the remaining progenitor cells, thus leading to telomere loss. Increased TRF1 expression may attenuate this.

In conclusion, we describe here an age-related loss of one of the key elements of telomere protection, TRF1. We further demonstrate that TRF1 decreased levels contribute to the aging phenotype, at least partially. In particular, transient and moderate TRF1 overexpression using recombinant adeno-associated viral vectors leads to beneficial effects both on molecular and physiological levels, ensuring better telomere protection and a prolonged health span.

CONCLUSIONS

1. TRF1 levels decrease with increasing age both in mice and humans.

2. AAV9-TRF1 vectors demonstrate high transduction efficiency and lead to increased TRF1 levels in multiple mouse tissues.

3. AAV9-TRF1 gene therapy delays physiological mouse aging. Namely, AAV9-TRF1 gene therapy prevents age-related decline in neuromuscular coordination, glucose tolerance, cognitive function, maintenance of subcutaneous fat, and chronic anemia.

4. AAV9-TRF1 treatment is not leading mice to be tumor prone and does not affect median survival.

5. AAV9-mediated TRF1 overexpression is maintained with age.

6. AAV9-TRF1 gene therapy does not have negative effects on telomere length and provides protection from the accumulation of short telomeres.

7. AAV9-TRF1 increases telomere protection in the hippocampus area of the brain of mice.

CONCLUSIONES

1. Los niveles de TRF1 disminuyen al aumentar la edad, tanto en ratones como en humanos.

2. Los vectores AAV9-TRF1 demuestran una alta eficacia de transducción y conducen a niveles aumentados de TRF1 en múltiples tejidos de ratón.

3. La terapia génica con AAV9-TRF1 retrasa el envejecimiento fisiológico del ratón. En este sentido, la terapia génica con AAV9-TRF1 previene la disminución relacionada con la edad en la función neuromuscular, la tolerancia a la glucosa, la función cognitiva, el mantenimiento de la grasa subcutánea y la anemia crónica.

4. El tratamiento con AAV9-TRF1 no hace que los ratones sean propensos a tumores y no afecta la supervivencia media.

5. La sobreexpresión de TRF1 mediada por AAV9 se mantiene con la edad.

6. La terapia génica con AAV9-TRF1 no tiene efectos negativos en la longitud de los telómeros y proporciona protección contra la acumulación de telómeros cortos.

7. AAV9-TRF1 aumenta la protección de los telómeros en el área del hipocampo del cerebro de los ratones.

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ANNEX

Published article is directly related to this Thesis

Derevyanko A, Whittemore K, Schneider RP, Jimenez V, Bosch F, Blasco MA. Gene therapy with the TRF1 telomere gene rescues decreased TRF1 levels with aging and prolongs mouse health span. // Aging Cell (2017) DOI: 10.1111/acel.12677.