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# Decline of hippocampal TRF2 during aging triggers ATM-dependent memory impairment

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# THÈSE DE DOCTORAT

Le déclin de TRF2 dans l'hippocampe au cours  
du vieillissement cause une déficience de  
la mémoire dépendante de ATM

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Présentée en vue de l'obtention du grade de Docteur en  
Sciences de la Vie et de la Santé d'Université Côte d'Azur

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# Le déclin de TRF2 dans l'hippocampe au cours du vieillissement cause une déficience de la mémoire dépendante de ATM

## Résumé

Le vieillissement est la cause principale de la grande majorité des pathologies, allant de l'athérosclérose aux tumeurs et à la démence. Il est donc incontestable que l'amélioration de la connaissance de ce phénomène multifactoriel au niveau cellulaire et moléculaire sera d'une valeur inestimable pour le traitement et la prévention d'un large éventail de pathologies humaines. Un facteur bien établi du vieillissement des cellules mitotiques est l'érosion des télomères médiée par la division cellulaire, un processus qui ne se produit pas dans les cellules post mitotiques telles que les neurones. En effet, on ne sait pas si les facteurs télomériques jouent un rôle dans le processus de vieillissement des neurones matures. Il a été récemment démontré que dans les myofibres post-mitotiques (cellules musculaires), l'expression du Telomere repeat-binding factor 2 (TRF2 - codé par le gène *Terf2*), un composant essentiel du complexe Shelterin de protection des télomères, diminue considérablement au cours du vieillissement, ce qui suggère que les modifications télomériques sont également impliquées dans le vieillissement des cellules post mitotiques. Nous avons donc décidé d'étudier si le même phénomène se produisait dans le neurone, un autre type de cellule post mitotique. De manière intéressante, notre groupe a montré que TRF2 possède des sites de liaison extra télomériques à proximité ou à l'intérieur de gènes impliqués dans les fonctions neuronales, ce qui suggère qu'il pourrait influencer leur expression. Ainsi, le but de notre projet est d'étudier les implications éventuelles de TRF2 dans le vieillissement neuronal. Pour cela, nous avons d'abord évalué l'expression de *Terf2* dans l'hippocampe murin au cours du vieillissement, en montrant qu'elle diminue avec le temps. Ensuite, nous avons étudié l'effet de la régulation négative de *Terf2* dans l'hippocampe murin sur la mémoire, un phénomène dépendant de l'hippocampe et notoirement altéré par le vieillissement. De manière remarquable, nous avons constaté que la régulation à la baisse de *Terf2* chez les jeunes souris entraîne un phénotype cognitif altéré affectant la mémoire épisodique et contextuelle. De plus, étant donné le rôle canonique de protection de

l'ADN par les télomères de TRF2 dans les cellules en division, nous avons évalué l'effet de sa régulation négative dans l'hippocampe sur l'apparition de lésions de l'ADN, en montrant que la région de l'hippocampe *Cornu ammonis* 1 et 3 (CA1 et CA3) et le Gyrus denté (DG) présentent une réponse accrue aux lésions de l'ADN (DDR), en particulier au niveau des télomères. En outre, nous avons évalué si l'inhibition de la DDR (inhibition du capteur de dommages à l'ADN ATM par le médicament spécifique KU60019) pouvait sauver le phénotype cognitif observé. En effet, nous avons pu montrer que l'inhibition de la DDR permettait de remédier au déficit cognitif. Enfin, nous avons évalué l'effet de la surexpression de TRF2 chez les souris âgées. De manière assez impressionnante, nous avons constaté que cette surexpression permettait de sauver complètement la mémoire épisodique et contextuelle. De manière assez frappante, nous avons pu montrer qu'au niveau cellulaire, la régulation à la baisse de *Terf2* entrave le transport axonal des vésicules (vésicules VAMP2) dans les cultures primaires de neurones hippocampiques et corticaux. De plus, l'analyse protéomique a révélé que la régulation négative de *Terf2* dans l'hippocampe affecte l'expression de plusieurs gènes ayant des fonctions neuronales essentielles, notamment la plasticité synaptique. Dans l'ensemble, ces résultats jettent une lumière nouvelle sur la dynamique intracellulaire du vieillissement neuronal, avec un rôle sans précédent pour une protéine télomérique et sa cible canonique, ATM. Ces connaissances nouvellement acquises constitueront un terrain fertile pour de futures recherches visant à prévenir, arrêter ou même inverser la perte de mémoire liée à l'âge.

**Mots clés :** Vieillissement, Télomères, Neurones, Hippocampe, Mémoire.

# Decline of hippocampal TRF2 during aging triggers ATM-dependent memory impairment

## Abstract

Aging is the leading cause of the vast majority of all pathologies, ranging from atherosclerosis to tumors and dementia. It is therefore undisputable that improving the knowledge of this multifactorial phenomenon at the cellular and molecular level will be of inestimable value for the sake of treating and preventing a broad spectrum of human conditions.

A well-established driver of mitotic cell aging is cell division-mediated telomere erosion, a process that does not occur in postmitotic cells such as neurons. Indeed, it is not known whether telomeric factors play a role in the aging process of mature neurons. It was recently shown that in postmitotic myofibers (muscle cells) the expression of Telomeric repeat-binding factor 2 (TRF2 – encoded by the *Terf2* gene), a pivotal component of the telomere-capping Shelterin complex, decreases dramatically during aging, suggesting that telomeric modifications are also involved in postmitotic cell aging. Therefore, we decided to investigate whether the same occurs in the neuron, another type of postmitotic cell. Interestingly, our group had shown that TRF2 has extratelomeric binding sites in the proximity or inside of genes involved in neuronal functions, suggesting that it might influence their expression. Thus, the aim of our project is to study eventual TRF2 implications in neuronal aging. For this purpose, we initially assessed *Terf2* expression in the murine hippocampus during aging, showing that this decreases over time. Afterwards, we investigated the effect of *Terf2* downregulation in the murine hippocampus on memory, a hippocampus-dependent phenomenon notoriously impaired by aging. Remarkably, we found that downregulating *Terf2* in young animals leads to an impaired cognitive phenotype affecting episodic and contextual memory. Moreover, given the canonical telomeric-DNA protection role of TRF2 in dividing cells, we assessed the effect of its hippocampal downregulation on DNA damage occurrence, showing that the region of the hippocampus *Cornu ammonis* 1 and 3 (CA1 and CA3) and the *Dentate gyrus* (DG) present increased DNA damage response (DDR), specifically at the telomeric level. Furthermore, we assessed whether inhibiting the DDR (inhibition of the DNA damage sensor ATM by the specific KU60019 drug) could

rescue the observed cognitive phenotype. Indeed, we could show a rescue of the cognitive deficit upon DDR inhibition. Finally, we assessed the effect of TRF2 overexpression in old mice. Impressively enough, we found that this completely rescues episodic and contextual memory. Quite strikingly, we could show that at the cellular level *Terf2* downregulation impairs vesicle axonal transport (VAMP2 vesicles) in primary cultures of both hippocampal and cortical neurons. Moreover, proteomic analysis revealed that hippocampal *Terf2* downregulation modulates the expression of several genes with pivotal neuronal functions, notably synaptic plasticity.

All in all, these findings shed new light on the intracellular dynamics of neuronal aging, with a stunning unprecedented role for a telomeric protein and its canonical target, ATM. This newly acquired knowledge shall provide fertile ground for future investigations with the goal to prevent, stop or even revert age-dependent memory loss.

**Key words:** Aging, Telomeres, Neurons, Hippocampus, Memory.

Dedicated to my Mother,  
whose sparkling eyes lit up the flame  
that guided me until Today.

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*“Ita enim senectus honesta est,  
si se ipsa defendit, si ius suum retinet, si nemini emancipata est,  
si usque ad ultimum spiritum dominatur in suos.”*

“For old age is honored only on condition that  
it defends itself, maintains its rights, is subservient to no one  
and to the last breath rules over its own domain.”

Marcus Tullius Cicero (*De Senectute*, 44 B.C.)

# 1. Brain aging

At the organismal level, aging is the gradual decline of all body functions over time, leading to decreased task performance and increased risk of developing a broad spectrum of diseases. As for the other tissues and organs of the body, brain functionality declines with aging. This phenomenon is characterized by impaired memory and learning capability, attention deficit, decreased sensorial perception and compromised motor coordination (Alexander et al., 2012) (Dykiert et al., 2012).

Brain aging affects cognitive performance at different levels ranging from memory (*e.g.*, working memory – the capacity to temporarily hold information necessary for decision-making processes) to executive functions. Indeed, elderly individuals frequently struggle to understand fast and complex wording, and retrieve or comprehend semantically elaborated information (Alexander et al., 2012). The speed of the age-driven cognitive decline is similar to that of the other organs, but with a marked acceleration when individuals reach their fifties (Mendonca et al., 2017). Once in their sixties, humans become more likely to develop neurodegenerative disorders such as Alzheimer's disease (AD) (Mattson, 2011) and Parkinson's disease (PD) (Aarsland et al., 2017).

Given that the brains of old individuals frequently show markers of neuronal pathologies (*e.g.*, hyperphosphorylated- $\tau$  tangles and amyloid- $\beta$  plaques for AD, and  $\alpha$ -synuclein inclusions for PD) even in the absence of an overt cognitive deficit (Elobeid et al., 2016), neurodegeneration might also be a consequence of normal brain aging, and the appearance of neurodegeneration in younger individuals might represent an accelerated form of brain aging. Unsurprisingly, the combination of human genetics and environmental factors determine the onset rate of brain aging symptoms and neuronal diseases (Hou et al., 2019).

In most of the western industrialized countries people are dying older and older, shifting forward the average age of the population, thereby increasing the number of individuals prone to develop the symptoms of aging and the above-mentioned neuronal pathologies. In the US alone, the population aged more than 65 is expected to increase from the 53 million of 2018 to 88 million in 2050 (“2019 Alzheimer's Disease Facts and Figures,” 2019). Worldwide, by the same time, the population aged more than 60 will reach 2.1 billion people. Among them, the population aged more than 80 is the fastest growing segment (United Nations, 2015). This prediction means that a consistent amount of the global population will face poor life quality, not being able to live autonomously and needing assistance. Their condition will in turn affect the life of people around

them and the society, them representing a considerable emotional and financial burden for their families and the healthcare systems.

This perspective makes it clear that advances in the understanding of brain aging with the hope to find ways to prevent, stop or even revert cognitive decline are of the utmost importance for the future of our societies.

The brain is by far the most complicated structure of the organism, but at a first attempt of description, it can be simply subdivided into three main regions, from the top to the bottom: forebrain, midbrain and hindbrain (Dryander, 1537) (Ackerman, 1992). Each of these regions is in turn composed of several distinguishable structures:

### *Forebrain*

The forebrain is the cerebrum (also referred to as cerebral cortex), the largest part of the human brain, which is associated with the higher functions such as thought and action. It is endowed with a six-layered wrinkled structure which allows the storage of a big surface in a relatively small volume, and is further divided into lobes: frontal, parietal, occipital and temporal lobes; respectively situated in the frontal, upper-rear, back, and lateral parts of the skull. Neuronal cell bodies, glial cells and unmyelinated axons make up the gray surface, whereas myelinated axons (nerve fibers) make up the white matter below it. A deep groove divides the cerebral cortex into two hemispheres that are connected between each other by a structure composed of bundles of axons named corpus callosum.

### *Midbrain*

The midbrain is situated below the cortex and above the hindbrain. It is composed of tectum, tegmentum, cerebral aqueduct, cerebral peduncles, several nuclei and fasciculi, the limbic system and the pituitary gland. The limbic system is also referred to as the “emotional brain”, consisting of thalamus, hypothalamus, amygdala and hippocampus. Therefore, the midbrain is that area of the brain devoted to the control of our behavior related to lower order emotional responses, such as feeding and reproduction.

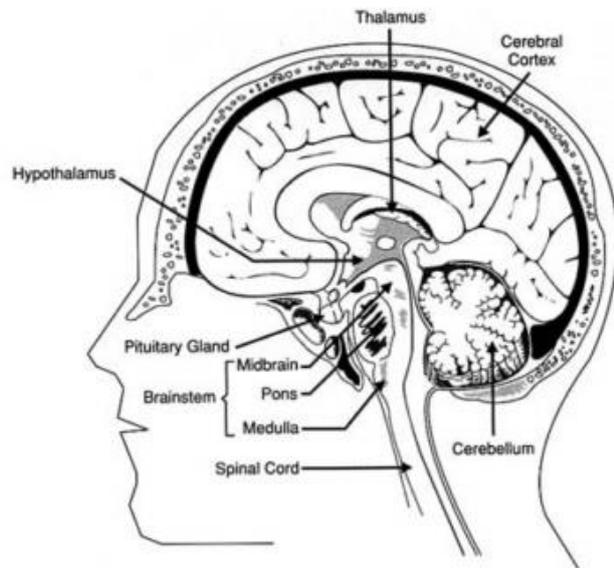
### *Hindbrain*

The hindbrain is located below the midbrain and is composed of pons, medulla, and cerebellum. The nerves contained in the pons serve as a bridge between several parts of the brain, *e.g.*, the cortex and the cerebellum. Below the pons is the medulla, part of the brain stem at the junction between

the brain and the spinal cord, which allows the transfer of signals from the brain to the spinal cord and vice versa. The cerebellum, located in the rear-lower part of the cranium, is divided into two hemispheres with a highly folded structure, much alike the cortex, and its function is mainly related to posture and movement.



**Figure 1. The anatomy of the brain (I)**  
 One of the first attempts of brain anatomical description from Dryander's anatomy. The ventricles [Ven] and the six-layered wrinkled structure of the cortex are highlighted [A-F] (Dryander, 1537).



**Figure 2. The anatomy of the brain (II)**  
 Schematic representation of the brain as described by Sandra Ackerman. Forebrain [Cerebral cortex], midbrain [with thalamus and hypothalamus], and hindbrain [Pons, medulla and cerebellum] are highlighted (Ackerman, 1992).

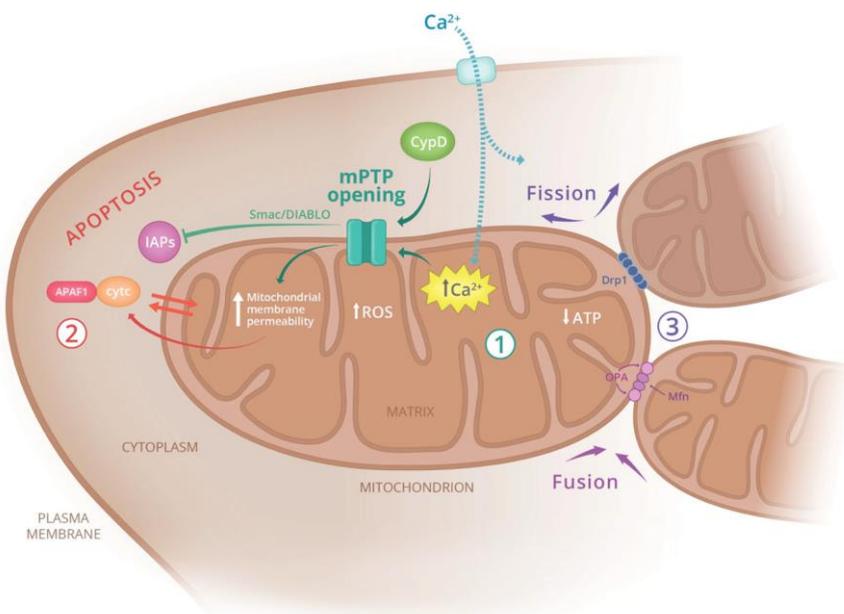
From an anatomical point of view, during aging the brain shrinks showing decreased gray and white matter volume and enlarged cerebral ventricles (Cohen et al., 2019), resulting from both dendritic regression and neuronal death (Dumitriu et al., 2010). Noteworthy, brain atrophy rates correlate with cognitive impairment and dementia (Jack et al., 2005). Importantly, environmental factors affect the rate of brain structural changes during aging. For example, physical aerobic exercise causes bigger hippocampal volume, whereas a chronic positive caloric balance leading to obesity correlates with hippocampal atrophy (Erickson et al., 2011) (Cherbuin et al., 2015). Furthermore, calorie restriction (CR) and intermittent fasting (IF) regimens in animal models are interventions that delay the age-driven cognitive impairment (W. Duan & Mattson, 1999) (Willette et al., 2010).

From a cellular and molecular perspective, brain aging presents the following hallmarks:

### *Mitochondrial dysfunction*

Mitochondria are the double-membrane organelles distributed throughout the whole cell cytoplasm (including soma, dendrites and axon) pivotal for the formation of the ATP necessary for all cell functions: growth, repair, recycling, and synaptic transmission (Mattson et al., 2008).

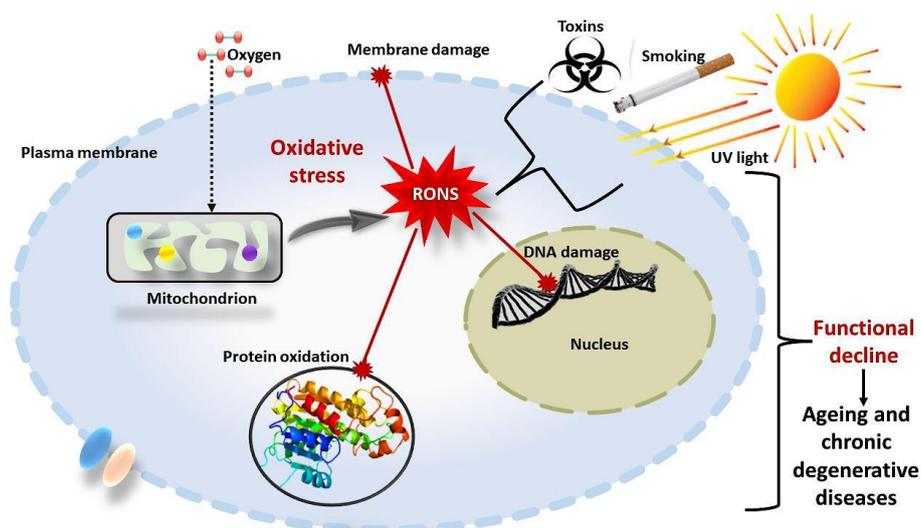
According to the metabolic state of the cell, mitochondria can grow, fuse to form big reticula or undergo a fixation process that allows them to be split into smaller units, therefore suitable for being phagocytosed and recycled via a specific type of autophagolysosomal degradation named mitophagy (Yun & Finkel, 2014). Analyses of mitochondria isolated from mice showed that aging causes either increased volume or fragmentation (Stahon et al., 2016), oxidative damage to mitochondrial DNA (Santos et al., 2013), impaired electron transport chain (ETC) function (Pollard et al., 2016), increased membrane depolarization (Lores-Arnaiz et al., 2016), defective  $\text{Ca}^{2+}$  handling (Pandya et al., 2015), and lower  $\text{Ca}^{2+}$  threshold for mitochondrial membrane permeability transition pore (mPTP) formation and consequent cell death (Brown et al., 2004) (Norat et al., 2020). Moreover, the decline of mitochondrial function with aging leads to decreased  $\text{NAD}^+$  cellular levels and a lower  $\text{NAD}^+/\text{NADH}$  ratio in neurons (Braidy et al., 2014), compromising the activities of  $\text{NAD}^+$ -dependent enzymes such as the deacetylases of the sirtuin family, well-established pro-longevity factors (E. F. Fang et al., 2017).



*Figure 3. Mitochondrial dysfunction*  
Mitochondria are pivotal organelles for a cell energy supply, especially for highly energy-demanding cells such as neurons. In the scheme, it is highlighted how, during aging, (1) increased reactive oxygen species (ROS) and poor  $\text{Ca}^{2+}$  handling may lead to (2) cytochrome C release and apoptosis or (3) defects in mitochondria structural remodeling via fission and fusion, which affects their activity and turnover (Norat et al., 2020).

## Oxidative damage

An age-driven oxidative imbalance (*i.e.*, increased reactive oxygen species [ROS] production and decreased antioxidant activities) causes the accumulation of dysfunctional proteins and mitochondria (Leyane et al., 2022). The main ROS produced in neurons are the superoxide ion ( $O_2^-$ ) generated by the mitochondrial ETC and diverse oxidases (Sabharwal & Schumacker, 2014); the hydroxyl radical ( $\cdot OH$ ) given by the reaction of hydrogen peroxide ( $H_2O_2$ ) with iron ( $Fe^{2+}$ ) or copper ( $Cu^+$ ); and nitric oxide (NO) produced in response to elevated intracellular  $Ca^{2+}$  levels (Halliwell, 2001).  $\cdot OH$  is highly reactive and can initiate the autocatalytic peroxidation of membrane lipids. The lipid peroxidation product 4-hydroxynonenal (HNE) and NO can modify cysteine, lysine, histidine, and tyrosine residues, impairing the function of several membrane proteins such as glucose transporters and neurotrophic factor receptors that are pivotal for cell metabolism and cell survival, respectively (Mattson, 2009) (Guix et al., 2005). The genetic downregulation of Superoxide dismutase 2 (SOD2), whose catalytic activity transforms toxic superoxide into hydrogen peroxide, causes the early onset of an age-related phenotype in a murine model (increased DNA damage, neurodegeneration and motor dysfunction), demonstrating that decreased antioxidant activities are sufficient to accelerate aging (Botto et al., 1998). Oxidized proteins are ubiquitinated for proteasomal degradation, whereas oxidized organelles and mitochondria are recycled via autophagolysosomal degradation (macro autophagy) (Nixon, 2013), but excessive oxidation can in turn impair these very cleansing processes (Keller et al., 2002) (Liebert et al., 2006).



**Figure 4. Reactive oxygen and nitrogen species**

Schematic representation of the accumulation and the effect of reactive oxygen and nitrogen species (RONS). RONS produced by mitochondrial respiration, oxidases and nitric oxide synthases can damage cellular membranes, proteins, organelles and nucleic acids, leading to the functional decline characteristic of aging and degenerative diseases (Leyane et al., 2022).

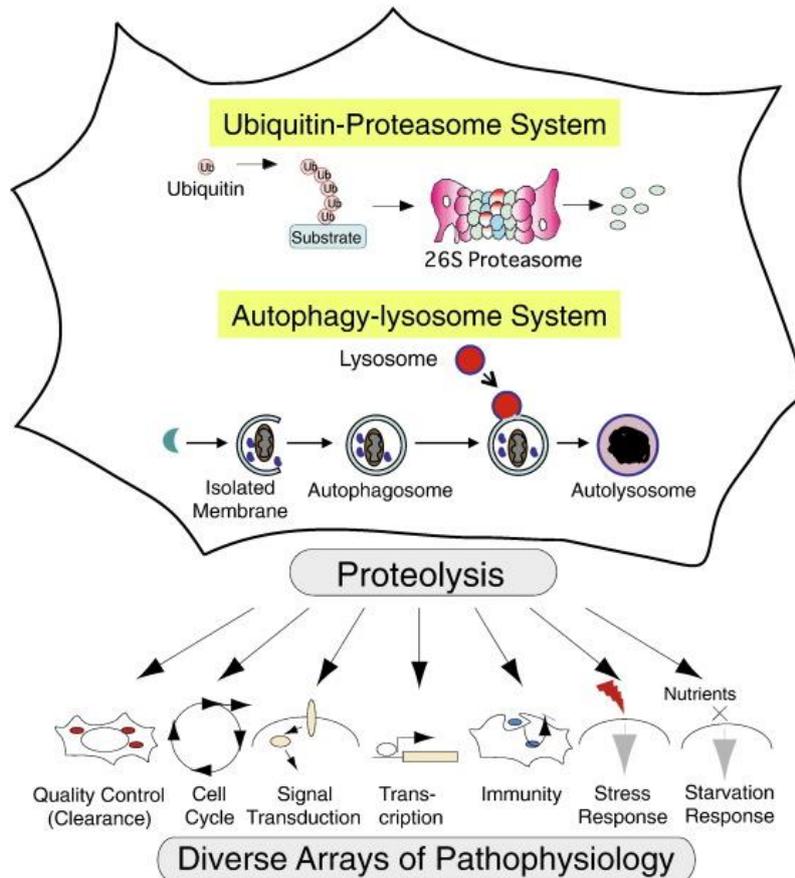
### *Impaired autophagy and proteasome function*

Neurons are postmitotic cells incapable of replicating that can persist for decades in the organism. Therefore, not being able to dilute their intracellular debris via mitosis, they are strictly dependent on the processes of autophagy and proteasomal activity for maintaining a proper functional state (K. Tanaka & Matsuda, 2014).

In the process of autophagy, damaged membranes or organelles in the cytoplasm are surrounded and enclosed by a double-layered phagosome (autophagosome) which then fuses with a low-pH-lysosome, giving rise to the autophagolysosome. In the lumen of the autophagolysosome the damaged content is degraded by hydrolytic enzymes.

For proteasomal activity, damaged proteins need to be targeted by ubiquitination, a process involving three different enzymes: E1, E2 and E3, that respectively activate, conjugate and bind ubiquitin to the target protein. Multiple ubiquitins are bound to one another forming a ubiquitin chain that is recognized and unfolded by the S19 proteasome subunit, and the ubiquitinated cargo is finally degraded in the barrel of the S20 proteasome subunit. Importantly, mutations in the E3 ubiquitin ligase Parkin, which transfers ubiquitin to lysine residues of the target proteins, are sufficient to cause early onset PD (Shimura et al., 2001).

Unfortunately, autophagy and proteasomal activity are impaired during aging as studies show intracellular accumulation of autophagosomes with undegraded cargos, and polyubiquitinated proteins over time (Nixon, 2013) (Graham & Liu, 2017). During aging, the low lysosomal pH suited for cargo degradation cannot be achieved due to oxidative damage to lysosomal ATPases (Colacurcio & Nixon, 2016), and accumulation of lipids into autophagosomes (Lipofuscin) is also observed (Sulzer et al., 2008). Importantly, stimulating autophagy by dietary energy restriction (DER), protein restriction, or rapamycin treatment in animal models favors longevity (Madeo et al., 2015). Interestingly, proteasome activity was shown to be specifically impaired during aging in some brain regions like the hippocampus and the cerebral cortex, while being unaffected in others such as cerebellum and brainstem (Keller et al., 2000).



**Figure 5. Proteasome and autophagy**

Intracellular cleansing mechanisms broadly classify into two distinct pathways consisting in the ubiquitin-proteasome and autophagy-lysosome systems, whose proper functioning, which is impaired during aging, is linked to the successful outcome of several cellular phenomena (K. Tanaka & Matsuda, 2014).

### **Dysregulated calcium homeostasis**

Calcium ( $\text{Ca}^{2+}$ ) regulates the structural and functional organization of neurons in space (neurite outgrowth and synaptic pruning; *i.e.*, neuronal plasticity) and time (short-term vs long-term memory) (Cohen et al, 2015). For an action potential to fire, the excitatory neurotransmitter glutamate from the presynaptic neuron has to bind to the postsynaptic  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors causing sodium ( $\text{Na}^+$ ) influx and an initial membrane depolarization. If the presynaptic stimulus is strong enough, the extent of the initial depolarization is sufficient to cause the activation of the *N*-methyl-D-aspartate (NMDA) glutamate receptors via the removal of the  $\text{Mg}^{2+}$  ion that blocks the influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$ , despite the binding of glutamate. The ensuing increased influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  activates voltage-gated  $\text{Na}^+$  channels at

the level of the soma which further activate other voltage-gated Na<sup>+</sup> channels in a unidirectional way, propagating the depolarization until the end of the axon. Therefore, Ca<sup>2+</sup> cytoplasmic concentration transiently rises and the Na<sup>+</sup>/potassium (K<sup>+</sup>) ATPase pumps are activated to restore the membrane potential, whereas Ca<sup>2+</sup> is actively removed by ATPase pumps spanning plasma, mitochondrial and endoplasmic reticulum membranes. Ca<sup>2+</sup> influx activates Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CAMKII) which in turn phosphorylates the transcription factor Cyclic-AMP response-element binding protein (CREB) (Cohen et al, 2015), driving the expression of genes involved in neuronal plasticity and stress resistance (Mattson, 2012). In aged hippocampal pyramidal neurons, Ca<sup>2+</sup> homeostasis is impaired, resulting in aberrant Ca<sup>2+</sup> cytoplasmic levels, leading to dysregulated protein phosphorylation and cytoskeletal dynamics, and aberrant gene expression (Thibault et al., 2001) (Toescu et al., 2004) (Gant et al., 2006) (Porte et al., 2008), whereas the genetic restoration of Ca<sup>2+</sup> homeostasis rescues the cognitive deficit in old rats (Gant et al., 2015). Moreover, aged hippocampal neurons are particularly vulnerable to Ca<sup>2+</sup>-driven excitotoxicity (Camandola & Mattson, 2011), which can lead to the activation of Ca<sup>2+</sup>-dependent proteases (Calpains) and caspase-mediated apoptosis (Nixon, 2003) (Mattson, 2000). Furthermore, Ca<sup>2+</sup> and ROS dysregulations are sustained by a reciprocal vicious relationship. Indeed, elevated Ca<sup>2+</sup> levels can boost the activity of ROS producing enzymes and ETC enzymes, increasing the production of ROS. It has been proposed that Ca<sup>2+</sup> induces a three-dimensional conformational change of the ETC complexes, thereby eliciting increased ROS production. Moreover, the NADPH oxidase 2 (NOX2) subunit Rac GTPase is activated by the Protein kinase C (PKC)-mediated phosphorylation, whose kinase activity is positively regulated by Ca<sup>2+</sup>. Furthermore, NOX5 was shown to directly bind Ca<sup>2+</sup>, which boosts its ROS producing activity. Finally, CAMKII (activated by Ca<sup>2+</sup>) can itself positively regulate NOX5 activity by phosphorylating its Ser475 residue.

In turn, ROS can induce structural conformational changes (*i.e.*, oxidation of redox-sensitive thiol groups) in plasma membrane (*e.g.*, L-type and T-type voltage-gated channels) and intracellular (*e.g.*, the ER Ryanodine receptors – RyR) calcium channels leading to the release of more Ca<sup>2+</sup> in the cytoplasm or impairing the calcium restorage into the ER (via the Sarco/endoplasmic reticulum Ca<sup>2+</sup> ATPase – SERCA), thereby fomenting the vicious cycle.

Increased levels of Ca<sup>2+</sup> and ROS can finally activate the mitochondrial membrane permeability transition pores (mPTP – Ca<sup>2+</sup>- and voltage-dependent channels) resulting in the disruption of the mitochondrial membrane potential ( $\Delta\Psi_M$ ), leading to mitochondrial membrane depolarization, ATP depletion and cell death (Görlach et al., 2015).

### *Impaired cellular stress response*

As a result of their normal electrochemical activity, neurons are continuously under stress (metabolic, ionic, oxidative). To cope with it, several signaling pathways have evolved. Three main stress signals eliciting rescue pathways are i) ATP consumption, ii) excessive  $\text{Ca}^{2+}$  cytoplasmic levels, and iii) ROS production.

i)  $\text{Na}^+$  and  $\text{Ca}^{2+}$  influx through the plasma membrane channels mediate synaptic transmission. After the neuron has fired, these cations must be brought back outside the cytoplasm to reestablish the membrane action potential. This process is mediated by ATPase pumps that actively transport  $\text{Na}^+$  and  $\text{Ca}^{2+}$  back outside the cytoplasm. The ensuing ATP consumption increases the AMP/ATP ratio leading to the activation of AMP-activated protein kinase (AMPK) which in turn, via phosphorylation, regulates the activity of proteins involved in metabolism, like glucose transporters and the mTOR pathway (Weisová et al., 2009) (Ikematsu et al., 2011) (S. C. Lin & Hardie, 2018).

ii)  $\text{Ca}^{2+}$  is a pivotal stress signalling mediator. As such, it can bind to the protein calmodulin leading to the activation of kinases (*e.g.*, CAMKII) that activate transcription factors like CREB and nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) (Cohen et al, 2015) (Saura & Cardinaux, 2017) (Snow et al., 2014). Importantly, CREB elicits the expression of Brain-derived neurotrophic factor (BDNF) and the DNA repair enzyme APE1 (J. L. Yang et al., 2010)

iii) In mitochondria,  $\text{Ca}^{2+}$  boosts oxidative phosphorylation and ROS production (Glancy & Balaban, 2012).  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  activate ROS-responsive transcription factors including NF- $\kappa\text{B}$  and Nuclear regulatory factor 2 (NRF2) (A. Y. Shih et al., 2005). NF- $\kappa\text{B}$  boosts the expression of the antioxidant enzyme SOD2, the  $\text{Ca}^{2+}$ -binding protein Calbindin, and the anti-apoptotic factor Bcl-2 (Sullivan et al., 1999) (Mattson & Meffert, 2006); NRF2 increases the expression of the antioxidant enzymes HO-1 [Heme oxygenase 1] and NQO1 [NAD(P)H dehydrogenase 1] (Son et al., 2010).

As these stress response pathways become deficient during aging, neurons get more and more vulnerable over time. Aging can affect these rescue pathways at different levels with decreased expression of the neurotrophic factors, their receptors, and their downstream signaling factors (Mattson et al., 2004) (Tapia-Arancibia et al., 2008) (Schliebs & Arendt, 2011).

Moreover, age-dependent membrane lipid peroxidation may be the cause of the activity decline of plasma membrane ATPases in synaptic terminals (Zaidi et al., 1998), and dysregulated  $\text{Ca}^{2+}$ /CAMKII signaling impairs mitochondrial dynamics (Jiang et al., 2015). Furthermore, hippocampal NO production and its downstream signaling are also perturbed with aging, and their restoration reverses age-driven cognitive decline (Zhang et al, 2017).

Importantly, chronic stress (mental or physical) has an aging-like effect at the neuronal level, impairing neuronal plasticity and causing neuronal degeneration via hyperactivation of the hypothalamic-pituitary-adrenal axis, leading to high glucocorticoid levels (cortisol in humans, and corticosterone in rodents), which inhibits the expression of BDNF, (McEwen & Morrison, 2013) the most abundant neurotrophin in the hippocampus.

### *Impaired neuronal networking*

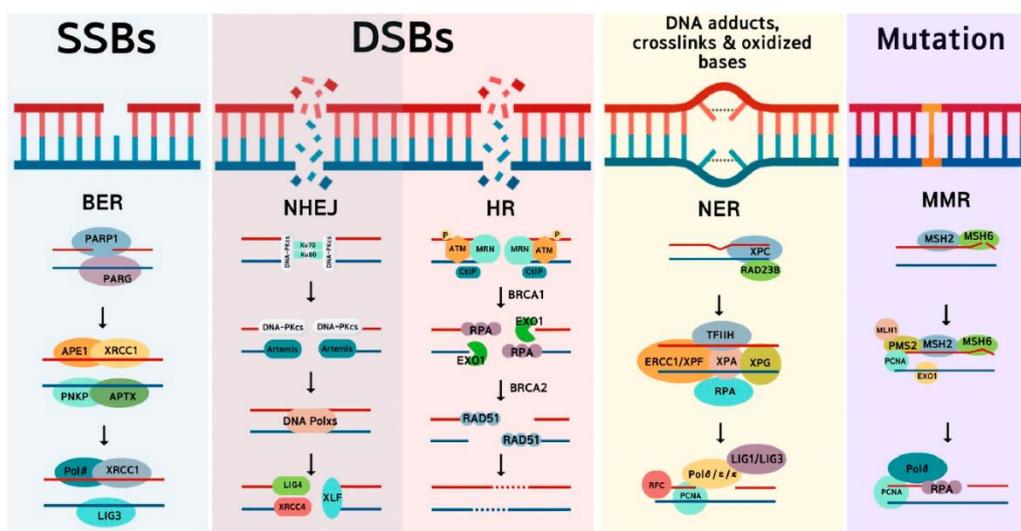
Neuronal circuits generally consist of excitatory glutamatergic neurons endowed with elaborated dendritic neurites and a long axon forming synaptic interactions with other excitatory glutamatergic neurons or inhibitory GABAergic interneurons. Structurally speaking, excitatory glutamatergic synapses “touch” the target neuron on dendrites that are distant from the cell body (soma). On the contrary, inhibitory GABAergic synapses directly reach the cell soma. Much alike the glutamatergic synapses, modulatory serotonergic and noradrenergic synapses from brainstem neurons, cholinergic synapses from forebrain neurons, and dopaminergic synapses from midbrain neurons are in contact with the target neuron at the dendritic level. Normal brain function requires the physical integrity and the proper integration of all this different neurotransmitter signaling circuits. Aging can compromise all of this. Indeed, impaired GABAergic signaling (in particular, due to GABA-A receptor impairment) in the aging brain causes excitatory imbalances, referred to as excitotoxicity (Heise et al., 2013) (Richardson et al., 2013) (McQuail et al., 2015) (Porges et al., 2017). Moreover, also hallmarks of brain aging such as mitochondrial dysfunction, oxidative stress, inflammation and impaired cellular stress responses, can get neuronal networks sensitive to excitotoxicity. Furthermore, the dysfunction of G protein-coupled receptors involved in serotonergic, noradrenergic, dopaminergic and cholinergic signaling, which are all pivotal for memory and learning, decision making and mood regulation, may increase the occurrence of age-associated neurodegenerative disorders (Collier et al., n.d.) (Schliebs & Arendt, 2011) (Mather & Harley, 2016). Oligodendrocytes are the brain cell type that forms myelin sheets around neuronal axons allowing the increase in speed of signal transmission. Myelinated axons constitute the white matter of the brain and allow inter- and intra-hemispheric communication (*e.g.*, *corpus callosum* and *fasciculi*, respectively). Noteworthy, an age-related mechanism involving DNA oxidation in oligodendrocytes causes axon demyelination (Tse & Herrup, 2017), resulting in compromised white matter structure, which is further exacerbated in individuals with cognitive impairment (Bennett & Madden, 2014) (H. Liu et al., 2017).

When an individual closes their eyes limiting the interactions with the surrounding environment, the activity of a network including precuneus (portion of the parietal lobule in front of the occipital lobe), posterior cingulate cortex, medial prefrontal cortex, and angular gyrus (posterior part of the inferior parietal lobule) is increased. This network, which is referred to as Default mode network (DMN), is thought to have an important role in remembering the past, imagining the future and mind-wandering. Aging affects DMN connectivity which is found further compromised in cognitively-impaired individuals (Leal & Yassa, 2013) (Sala-Llonch et al., 2015). Moreover, at resting state, young adults activate fronto-parietal and salience networks (collection of brain regions that cooperate to evaluate the importance of internal and external stimuli and act upon them) while older adults do not, which correlates with decreased grey matter volume and impaired white matter integrity (Marstaller et al., 2015). Furthermore, the functional connectivity between DMN and hippocampus correlates with successful memory performance in older adults (Li et al., 2015).

### *Impaired DNA damage response*

During a cell lifespan, the DNA (nuclear and mitochondrial) is continuously damaged due to the presence of ROS, resulting from the cell metabolic activity. In neurons the DNA is further damaged because of their excitatory synaptic activity (J. L. Yang et al., 2010). In young functional cells, when DNA damage occurs is corrected, according to the type of lesion, by one of the following mechanisms: homologous recombination (HR), non-homologous end-joining (NHEJ), mismatch repair (MR), nucleotide excision repair (NER), and base excision repair (BER) (Moon et al., 2023). The latter is pivotal for repairing oxidative DNA damage in postmitotic cells like neurons and involves the concerted activities of glycosylases (OGG1, UDG, NEIL1) that recognize and excise the altered base (usually, either alkylated, deaminated or oxidized), an AP-endonuclease (APE1) excising the abasic site, a polymerase (Pol $\beta$ ) adding new undamaged nucleotides to the DNA strand, and a DNA ligase to seal the nick of the phosphodiester backbone (Leandro et al., 2015). Aging positively correlates with increased mitochondrial and nuclear DNA damage and negatively correlates with the expression and activity of proteins involved in DNA repair. In humans, specific DNA regions that correspond to promoters of genes involved in synaptic plasticity and mitochondrial functions result particularly affected by oxidation during aging (Lu et al., 2004). In the murine hippocampus, frontal cortex, brainstem and cerebellum, aging inversely correlates with OGG1 and UDG activities (Imam et al., 2006). Aging also correlates with decreased expression of Pol $\beta$ , which is rescued by a calorie restriction (CR) regimen (Cabelof et al., 2003). Being neurons post mitotic, non-replicative cells, BER

is their main way to repair damaged DNA. Nevertheless, NER and transcription-coupled repair activities were also shown to be decreased in the aging brain, which might account for decreased DNA repair activities in non-neuronal cells such as neural stem cells and glial cells (Jaarsma et al., 2011). The fact that brain cells of individuals with premature aging syndromes such as Cockayne, Werner and Ataxia telangiectasia exhibit impaired activity of proteins involved in DNA repair (CSB, Werner and ATM, respectively) shows that defects in DNA repair mechanisms can by themselves cause premature aging (Scheibye-Knudsen, 2016). Purkinje cells (a cerebellar cortex-specific neuronal cell type), cochlear and retinal neurons are dysfunctional in Cockayne syndrome patients (Weidenheim et al., 2009), and CSB-deficient mice exhibit premature hearing loss and mitochondrial dysfunction in hippocampal and cortical neurons (Paul Nagtegaal et al., 2015) (Thomsen et al., 2018). Moreover, AD-like cognitive deficits characterize Werner syndrome (Rekik et al., 2017). Furthermore, ATM patients exhibit impaired control of body movements as a consequence of Purkinje and granule neurons progressive death (Rothblum-Oviatt et al., 2016). Remarkably, genetically inducing BER decreases neurodegeneration and cognitive deficits in a progeroid (DNA excision repair-deficient) mouse model by decreasing the accumulation of oxidative DNA damage (Vermeij et al., 2016). Very interestingly, the stimulation of hippocampal neurons specifically induces the formation of DSBs via Topoisomerase II $\beta$  activity in the promoters of a subset of immediate early genes (*e.g.*, *Fos*, *FosB*, *Npas4* and *Egr1*), which causes their expression in an ATM-independent fashion. (Madabhushi et al., 2015).



**Figure 6. The variety of DNA damage and associated responses**  
 Exogenous and endogenous insults can cause single and double stand breaks (SSBs and DSBs), DNA adducts and mutations that are accordingly repaired with the suitable corresponding DNA damage responses [BER, Base excision repair; NHEJ, Non-homologous end-joining; HR, Homologous recombination; NER, Nucleotide excision repair; MMR, Mismatch repair] (Moon et al., 2023).

### *Increased inflammation*

With brain aging, glial cells (in particular microglia) usually get into an activated state characterized by the production of pro-inflammatory cytokines such as Interleukin 1 $\beta$  (IL-1 $\beta$ ), Interleukin 6 (IL-6), and Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (Cribbs et al., 2012). Moreover, the complement cascade (an innate immunity-related sequence of events involving several protein-protein interactions and cleavage, leading to membrane disruption and target cell death) is also boosted and plays a role in AD development and ischemic stroke occurrence (Hong et al., 2016) (Arumugam et al., 2009). Furthermore, activated microglia produce huge amounts of NO via an Inducible NO synthase (iNOS), which causes severe oxidative damage to neurons. Finally, Toll-like receptors (TLRs), the innate immunity receptors binding to PAMPs (pathogen-associated molecular patterns) and DAMPs (danger-associated [tissue damage-related] molecular patterns), are heavily implicated in increased inflammation during brain aging (Okun et al., 2011). Indeed, induction of TLR4 activation in an experimental model of age-driven neurodegeneration further worsens the situation, whereas microglia inhibition has a protective outcome (Colonna & Butovsky, 2017).

Nonetheless, when properly regulated, immune activation is pivotal for neuroplasticity and stress resistance. For example, i) TNF- $\alpha$  is implied in hippocampal plasticity and the ensuing learning and memory formation (Snow et al., 2014), ii) the complement regulates synaptic remodeling in the developing and adult brain (B. Stevens et al., 2007), iii) Neurons express TLRs that are pivotal for neuronal development and adult neuroplasticity (Okun et al., 2011), iv) TNF- $\alpha$  receptor-deficiency in hippocampal murine neurons causes increased epileptic seizures occurrence (Bruce et al, 1996), and, finally, v) TLR2 and TLR4 regulate appetite, the parasympathetic nervous system and therefore bodyweight (Okun et al., 2014).

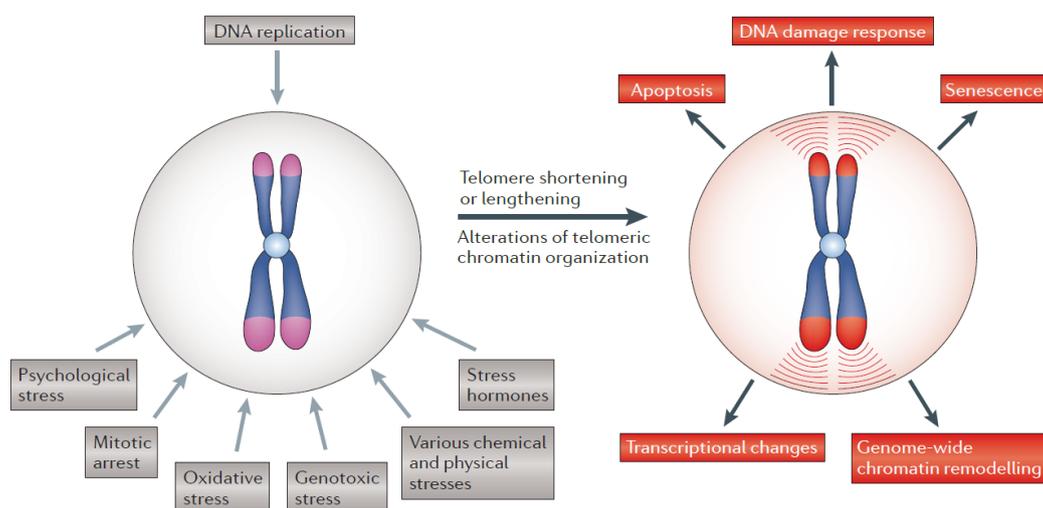
### *Impaired neurogenesis*

The overwhelming majority of neurons in the mammalian brain are generated during embryogenesis or early development. Nonetheless, two areas of the murine brain, namely the dentate gyrus of the hippocampus and the olfactory bulb, can respectively give rise to newborn granule neurons and interneurons throughout the course of a lifetime (Ming & Song, 2011). Dentate granule neurons play a pivotal role in spatial memory formation (the memory of the spatial localization of objects relative to one another in the environment). Importantly, it has been reported that hippocampal and olfactory neurogenesis decrease during aging, which may lead to cognitive and olfactory impairment (Lazarov et al., 2010). Interestingly, neuronal progenitors exhibit impaired mitochondrial oxidative

metabolism during aging (Stoll et al., 2011), and genetically inducing ETC impairment in hippocampal neuronal progenitors mimics the age-related neurogenesis impairment (Beckervordersandforth et al., 2017).

### *Telomere attrition and cell senescence*

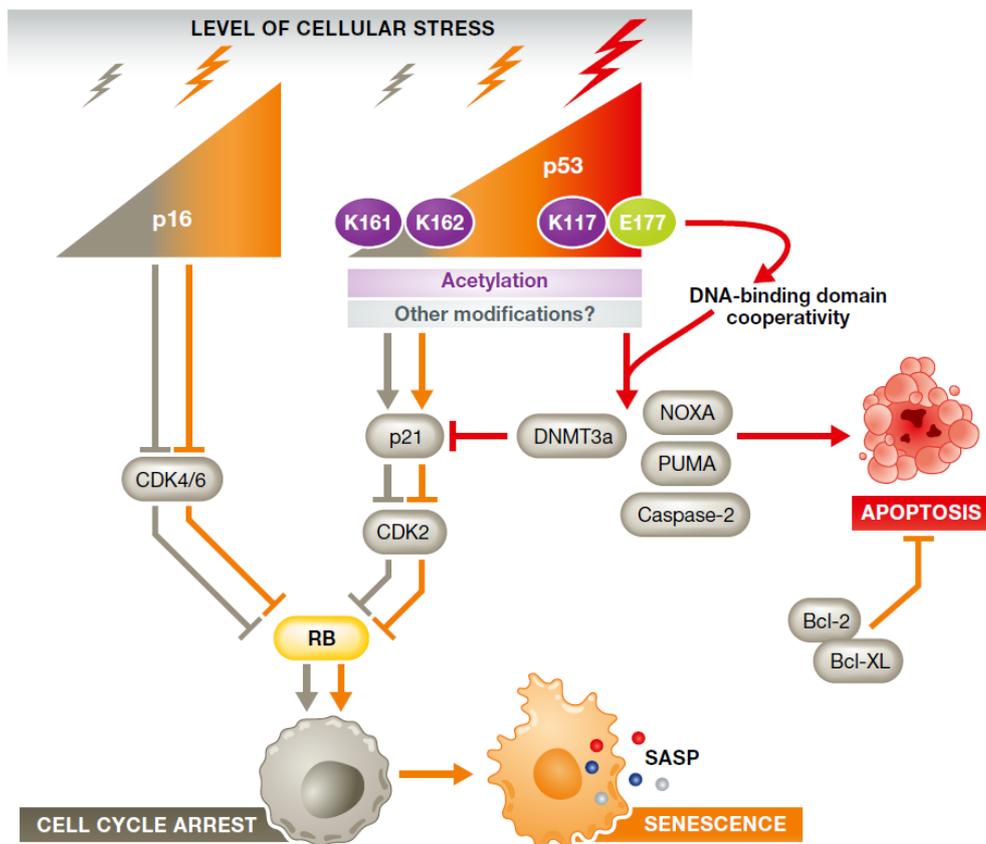
During their lifespan, mitotic cells go through several rounds of replication and in doing so they lose DNA bases at the extremities of their linear chromosomes, that are referred to as telomeres. Telomeres are nucleoprotein structures consisting of T<sub>2</sub>AG<sub>3</sub> hexanucleotide DNA repeats bound by a protective protein complex named Shelterin (Ye et al., 2014). When telomeres shorten over a certain threshold (Hayflick limit), Shelterin cannot protect them anymore, the DNA damage response is triggered and the cell either dies via apoptosis or stops permanently into a senescent state (J. Zhang et al., 2016) (Childs et al., 2014). Since mature neurons are postmitotic cells, they do not undergo cell division-mediated telomere erosion, but their telomeres can still be damaged by oxidative stress and other environmental insults (*e.g.*, radiations). Interestingly, newborn neurons are very sensitive to telomere damage-induced apoptosis (Cheng et al., 2007). Furthermore, dentate gyrus neurogenesis and spatial learning are reduced in telomerase-deficient mice (Rolyan et al., 2011), and telomerase reactivation rescues neurogenesis and olfactory capacity (Jaskelioff et al., 2011).



**Figure 7. The outcomes of age-associated telomere alterations**

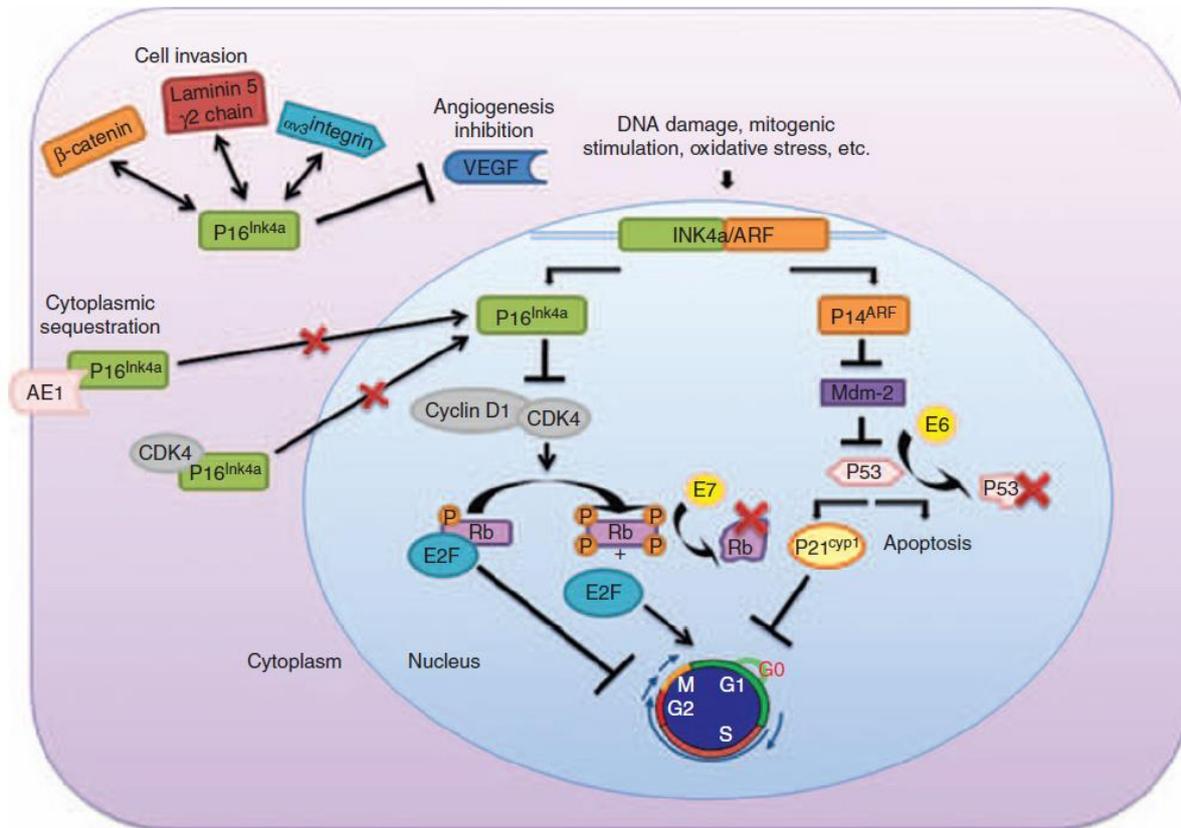
*During aging, telomeres suffer several stresses in both mitotic and post-mitotic cells. The ensuing telomeric damage can lead to genome-wide chromatin remodeling and transcriptional changes, ultimately resulting in apoptosis or senescence (Ye et al., 2014).*

Upon senescence, cells stop dividing, start to permanently express p21 and p16, get resistant to apoptosis and produce pro-inflammatory cytokines (Childs et al., 2014) (Romagosa et al., 2011). Some neural progenitors and glial cells might face senescence during aging as suggested by increased expression of p16 and reduced numbers of proliferating progenitors in the subventricular zone (Molofsky et al., 2006). Noteworthy, when human neural progenitors isolated from a developing fetal cortex are seeded into cultures (primary cultures), they can only undergo a limited number of cell divisions before facing senescence (L. S. Wright et al., 2006).



*Figure 8. The destiny of a stressed cell*

*Schematic of the pathways elicited in a stressed cell, including p16-pRB and p53-p21. According to the extent of the damage, the cell can express p16 and p53, which lead the cell to either cell cycle arrest, senescence or also apoptosis (Childs et al., 2014).*



**Figure 9. The p16 and p21 pathways**

On the one hand, p16 binds and inactivates the Cyclin-dependent kinases (CDK)4/6, thereby impeding Retinoblastoma protein (Rb) phosphorylation and thus inducing cell cycle arrest. On the other hand, p14 inhibits MDM2 resulting in p53 stabilization, which can either drive to cell cycle arrest via p21 that binds and inactivates CDKs, or to apoptosis, according to the extent of the DNA damage (Romagosa et al., 2011).

### Impaired metabolism

Brain metabolism of both glucose and lipids gets impaired with aging. As a consequence of cells getting less and less efficient in glucose transportation upon insulin signaling, glucose concentrations in the blood increase during aging. Insulin resistance (*i.e.*, elevated fasting blood glucose and insulin levels) is one of the greatest risk factors for diabetes, atherosclerotic diseases and stroke. Importantly, peripheral insulin resistance is also associated with cognitive deficit during aging (Thambisetty et al., 2013). Moreover, positron emission tomography (PET) imaging shows a decrease of glucose uptake in temporal, parietal, frontal lobes, and motor cortex neurons which correlates with both aging and cognitive impairment (Goyal et al., 2017) (Kato et al., 2016). Mechanistically, insulin binding causes the activation of its receptor's tyrosine kinase domain, which leads to the phosphorylation of the Insulin receptor substrate 1 (IRS-1). Upon insulin resistance, IRS-1's tyrosine

residue phosphorylation is decreased, whereas phosphorylation at its Ser312 residue is increased. Indeed, *postmortem* analysis of elderly brains shows increased Ser312 phosphorylation (Moloney et al., 2010) (Yarchoan et al., 2014). Furthermore, during aging the neuronal glucose transporter GLUT3 activity can be impaired by oxidation and the ensuing HNE formation (Mark et al., 1997).

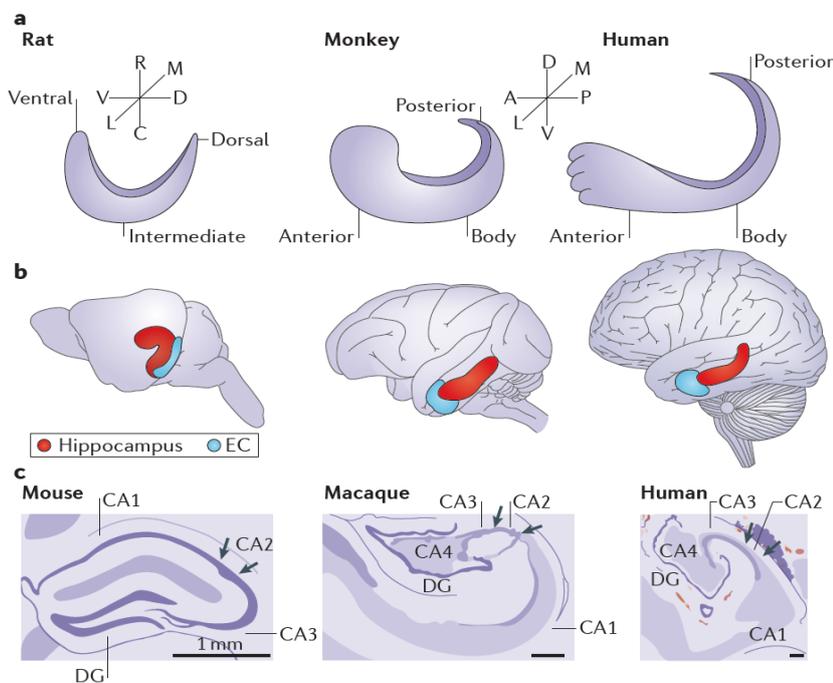
Dyslipidemia (*i.e.*, elevated blood levels of low-density lipoproteins and triglycerides) is a major risk factor for vascular dementia and stroke. Aging affects the metabolism of several lipid molecules leading to the accumulation of ceramides (Cutler et al., 2004), lipid-laden cells (Shimabukuro et al., 2016), and the decline of omega-3 fatty acid levels (Denis et al., 2013). Moreover, there is genetic evidence for a link between impaired lipid metabolism and cognitive deficit during aging: the  $\epsilon 4$  allele of the gene encoding for the Apolipoprotein E, which transports cholesterol and other lipids, is the major genetic risk factor for late-onset sporadic AD (Lane-Donovan et al., 2014). The mechanism behind it is not clear, but it might be due to APOE  $\epsilon 4$  lacking the antioxidant properties of APOE  $\epsilon 2$  and APOE  $\epsilon 3$  [HNE-scavenging activity] (Pedersen et al., 2000), and/or to the altered trafficking of proteins (*e.g.*, APP – Amyloid precursor protein) through the endosomal pathway (Lane-Donovan et al., 2014).

After an initial introduction about the aging of the brain as a whole, the reader is now kindly invited to dive deeper into the brain ocean and meet one of its most marvelous creatures: the Hippocampus.

## 2. The Hippocampus: The Cradle of Memory

Part of the limbic system, the hippocampus is that seahorse-shaped structure (via the Latin, from the Greek: ἵππόκαμπος; indeed, seahorse) situated below the medial temporal lobe of the cerebrum, deep inside the brain (Zeidman & Maguire, 2016).

The hippocampus is the brain region devoted to the formation of declarative memories (episodic and semantic), spatial learning, and the regulation of emotional behavior (El-Falougy & Benuska, 2006). Interestingly, *in vivo* studies have shown a longitudinal distribution of hippocampal functions along its major axis (Bannerman et al., 2002) (Strange et al., 2014). The maintenance of its structure and functionality is pivotal for memory and learning but, unfortunately, this brain region is particularly vulnerable to the aging process. Moreover, structural and functional changes of the hippocampus correlate with the occurrence and severity of cognitive decline in both animals and humans. Noteworthy, this cognitive impairment can be mimicked in animal models with bilateral hippocampal damage (Geinisman et al., 1995). Importantly, hippocampal aging comes along with several structural, cellular, and molecular alterations that will be described hereafter.



**Figure 10. The hippocampus in different mammalian species**

The hippocampus (a) orientation and (b) localization in the rat, monkey and human brain. (c) Hippocampal cross-sections of mouse, macaque and human brains. The typical hippocampal subfields, DG and CA1/2/3/4, are indicated [R = Rostral, C = Caudal, V = Ventral, D = Dorsal, L = Lateral, M = Medial, A = Anterior, P = Posterior; DG = Dentate gyrus, CA = Cornu ammonis] (Strange et al., 2014).

## 2.1. Structural, cellular and molecular aspects of hippocampal aging

### *Volume loss*

Interestingly, a correlation between the impairment of hippocampus-dependent activities and a decrease in hippocampal volume was shown in female rats (Driscoll et al., 2006). Nonetheless, a study with old male mice showed an insignificant age-associated decrease in hippocampal volume, but found a significant axonal degeneration (Von Bohlen und Halbach & Unsicker, 2002). In human cross-sectional studies results are also contradictory. For example, hippocampus-dependent memory deficits were observed in elderly subjects, which correlated with bilateral reduction of hippocampal volume (Driscoll et al., 2003), whereas other studies reported no significant change in hippocampal volume during aging (Du et al., 2006), and a single study observed a significant reduction but only in men (Pruessner et al., 2001). Moreover, some studies showed no clear correlation between structural and functional hippocampal changes and memory impairment during aging (Persson et al., 2012). Nonetheless, in a five-year longitudinal study, a mild reduction of the hippocampal volume was detected in individuals below the age of 50, whereas a remarkable volume loss (1.2% per year) was observed in individuals above that age (Raz et al., 2004). On the same line, another 30-month-long longitudinal study showed an exponential reduction in the CA and DG volume of individuals older than 49 (Raz et al., 2010). Intriguingly, it was also shown that distinct portions of the hippocampus have different rates of volume loss (*i.e.*, anterior vs posterior). It seems that, due to its higher connectivity with the subcortical and hypothalamic nuclei, and therefore being more affected by stress hormones and by the hypothalamic-pituitary axis, the anterior part of the hippocampus is more prone to age-related atrophy (Gordon et al., 2013).

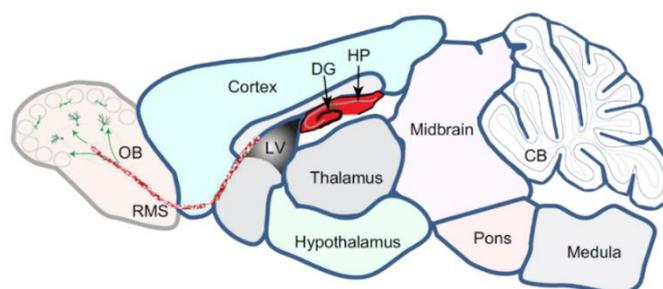
### *Neuronal loss and impaired neurogenesis*

The decrease in hippocampal volume could be due to the neuronal loss occurring during aging. Accordingly, a significant decrease in NeuN-positive cells (NeuN being a bonafide marker of mature neurons) and non-neuronal cells was observed during aging in mice (Fu et al., 2015). Likewise, a decrease of the neuronal metabolite N-acetyl aspartate (NAA), suggesting hippocampal neuronal loss, was observed in humans (Driscoll et al., 2003).

Alternatively, but not mutually exclusive, hippocampal volume loss could be due to decreased neurogenesis during aging. Indeed, the sub-granular zone (SGZ) of the hippocampus is the only brain region, together with the olfactory bulb, to conserve proliferative capacity over age (Ming & Song, 2011). The newborn neurons generated in the SGZ migrate to the Granular cell layer of the DG where

they start to develop neurites that allow their integration in the existing neuronal networks (Bruehl-Jungerman et al., 2007). Adult neurogenesis was originally demonstrated in humans by using hippocampal tissues of deceased cancer patients that were treated with 5-bromo-2'-deoxyuridine (BrdU, which incorporates in dividing cells) for diagnostic purposes (Eriksson et al., 1998). A decade ago, a study on individuals that were exposed to high levels of radioactive carbon-14 isotope ( $^{14}\text{C}$ ) confirmed that neurogenesis occurs in the human hippocampus with a rate of 700 neurons per day (Spalding et al., 2013). On the other hand, the rodent brain was shown to be able to produce as many as 9000 new neurons (granule cells) per day in the dentate gyrus (Cameron & McKay, 2001). Interestingly, while most human DG neurons is believed to be replaced during aging (Ernst & Frisén, 2015), only a 10% of the rodent neurons are (Gil-Mohapel et al., 2013). Indeed, adult neurogenesis seems to be only mildly impaired by aging in humans (Spalding et al., 2013), whereas it is greatly affected in rodents (Ninkovic et al., 2007). Notwithstanding, another study showed that the levels of doublecortin (marker of newborn neuroblasts) comparably decline in humans and rodents during aging (Knoth et al., 2010).

Impaired adult neurogenesis affects memory, spatial learning (W. Deng et al., 2010) and emotional behaviors (Samuels & Hen, 2011), and aging negatively correlates with it in several mammalian species, including mice (Bondolfi et al., 2004) and humans (Knoth et al., 2010). The rate of neurogenesis exponentially declines with aging, with fewer and fewer newborn cells being generated over time and smaller and smaller proportions of them maturing into functional neurons, all of which has a negative impact on longevity (Lazic, 2012). Remarkably, a strong reduction in cell proliferation and differentiation can be detected between 1.5 and 3 months of age in C57BL/6J mice. By the time mice are 24-month-old, a striking 98-99% decrease in the expression of all proliferative markers can be observed (Gil-Mohapel et al., 2013).



**Figure 11. Adult neurogenesis in the murine brain**

Adult neurogenesis is normally restricted to two main neurogenic regions, namely the Sub-granular zone (SGZ) of the Dentate gyrus (DG) in the hippocampus (HP), where new granule neurons are generated; and the Sub-ventricular zone of the lateral ventricles (LV), where new neurons are generated to then migrate via the Rostral migratory stream (RMS) to the olfactory bulbs to become interneurons (Ming & Song, 2011).

The age-related impaired neurogenesis might derive from the increased neuroinflammation that occurs with aging (elevated levels of pro-inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$  and IL-6) (Bodles & Barger, 2004) and a decreased release of growth factors by astrocytes, *e.g.*, Fibroblast growth factor-2 (FGF-2), Insulin growth factor-1 (IGF-1), and Vascular endothelial growth factor (VEGF) (Shetty et al., 2005). Noteworthy, age-related memory impairment in both rodents and humans correlates with reduced neurogenesis (Driscoll et al., 2003); vice versa, hippocampal neurogenesis correlates with learning capability (Gould et al., 1999), and decreased neurogenesis impairs memory (Shors et al., 2001). Moreover, it was shown that the steep neurogenesis decline that mice undergo during early adulthood implies a decreased use of spatially precise strategies in favor of compensative, more imprecise ones (Gil-Mohapel et al., 2013).

Furthermore, age-related impaired neurogenesis in the DG occurs simultaneously to structural modifications of the CA1 and CA3 regions, *i.e.*, decreased dendritic branching (Markham et al., 2005) and numbers of spines and synapses (Adams et al., 2008) (L. Shi et al., 2005). Importantly, the synaptic alterations that occur in the hippocampus during aging may be explained by the tampered expression of proteins involved in synaptogenesis and synaptic stabilization. For example, Postsynaptic density protein 95 (PSD-95), Synapsin I, Synaptophysin, AMPA and NMDA receptor subunits are all decreased during aging (Nyffeler et al., 2007) (Vanguilder et al., 2010). On top of this, a concomitant age-related upregulation of Extracellular matrix (ECM) proteins (*e.g.*, Hyaluronan, Proteoglycan link protein 1, and Neurocan) may interfere with pathways involved in synaptic transmission and contribute to the age-related cognitive decline (Végh et al., 2014).

### *Neurovascular alterations*

Neurovascular and inflammatory alterations may be, at least partially, the cause of decreased neurogenesis and neuronal loss observed during aging. Indeed, studies on rats showed that hippocampal vasculature and cognitive functions simultaneously decline during aging (R. Zhang et al., 2012). Moreover, a study on monkeys observed an age-related reduction in hippocampal blood flow accompanied by decreased neuronal functions, as measured by the decreased expression of the Immediate early gene (IEG) *Arc* (Small et al., 2004). Furthermore, a study on mice showed an age-dependent alteration of the basement membrane and pericyte loss, which can account for Blood-brain barrier (BBB) impairment and vascular leakage (Soto et al., 2015). Noteworthy, the same age-related BBB alterations were also observed in the CA1 and DG of human hippocampi, which correlated with cognitive decline (Montagne et al., 2015). Importantly, when the BBB is damaged,

neurotoxic proteins can get access to the Central nervous system (CNS) causing neuroinflammation, oxidative stress and cell death, leading to cognitive decline (Enciu et al., 2013). Intriguingly, when compared to the other brain structures, the hippocampus is remarkably prone and sensitive to BBB breakdown during aging (Montagne et al., 2015). This might be due to the hippocampus being particularly vulnerable to the endothelial dysfunction caused by pathological phenomena such as increased (stress-related) glucocorticoid concentrations (Ekstrand et al., 2008) and accumulation of Amyloid- $\beta$  (A $\beta$ ) aggregates (Iadecola, 2015).

### *Neuroinflammation*

From an immune perspective, the expression of pro-inflammatory genes whose products are toll-like receptors and complement components, is increased in the aging hippocampus (Cribbs et al., 2012). This alteration is most likely due to an hippocampal age-related overactivation of the microglia, as found in both rats and humans (Griffin et al., 2006). Microglia are abundant in the hippocampus (Lawson et al., 1990) and although their role as protective innate immune cells is pivotal, their overactivation observed during aging is detrimental and causes excessive/chronic neuroinflammation leading to neuronal damage (Von Bernhardi et al., 2010). Therefore, dysregulated microglia activation is likely to contribute to the age-related cognitive impairment. Very interestingly, microglia overactivation in another brain region, the hypothalamus, may itself affect hippocampal neurogenesis due to an impaired hypothalamic production of gonadotropin-releasing hormone (GnRH), leading to systemic aging and therefore indirectly impacting the hippocampus (G. Zhang et al., 2013).

### *Impaired synaptic plasticity*

Another pivotal neuronal feature affected by age is synaptic plasticity, *i.e.*, the capacity of neurons to newly create or modify the strength of their interactions in an activity-dependent fashion (Citri & Malenka, 2008). Accordingly, long-term synaptic plasticity consists of long-lasting (from several minutes to days and years), activity-dependent changes in synaptic efficacy (Abraham et al., 2002). There are two distinct forms of long-term synaptic plasticity: Long-term potentiation (LTP) and Long-term depression (LTD), being LTP the strengthening of a synapsis (Bliss & Gardner-Medwin, 1973) and LTD the weakening of it (Dudek & Bear, 1993), both potentially lasting from minutes to decades. Impaired hippocampal LTP correlates with aging in rodents, leading to defective long-term memory consolidation (Barnes & McNaughton, 1985) (Lister & Barnes, 2009). Indeed, studies with rats

showed that this age-dependent decline in LTP correlates with impaired performances in spatial memory tests (Barnes & McNaughton, 1980). Importantly, old rats also exhibit lower thresholds for LTD and therefore major hippocampal depotentiation (Norris et al., 1996). Altogether, these studies showed that old rats hardly consolidate new memories and easily erase past ones, due to increased LTP and decreased LTD thresholds, respectively (Barnes et al., 2000).

Other factors contributing to the decline of rodent hippocampal synaptic transmission during aging and the ensuing cognitive impairment are a decreased number of hippocampal synapses, structural alterations of dendrites and dendritic spines (atrophy), and the reduced expression of several synaptic proteins such as Synaptophysin (Gureviciene et al., 2009) (Rogers et al., 1984) (R. Wang et al., 2007). Analogous changes have been reported also in the aging human hippocampus (Geinisman et al., 1995) (Mattson & Magnus, 2006).

Noteworthy, the distinct regions of the hippocampus are differently affected by aging with respect to synaptic plasticity. For example, in old rats, the synapses of the Perforant path (PP) starting from the Entorhinal cortex (EC) and projecting to the Granule cells (GCs) of the DG, show a particularly marked decrease in NMDA receptor responsiveness, fewer synaptic interactions, and a higher LTP threshold (Barnes et al., 2000). The age-associated degeneration of the PP synapses was also confirmed in humans by means of *in vivo* imaging analysis, and it correlates with memory impairment (Yassa et al., 2010).

Since glutamate transmission has a pivotal role in the LTP and LTD processes, it has been proposed that dysregulated glutamate neurotransmission and excitotoxicity might be the leading cause of the above-mentioned age-driven synaptic plasticity impairment (Michaelis, 1998). Moreover, the age-associated increase in neuroinflammation might also contribute to impaired neuroplasticity. Indeed, proinflammatory cytokine production (*e.g.*, IL-1 $\beta$ ), can interfere with the activity of neurotrophic factors such as BDNF, a well-known hippocampal plasticity modulator (Patterson, 2015). Furthermore, age-related hormonal changes such as diminished insulin and leptin levels are also associated with decreased synaptic plasticity (McGregor et al., 2015).

Finally, the levels of the Vesicle-associated membrane protein 2 (VAMP2) also decrease with age in the murine hippocampus. Together with Syntaxin-1 and Synaptosomal-associated protein 25 kDa (SNAP25), VAMP2 forms the Snap receptor (SNARE) complex, which is pivotal for neurotransmitter release in the synaptic cleft upon Ca<sup>2+</sup> influx in the presynaptic terminal. Indeed, *in vitro* experiments showed that downregulation of VAMP2 leads to a decreased rate of neurotransmitter release. Moreover, *in vivo* experiments revealed that VAMP2 knock-down impairs memory and learning as

assessed by behavioral tests, suggesting that the VAMP2 age-related decline plays a pivotal role in the impairment of synaptic plasticity (Orock et al., 2020).

### *Overproduction of reactive oxygen and nitrogen species*

One of the hallmarks of cellular aging is the increased levels of free radicals as initially proposed by Harman in 1956 (HARMAN, 1956): aging leads to uncontrolled oxidative processes and decreased levels of endogenous antioxidant defensive mechanisms, which cause the accumulation of Reactive oxygen species (ROS) and Reactive nitrogen species (RNS), ultimately leading to the damage of cellular molecules (*i.e.*, nucleic acids, lipids and proteins) that in turn, in a positive feedback loop, elicit further mitochondrial dysfunction and ROS production (Balaban et al., 2005). Nonetheless, oxidant molecules play pivotal roles in signaling and synaptic plasticity (Finkel et al., 1998). For example, the redox state of the cell is crucial for proper NMDA receptor functioning at hippocampal synapses during LTP events (Bodhinathan et al., 2010), and for adult neurogenesis in the SGZ of the DG (T. T. Huang et al., 2012). In the hippocampi of old rats, oxidative damage to cellular lipids correlates with impaired LTP (Lynch et al., 1998). Therefore, an age-associated imbalance of the intracellular redox state may be detrimental for structural and functional plasticity. Noteworthy, among all the brain regions, the hippocampus is the preferential localization of a dysregulated redox state in old mice (Stebbing et al., 2016).

Imbalance of the intracellular redox state is due to several factors, *e.g.*, increased levels of enzymes involved in energy production (S. Yang et al., 2008) and increased oxidative stress and lipid peroxidation (Cini & Moretti, 1995). Moreover, protein oxidation was also detected in both human and murine memory-impaired old brains (Nicolle et al., 2001). Furthermore, also cell membranes are oxidatively damaged, and mitochondria get dysfunctional with hippocampal aging. This affects transport processes and the levels of metabolic substrates (Speakman et al., 2004), which in turn impairs hippocampal function (Reddy, 2009). Finally, in the hippocampus of both rodents and humans, a decrease of enzymatic (*e.g.*, superoxide dismutase, catalase and other reductases) and non-enzymatic antioxidant activities was detected during aging (Venkateshappa et al., 2012). This decrease in antioxidant capacity leads to vulnerability against oxidative insults and neurodegeneration. Hopefully, antioxidant treatment in mice can alleviate the effect of the elevated hippocampal ROS levels and the ensuing cognitive decline associated with age (Souza et al., 2015).

Noteworthy, Apolipoprotein D (a neuroprotective antioxidant) is upregulated in human hippocampal neurons upon increased age-associated oxidative stress (Martinez et al., 2013), most likely reflecting a coping mechanism.

ROS accumulation during aging causes misfolding or partial unfolding of proteins that are checked for quality control by the protein degradation pathway involving the proteasome. Unfortunately, this protein quality control system is itself impaired in the hippocampus of both rodents and humans affected by cognitive decline (Squier, 2001) (VanGuilder & Freeman, 2011) (S. Yang et al., 2008). Moreover, the expression of protein chaperons such as Calnexin and heat shock proteins, and also of proteasome subunits, is decreased in the hippocampus of old rats (Squier, 2001) (S. Yang et al., 2008). Nevertheless, a canine study showed a compensatory increased expression of some chaperone proteins such as Heat-shock protein 90 (HSP90) during aging, probably a coping mechanism to contrast the age-dependent protein misfolding and boost protein quality control (Romanucci & Della Salda, 2015). All in all, old animals are less capable of dealing with protein misfolding because of the decreased activity of mechanisms involved in quality control of proteins. Moreover, the thermodynamic state of misfolded proteins causes them to aggregate (*e.g.*, A $\beta$  aggregates), which increases cellular stress and the accumulation of neurofilaments with neurodegenerative outcomes (Paz Gavilán et al., 2006).

### *Impairment of the hypothalamic-pituitary-adrenal axis*

Another function affected by aging in both rodents and humans is that of the hypothalamic-pituitary-adrenal (HPA) axis (Ferrari & Magri, 2008), accounting for the major neuroendocrine responses of the brain, whose activation leads to glucocorticoid production by the adrenal glands (S. J. Lupien et al., 2009). Structural hippocampal alterations correlate with an impaired HPA axis function, characterized by its hyperactivation and an ensuing excessive or prolonged stress response (Rodrigues Siqueira et al., 2005). Glucocorticoids (*i.e.*, Cortisol in humans and Corticosterone in rodents) can bind to two different nuclear receptors. One is the Mineralocorticoid receptor (MR), a high-affinity receptor that can be activated by basal concentrations of these hormones. The other is the Glucocorticoid receptor (GR), a low-affinity receptor that needs high concentrations of glucocorticoids to be activated (Funder et al., 1997). Activation of MRs by moderate concentrations of glucocorticoids has a positive effect on synaptic transmission and plasticity (Gass et al., 2000), whereas the activation of GRs by excessive concentrations of glucocorticoids has a deleterious outcome impairing synaptic plasticity (Krugers et al., 2006), trophic support (Suri & Vaidya, 2013)

and adult hippocampal neurogenesis (Anacker et al., 2013). Indeed, glucocorticoids mediate glutamate release, therefore acute or prolonged activity of these hormones is implicated in excitotoxicity and oxidative stress (Popoli et al., 2012). Moreover, elderly individuals are more responsive to Cortisol than young ones, which supports the idea that age-related changes of the hippocampus can exacerbate the age-associated impairment of the HPA axis (Belvederi Murri et al., 2014) (S. Lupien et al., 1994). Increased levels of glucocorticoids can thus damage the hippocampus and may therefore elicit the activation of the immune system (priming of microglia) further contributing to hippocampal aging (Barrientos et al., 2015). Finally, variations in the glucocorticoid-dependent transcriptome can also boost hippocampal aging by modifying the expression of genes involved in several neuronal and metabolic processes (K. C. Chen et al., 2013) (Gassen et al., 2017).

### *Decreased levels of neurotrophins*

For their growth, survival and functioning, neurons need the presence of a specific type of growth factors referred to as neurotrophins. There are four of them which are also structurally related, namely: Brain-derived neurotrophic factor (BDNF), Nerve growth factor (NGF), Neurotrophin-3 (NT-3), and Neurotrophin-4/5 (NT-4/5) (Skaper et al., 2008). Among them, BDNF is the most abundant in the hippocampus (Erickson et al., 2012) and is pivotal for neurogenesis and neuronal survival (Pencea et al., 2001), synaptic plasticity (J. I. Tanaka et al., 2008) and memory formation (Erickson et al., 2012). Impairment of the signaling mediated by neurotrophic factors (especially BDNF) correlates with cognitive impairment (Erickson et al., 2010), decreased LTP and spatial memory (Rex et al., 2006), diminished neurogenesis, and depression, which can be rescued by exogenous BDNF administration (Schmidt & Duman, 2010).

BDNF protein levels decrease in the hippocampus of both old rats (Calabrese et al., 2013) and monkeys (M. Hayashi et al., 2001). In the rat, the age-related decrease of BDNF protein levels correlates with a decline of its mRNA expression (Perovic et al., 2013), especially in the CA1 and CA3 regions (T. R. Chapman et al., 2012). Moreover, in the hippocampus of aged rats it was reported a decreased processing of the pro-neurotrophin precursors, with the ensuing accumulation of pro-BDNF and pro-NGF, and decreased levels of mature BDNF and NGF (Silhol et al., 2008). Furthermore, also the expression of the Neurotrophin receptor tyrosine kinase 2 (TrkB) was reported to be decreased in the hippocampus of old animals (Perovic et al., 2013). Noteworthy, the age-associated increase of oxidative stress might contribute to BDNF decrease, since antioxidant treatment can rescue the BDNF deficits in the hippocampus of old mice (Souza et al., 2015).

Another important factor for the hippocampus known to decline with aging is Vascular endothelial growth factor (VEGF), which is pivotal for neurogenesis (Shetty et al., 2005), acting both as a neurotrophic factor and an angiogenesis inducer (L. Cao et al., 2004) (Sun et al., 2003). VEGF might indeed manage the cross-talk between neural progenitors and endothelial cells to allow a synchronized growth of blood vessels and neurogenesis to guarantee proper metabolic support to newborn neurons (Kirby et al., 2015). Indeed, VEGF overexpression in adult rats elicits a 2-fold increase in DG neurogenesis and, remarkably, VEGF inhibition completely impairs cognitive stimulation-induced neurogenesis (L. Cao et al., 2004). Moreover, VEGF is involved in the modulation of synaptic plasticity by increasing dendritic spine number, synaptic transmission and LTP (Y. F. Huang et al., 2010) (B. W. Kim et al., 2008) (Licht et al., 2011). Therefore, VEGF can be fully considered a neuroprotective factor also taking into account the fact that it rescues neurologic functionality after hippocampal damage (Nicoletti et al., 2008). The age-related decline in VEGF signaling might thus be causal for hippocampal impairment due to the reduced perfusion and trophic supply to hippocampal neural progenitors that it causes. Underlining the importance of neurotrophins for hippocampal fitness, the expression of other neurotrophic factors such as NGF (Lärkfors et al., 1987), FGF-2, and IGF-1 (Shetty et al., 2005) is also decreased in the hippocampus of old rats.

Decreased BDNF levels and TrkB mRNA expression in serum (Erickson et al., 2010) and cerebrospinal fluid (CSF) of aged humans correlate with cognitive deficit (impaired spatial memory) (G. Li et al., 2009). Interestingly, diminished BDNF serum levels also correlate with gray matter loss and decreased hippocampal volume. Thus, it sounds plausible that therapies based on neurotrophins and growth factors might improve age-related hippocampal impairments. Indeed, rescuing the hippocampal levels of neurotrophic factors such as FGF-2 and IGF-1 via direct infusion or overexpression increases hippocampal neurogenesis in old rats (Jin et al., 2003) (Pardo et al., 2016). Moreover, it is by now well-established knowledge that these very factors are pivotal for the pro-neurogenic effects of non-invasive therapies such as calorie restriction (CR), physical activity and cognitive stimulation (J. Lee et al., 2002) (Cotman et al., 2007). Therefore, neurotrophin treatment combined with appropriate lifestyle interventions may provide a promising therapeutic approach to counteract the age-related hippocampal decline.

### *Epigenetic alterations*

Epigenetic changes, *i.e.*, the biochemical modifications that DNA and DNA-bound proteins undergo during a cell lifespan (*e.g.*, DNA de-/methylation and histone de-/acetylation and de-/methylation),

can modify patterns of gene expression. Usually, DNA methylation (catalyzed by DNA methyltransferases – DNMTs) leads to gene repression, whereas histone acetylation (catalyzed by Histone acetyl transferases – HATs) boosts gene expression (Barrett & Wood, 2008).

Epigenetic modifications affecting genes involved in synaptic plasticity (*e.g.*, the IEGs *Arc*, *Zif268*, *Narp*, *Homer1a* and *Bdnf*) are associated with age-related memory impairment (Blalock et al., 2003) (Penner et al., 2010). For example, the transcription of *Arc* occurs during neuronal activity and LTP events leading to memory consolidation (Miyashita et al., 2008), whereas the expression of IEGs such as *Zif268*, *Homer1a*, and the same *Arc* is decreased in the hippocampus of old rats with memory deficits (Blalock et al., 2003). The decrease in these IEGs expression is likely due to modifications of the DNA methylation pattern (Penner et al., 2016). Indeed, an age-related increase in the methylation within the promoter of the IEG *Early growth response 1 (Egr1)* correlates with a decreased transcription of the gene in the DG of old rats upon spatial memory testing (Penner et al., 2016).

Age also affects histone methylation and acetylation with a negative impact on synaptic plasticity and memory. For example, an age-dependent decrease in the acetylation of histone H4 in the CA1 and DG regions correlates with impaired spatial and contextual memory, reduced LTP and diminished BDNF/TrkB signaling (Deibel et al., 2015) (Peleg et al., 2010). Remarkably, treatment with Histone deacetylase (HDAC) inhibitors could improve memory and learning in such a context. Analogously, histone H3K9 trimethylation leads to gene repression, whereas inhibition of its specific methyltransferase SUV39H1 rescues spine density and spatial learning in the hippocampus of old mice (Snigdha et al., 2016). Moreover, other age-related changes in histone H3 methylation levels (a decrease in H3K4 trimethylation and an increase in H3K27 trimethylation) correlate with decreased levels of doublecortin mRNA, leading to fewer doublecortin-positive cells in the DG (Kuzumaki et al., 2010). Therefore, since doublecortin is only expressed in neuroblasts, age-related epigenetic modifications might also affect adult neurogenesis.

### *Senescence*

Replicative senescence, as first described by Hayflick and Moorhead (Hayflick and Moorhead, 1961), is the phenomenon whereby a given mitotic cell loses the ability to replicate after reaching a certain number of cell divisions due to the shortening of its telomeres, *i.e.*, the nucleoprotein structures located at the extremities of eukaryotic chromosomes that cannot be fully replicated upon each mitotic event (Allsopp et al., 1995) (Olovnikov, 1996). Upon reaching the threshold of telomere

shortening (Named after one of its discoverers, “Hayflick limit”), the cell either dies via apoptosis or stops permanently into a senescent state, in both cases following the activation of pathways involving tumor suppressor protein p53 and Retinoblastoma protein (pRb) (Chin et al., 1999) (Deng et al., 2008). Indeed, it was postulated that cellular senescence evolved to counteract tumorigenesis, interrupting the uncontrolled proliferation of cancerous cells (Campisi, 2001).

The telomeric status is pivotal for the aging process. In fact, multiple premature aging syndromes and age-related pathologies are consequence of accelerated telomere shortening and dysfunction (Blasco, 2007) (Burtner & Kennedy, 2010) (Rudolph et al., 1999). Degenerative processes associated with telomere shortening can be reversed upon telomere rescue via the reactivation of Telomerase (a reverse transcriptase enzyme that elongates telomeres by adding telomere repeats *de novo*), whose expression is turned off in all the tissues of the organism with the exception of the germ and stem cell niches. Therefore, it is extremely likely that telomeres have a pivotal role in stem cell aging (Jaskelioff et al., 2011). Telomere shortening causes tissue failure by making it impossible for cells to replicate when tissues need to be renewed. Yet another way by which telomere erosion negatively impacts tissues is via eliciting a so called Senescence-associated secretory phenotype (SASP) (Campisi & D’Adda Di Fagagna, 2007), which can give rise to a pro-inflammatory extracellular environment due to the cell releasing cytokines and chemokines such as TNF- $\alpha$  and IL-6, proteases, insoluble proteins and ECM components (Coppé et al., 2010) (B. Wang et al., 2020). Therefore, SASP contribute to the age-related chronic inflammation that leads to tissue dysfunction and pathology (Tchkonia et al., 2013), and senescent cells (SCs) might boost neurodegenerative processes by releasing SASP molecules (Chinta et al., 2015). Importantly, depletion of SCs by senolytic drugs (compounds that specifically target senescent cells for death) prevents the occurrence of degenerative phenotypes observed in old mice such as osteoporosis, lordokyphosis, cataracts and lipodystrophy (Baker et al., 2011) (Y. Zhu et al., 2015).

Interestingly, upon oxidative stress and DNA damage, cultured human astrocytes undergo senescence and exhibit SASP (Bitto et al., 2010) which, *in vivo*, may reduce their neuroprotective activity and impair the synaptic function of neighboring neurons, ultimately leading to AD (Pertusa et al., 2007) (Bhat et al., 2012). Moreover, other pivotal CNS cell types (microglia, oligodendrocytes and endothelial cells) may also undergo senescence (Chinta et al., 2015). Microglial senescence might play a crucial role in inflammaging (*i.e.*, the increasing inflammation due to aging) (Rawji et al., 2016); oligodendrocytes senescence might impair axon myelination and affect signal

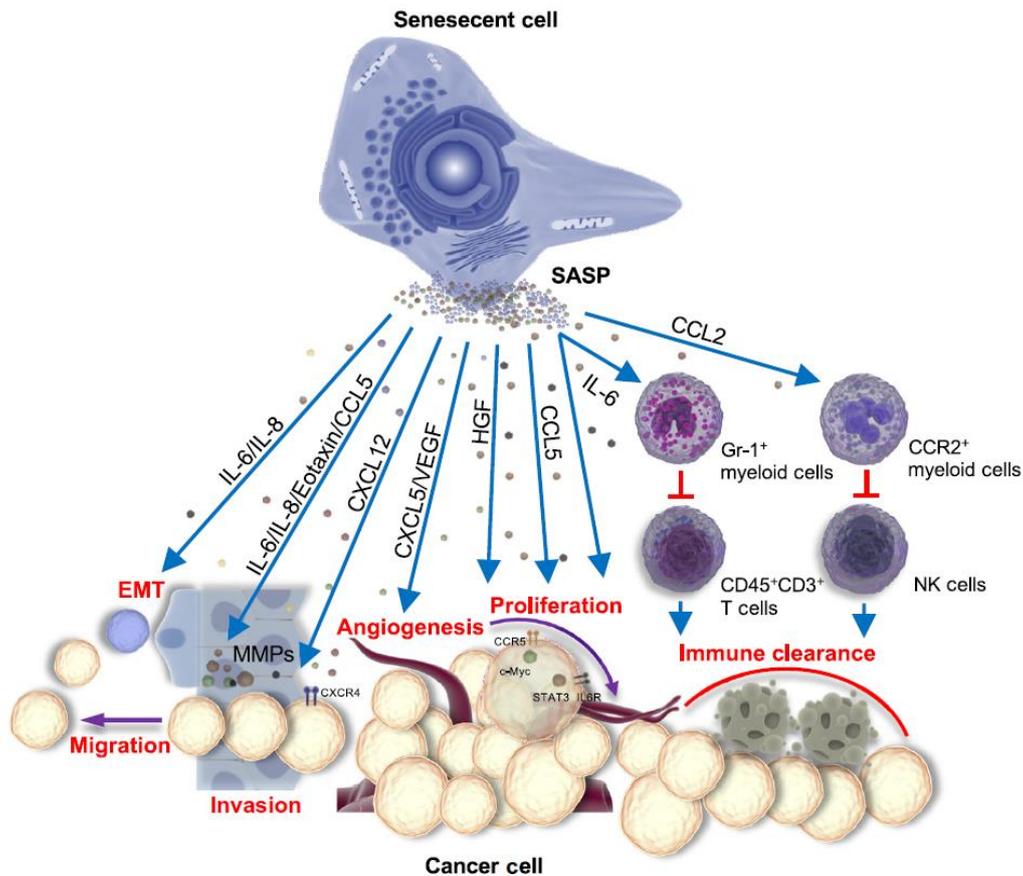
transmission; and endothelial cell senescence might disrupt BBB integrity (Chinta et al., 2015) (Crowe et al., 2016).

DNA damage can elicit senescence in both humans and rodents (Uday Bhanu et al., 2010) (C. M. Dong et al., 2014) (Jurk et al., 2012), and *in vitro* studies suggest that senescent neurons might accumulate in the hippocampus during aging thus contributing to its age-related dysfunction (W. Dong et al., 2011) (Geng et al., 2010). Adult hippocampal neurogenesis drastically decreases during aging (Ben Abdallah et al., 2010), therefore, hypotheses exist that the increasing number of SCs with age might contribute to this phenomenon. Nonetheless, experimental evidence in rat DG suggests that the decline of adult hippocampal neurogenesis is due to Neural stem cells (NSCs) entering a quiescent state (Hattiangady & Shetty, 2008) via a Notch-dependent mechanism (Lugert et al., 2010). Very interestingly, physical exercise, a strong activator of adult hippocampal neurogenesis, can awaken quiescent cells reestablishing the NSC pool (Lugert et al., 2010). Moreover, modifying the composition of the systemic environment (via heterochronic parabiosis) has a rejuvenating effect on the hippocampus of the old animal; *i.e.*, improved neurogenesis, synaptic plasticity, learning and memory (Villeda et al., 2011) (Villeda et al., 2014).

Nevertheless, an alternative theory proposes that the decline of adult hippocampal neurogenesis stems from the exhaustion of the NSC pool (Van Deursen, 2014). According to this hypothesis, during aging each NSC might only be used once to become an astrocyte (Encinas et al., 2011). Another alternative scenario could imply telomere attrition/DNA damage as the leading cause for NSCs to become senescent (Behrens et al., 2014). Indeed, exposing NSCs to DNA damage-inducing agents such as hydroxyurea (C. M. Dong et al., 2014) and ionizing radiations (T. Li et al., 2015) causes a senescent phenotype. Moreover, other than senescent NSCs, also senescent postmitotic cells such as neurons and astrocytes might accumulate in the aging hippocampus leading to the functional decline of this delicate brain region (Crowe et al., 2016) (Golde & Miller, 2009).

Preventing cell senescence or removing senescent cells via senolytic strategies may avoid the decline in hippocampal plasticity observed in old individuals (Kirkland & Tchkonja, 2015). Indeed, senolytic approaches were proposed as therapies to delay and prevent functional decline (Geiger, 2016). Moreover, the use of antioxidant and anti-inflammatory molecules was shown to delay cellular senescence and improve hippocampal functions in old rodents (P. H. Shih et al., 2010) (X. L. Zhang et al., 2007) (J. Zhu et al., 2014). Taking advantage of the different morphology and expression profile of senescent cells for developing antibodies against senescence-specific antigens, or targeting the mRNA of senescence-specific genes might be successful strategies (Tchkonja et al., 2013). Indeed,

recent studies proved that the depletion of SCs leads to vascular improvement (Roos et al., 2016), rejuvenation of the hematopoietic stem cell pool (Chang et al., 2016), and amelioration of cataracts, sarcopenia and lipodystrophy (Baker et al., 2011). All in all, it seems plausible that SCs depletion may rescue from the age-associated dysfunction of the hippocampus by decreasing neuroinflammation, while boosting neurogenesis and synaptic plasticity.

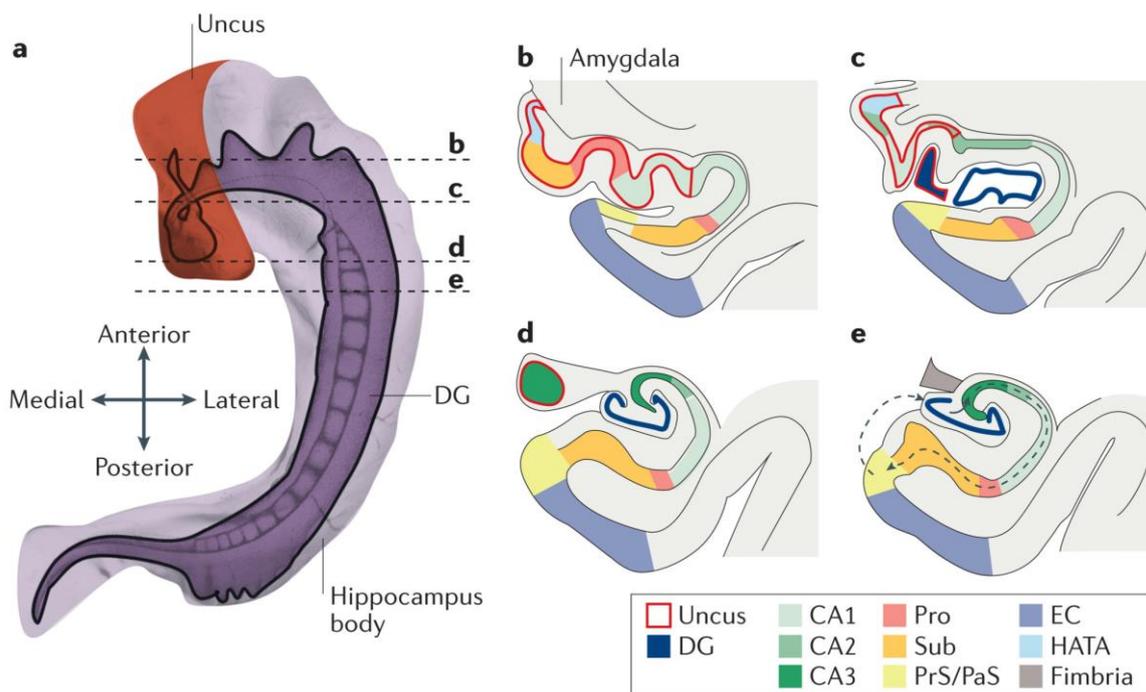


**Figure 12. The senescence-associated secretory phenotype (SASP) and its outcomes**

Upon senescence, cells release a panel of molecules that can affect several phenomena including immune cell recruitment, angiogenesis and cell proliferation, therefore favoring inflammation and ultimately fibrosis and tumorigenesis (B. Wang et al., 2020).

## 2.2. Functional topography of the hippocampus: the anatomy of memory

The hippocampal curved, croissant-like structure is shared by all mammalian species, with a dorso-ventral orientation in rodents, corresponding to a postero-anterior orientation in primates and humans. The dorsal and ventral portions of the hippocampus have different degrees of connections with cortical and subcortical areas of the brain; therefore, this raised the question as to whether different hippocampal regions abide to different functions, and whether the hippocampal function is homogeneously controlled along its major axis.



**Figure 13. Anatomy of the human hippocampus**

(a) Overview of the human hippocampal structure. (b-e) Cross-sections along the longitudinal axis of the hippocampus [DG, Dentate gyrus; CA, Cornu ammonis; Pro, prosubiculum; PrS/PaS, presubiculum and parasubiculum; EC, entorhinal cortex; HATA, hippocampal-amygdaloid transition area] (Zeidman & Maguire, 2016).

The case of the patient H.M., who underwent bilateral hippocampal resection (in an attempt to cure his epilepsy), was pivotal for understanding the role of the hippocampus in memory formation (SCOVILLE & MILNER, 1957). This surgical procedure severely affected declarative memory and, in particular, episodic memory. Indeed, the patient could not form and retain any new memory related to new episodes occurring in his life. Following studies discovered the existence of hippocampal Place cells and found a role in spatial memory for this seahorse-shaped brain region (O'Keefe et al., 1971) (O'Keefe & Speakman, 1987). Later on, the additional discovery of an

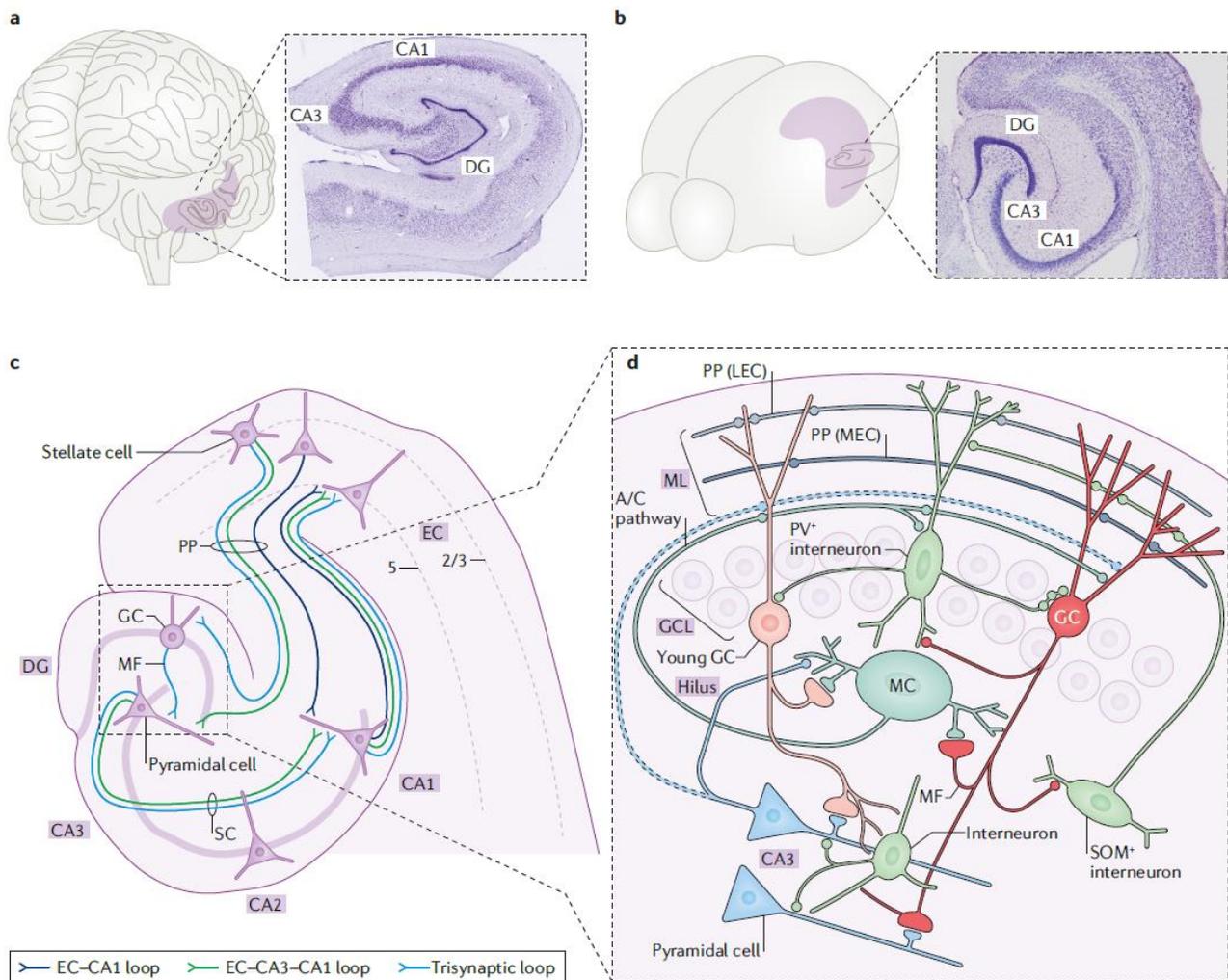
hippocampal role in emotional memory (Kim and Fanselow, 1992) raised the hypothesis that different types of memory could depend on different intrahippocampal circuits. Now the question was whether these circuits were superimposed or not.

Topologically speaking, hippocampal circuits that are functionally distinct appeared also to be separated along the major hippocampal axis. Indeed, first electrophysiological studies in rodents found that the responses dependent on the dorsal hippocampus were dissociated from those dependent on the ventral part, and vice versa (Elul, 1964) (Racine et al., 1977). Moreover, early lesion studies (studies aimed at lesioning a specific region of the hippocampus in order to discover its function based on the effect of its impairment on specific tasks and behaviors) found that dorsal and ventral hippocampal lesions affect behavior differently (Nadel, 1968) (R. Stevens & Cowey, 1973). More detailed, subsequent studies that took into account also the precise location and size of the lesions, found that the dorsal hippocampus is pivotal for spatial learning (E. Moser et al., 1993). Furthermore, based on the fact that the ventral hippocampus has a denser connectivity with the amygdala (Van Groen & Wyss, 1990) and the hypothalamus (Risold and Swanson, 1996), it was hypothesized that it could have a role in emotional responses (J. A. Gray & Mcnaughton, 2000). Ever since those studies, the most accepted idea was that the anterior hippocampus was devoted to emotional responses, whereas the posterior part would be responsible for cognitive functions and in particular spatial memory (Bannerman et al., 2004) (Fanselow and Dong, 2010). Despite that, it was later reported that the hippocampal long axis connectivity with cortical and subcortical areas of the brain follows a gradual distribution (Amaral and Witter, 1989), which led to the hypothesis that also the functional specificity of the hippocampus might follow a gradient-like distribution along its major axis (Kjelstrup et al., 2008). Nevertheless, first gene expression analyses revealed a discretized hippocampal partitioning along its long axis (Thompson et al., 2008). Therefore, the functional topography of the hippocampus resulted more complex than initially thought (M. B. Moser & Moser, 1998b) (Bartsch & Wulff, 2015).

Importantly, the longitudinal dimension of the hippocampus is not the only one to be considered for the understanding of the function-anatomy connection. Indeed, the hippocampus is characterized by the presence of anatomically distinct, well-defined, subfields. Namely, the *Cornu ammonis* (CA) 1, the less studied CA2 and CA4, the CA3, and the *Dentate gyrus* (DG). For example, the DG, endowed with adult neurogenesis capacity, is pivotal for spatial discrimination (Clelland et al., 2009) (Trouche et al., 2009) (Masachs et al., 2021). Recent findings also extended its role to contextual discrimination and episodic memory (Hainmueller & Bartos, 2020). The CA1 seems to be pivotal for

temporal pattern separation (Gilbert et al., 2001), although it was also shown that the CA1-restricted ablation of the NMDA receptor (pivotal for LTP) impairs spatial memory (Tsien et al., 1996). The CA3 appears more as an intermediate structure involved in both episodic memory (Kesner, 2007b) and spatial processing (Hunsaker et al., 2008). Indeed, DG and CA3 cooperate for the encoding process of spatial memories (Jermain et al., 2006) (Kesner, 2007a). Interestingly, DG and CA3 are simultaneously involved in successful memory storage and recall: the capacity of properly retrieving a memory even when the stimulus is not exactly the same that created it (pattern completion – mediated by the CA3) while being able to make that distinction (pattern separation – mediated by the DG) (Neunuebel & Knierim, 2014). Moreover, ablation of the NMDA receptor subunit NR1 impairs spatial working memory (but not spatial reference memory) when it is specifically restricted to either the DG or the CA3, but has no effect when it is limited to the CA1 (Niewoehner et al., 2007). Furthermore, CA3 and CA1 both contribute to contextual memory formation, but only the CA1 is necessary for its retrieval (Ji & Maren, 2008). Time-wise, the CA1 and CA3 seem to be respectively involved in intermediate-term memory and short-term memory formation (Kesner et al., 2004). A study aimed at evaluating the role of the entorhinal efferent pathways (medial and lateral perforant paths – referred to as mPP and IPP, respectively) into the dorsal hippocampus with respect to spatial and non-spatial memory found out that the mPP targeting the DG is not involved in novel object recognition, whereas the mPP and IPP targeting the CA1 are not involved in novel object recognition and spatial discrimination, respectively. On the other hand, both mPP and IPP targeting the CA3 resulted always necessary for both types of memories (Hunsaker et al., 2007). Another study assessed the role of the CA3 most important afferent inputs, the PP-CA3 and the DG-mediated mossy fibers(MFs)-CA3 on spatial memory, distinguishing between encoding and retrieval. Interestingly, DG-lesioned animals exhibited impaired memory encoding but intact memory retrieval, whereas, vice versa, PP-CA3-lesioned animals showed intact memory encoding but impaired memory retrieval (I. Lee & Kesner, 2004).

Therefore, each diverse memory type is characterized by the different contribution of the distinct hippocampal subfields, by their interactions, and by their location along the hippocampal major axis.

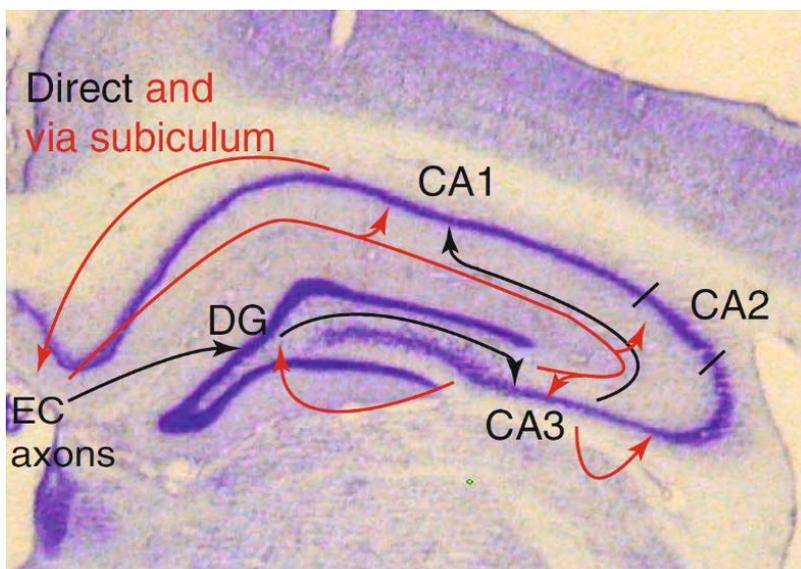


**Figure 14. The anatomy of memory**

(a) Human and (b) mouse brain Nissl-stained cross-sections of the hippocampus. (c) Schematic of the murine hippocampal structure and its main synaptic connections. Dentate gyrus (DG) granule cells (GCs) send mossy-fiber (MF) axons to the CA3. CA3 pyramidal cells send Shaffer-collateral (SC) projections to the CA1, which then projects back to the entorhinal cortex (EC). All of these areas receive perforant-path (PP) inputs from the EC, hence creating multiple parallel loops by which information can flow through the hippocampus. (d) Magnified view of the DG microcircuitry, its intrinsic connections and outputs to the CA3. GCs form the densely-packed GC layer (GCL), which also houses parvalbumin-expressing (PV<sup>+</sup>) interneuron basket cells and axo-axonic cells. Enwrapped by the GC layer are the CA3 area and the DG hilus, which contains glutamatergic mossy cells (MCs) and other types of GABAergic interneurons, such as those expressing somatostatin (SOM<sup>+</sup> interneurons). GCs make synaptic contacts on all of the aforementioned cell types (except for other GCs) via their MF axons [A/C, associational-commissural; LEC, lateral entorhinal cortex; MEC, medial entorhinal cortex; ML, molecular layer] (Hainmueller & Bartos, 2020).

### *Hippocampal-cortical connectivity*

In rodents, a dorso-lateral to ventro-medial gradient originating from the entorhinal cortex (EC) projects with a similar dorsoventral gradient onto the hippocampus (Dolorfo & Amaral, 1998) (Van Strien et al., 2009) (Knierim, 2015). The cortex-to-EC projections are topographically arranged in a way that is maintained in the EC-to-hippocampus projections. Taking the rat as a model, signals from the infralimbic and prelimbic cortices, passing by the ventromedial EC, mainly reach the ventral hippocampus (Jones and Witter, 2007). On the other hand, projections originating in the prelimbic cortex pass through intermediate parts of the EC to reach the hippocampus at an intermediate dorsoventral level. The remaining areas of the cingulate cortex (*i.e.*, anterior cingulate and retrosplenial cortices) mainly target dorsolateral parts of the EC, which in turn target the dorsal hippocampus (Jones and Witter, 2007). Therefore, cingulate areas that are devoted to emotional regulation (infralimbic and prelimbic cortices) mainly target hippocampal ventral regions, whereas cingulate areas involved in spatial learning and memory (retrosplenial cortex) project mostly to dorsal regions. Noteworthy, this projection transitions are not discrete but, instead, follow a graded distribution. Moreover, reciprocally, the topographical organization of CA1/subiculum-to-EC projections is akin to that of EC-to-hippocampus projections (Witter, 1993).



**Figure 15. Entorhinal cortex-hippocampal interconnectivity**

Coronal section of the murine hippocampus. The black arrows highlight the classic "trisynaptic loop" connecting sequentially EC, DG, CA3 and CA1. The red arrows highlight other "via subiculum" important connections showing the EC direct connections to the three CA areas, the CA3 recurrent collateral circuitry, and the CA3-DG and CA1-EC feedback projections (Knierim, 2015).

### *Hippocampal-subcortical connectivity*

The major subcortical target of the hippocampus is the Lateral septum (LS) region (involved in emotional, motivational and spatial behaviors), in the lower, posterior part of the medial surface of the frontal lobe (Risold and Swanson, 1996). The dorsal half of the hippocampus projects to a small dorsal part of the LS, whereas the more ventral the hippocampal area, the more are the projections originating from it, and the more ventral and larger the target areas of the LS are. Moreover, hippocampal areas adjacent to one another project on distinct but overlapping regions of the LS. Therefore, even though individual LS neurons receive inputs from different pyramidal neurons that are located far away from one another along the hippocampal dorsoventral axis, the resultant final hippocampus-to-LS projection, as a whole, is topographically graded (Risold & Swanson, 1997). Noteworthy, the same pattern is conserved for the LS-to-hypothalamus projections, which means that different hippocampal areas project onto different hypothalamic areas that are responsible for goal-oriented behavioral, endocrine and autonomic responses (Risold and Swanson, 1996). Hippocampal connectivity with the nucleus accumbens [NAc – that region of the basal forebrain situated in front of the hypothalamus, devoted to the processing of rewarding and reinforcing stimuli, which respectively elicit approach responses and strengthen stimuli-responses tendencies] (Groenewegen et al., 1987) and the amygdala [one of the two nut-shaped structures situated in the temporal lobes of the cortex, involved in the processing of emotions, in particular fear] (Kishi et al., 2006) presents progressively more ventral hippocampal portions targeting progressively more medial regions of these two brain areas.

Noteworthy, the above-mentioned topographical gradients form with neurogenesis during the embryonic stage (Bayer & Altman, 1987). Even though neurogenesis happens simultaneously along the dorsoventral axis of the hippocampus, the dorsal hippocampus projects to target areas where neurons were born earlier, whereas the more ventral the hippocampal area considered, the later were born the cells of its target area. *E.g.*, the dorsal hippocampus projects to a LS zone containing earlier-formed and medially-placed neurons, whereas the ventral hippocampus (spatially further away from the LS) targets later-formed and laterally-placed LS neurons (Bayer & Altman, 1987).

Finally, the density of modulatory projections targeting the hippocampus also changes along its major axis, but it is not clear yet whether it is in a graded or discretized way. What is evident is an increasing gradient of the monoamine system (including dopaminergic, noradrenergic, serotonergic, and histaminergic circuitries) projecting to the ventral portion of the hippocampus. Therefore, generally speaking, the dorsoventral organization of the projections reaching the

hippocampus does not follow a discrete dorso-ventral pattern, but rather a graded distribution along the axis.

### *The hippocampal transcriptomic profile*

Genome-scale *in situ* hybridization studies showed a discretized dorso-ventral pattern of gene expression in the murine hippocampus (Thompson et al., 2008) (Lein et al., 2007). Noteworthy, a given genetic domain is not characterized by the expression of any single gene, but by the expression of a combination of them (Thompson et al., 2008). Therefore, the overlapping expression of many genes with common expression boundaries identifies well-defined genetic domains (Thompson et al., 2008). Several distinct molecular subdomains, each one characterized by a unique combination of expressed genes, are present along the major hippocampal axis. The CA3 region is divided into nine genetic subdomains (Thompson et al., 2008), while the CA1 (H. W. Dong et al., 2009) and DG (Fanselow and Dong, 2010) regions are divided into three major domains each, *i.e.*, dorsal, intermediate and ventral (the ventral CA1 can in turn be split into four subdomains). Noteworthy, the molecular characterization of the hippocampal long axis does not seem to identify a dorsal versus ventral partition, but rather a tripartite model which includes an intermediate part between the dorsal and ventral ones, as also corroborated in rats (O'Reilly et al., 2014). It has not been determined yet whether this transcriptomic pattern translates into specific functions for the identified domains. The gene expression profiles (*e.g.*, adhesion molecules and ion channels) of the different genetic domains may account for the differences that were detected along the hippocampal major axis with respect to neuronal excitability (Dougherty et al., 2012) and synaptic plasticity (Maggio & Segal, 2007). For example, the expression of neurotransmitter receptors varies along the hippocampal major axis for most of the neurotransmitter systems (Dougherty et al., 2013). Indeed, the combination of genetic and anatomical studies in rodents revealed that neuronal circuits within the hippocampus (Deguchi et al., 2011) and between the hippocampus and the LS (H. W. Dong et al., 2009) exhibit characteristic transcriptomic patterns, indicating an overlap between anatomic and transcriptomic domains. Importantly, it must be taken into account that there are differences between the gene regulation of the murine and human hippocampi (Hawrylycz et al., 2012).

Now the point was understanding how to reconcile the transcriptomic data (indicating clear expression boundaries) with the anatomical data (suggesting extrinsic gradients of connectivity) along the major axis of the hippocampus. Noteworthy, at the level of individual genes, there are

different expression patterns, *i.e.*, graded, discrete and abrupt transitions (Thompson et al., 2008). Moreover, even though collectively the extrinsic hippocampal connectivity shows a graded distribution, sharp boundaries also exist. With respect to intrinsic connectivity, the two principal longitudinal nerve fibers in the hippocampus (*i.e.*, the longitudinal axon collaterals of CA3 pyramidal cells, and the longitudinal axons of DG mossy cells) exhibit stunning divergence within the dorsal two-thirds and the ventral one-third of the rat hippocampus, although a few fibers still cross between the two portions (Amaral and Witter, 1989) (X. -G Li et al., 1994) (Ishizuka et al., 1990) (Swanson et al., 1978) (Fricke & Cowan, 1978). This means that the intrinsic connectivity between these two areas is remarkably partitioned. Moreover, in monkeys, the posterior two-thirds of the hippocampus present a higher degree of intrinsic connectivity if compared to the anterior one-third (Kondo et al., 2009), though the intrinsic connectivity boundary is less evident than in rodents. In humans, sharp transitions in electrophysiological properties along this axis were detected making use of multicontact depth electrodes. Noteworthy, an abrupt decrease in coherence at the anterior one-third/posterior two-thirds transition was observed (Staresina et al., 2012). Remarkably akin to what happens in humans, theta-wave (neural oscillation – rhythmic or repetitive patterns of neural activity that underly learning, memory and spatial navigation) coherence in rats is high between dorsal and intermediate regions, but notably lower between dorsal and ventral portions (Patel et al., 2012).

Overall, these data suggest the existence of diverse longitudinal anatomical and transcriptomic patterns, both graded and discrete, that can at times overlap.

### 2.3. From structure to function

#### *Spatial learning in rodents*

Spatial learning is the process by which an organism acquires a mental representation of its environment allowing its conscious exploration. This phenomenon takes place thanks to a particular type of neurons named place cells. Place cells are pyramidal neurons of the hippocampus that fire when an animal is in a particular place of its environment, which is referred to as place field. Although the experience of a particular environment activates a particular cluster of place cells, they represent locations in a non-topographic way, meaning that two neighboring place cells are not necessarily activated in neighboring zones of a place field (O'Keefe and Nadel, 1978). Indeed, a single local cluster of place cells in the dorsal hippocampus can cover most of a spatial environment (Wilson and McNaughton, 1993). Initial lesion studies hinted for a pivotal role in spatial memory for the

dorsal first quarter (or even less) of the whole hippocampus (M. B. Moser et al., 1995). Interestingly, if the hippocampus is undamaged the dorsal two-thirds (thus, also a ventral portion) are necessary for spatial learning (M. B. Moser & Moser, 1998a). Therefore, on the one hand, when the hippocampus is integer extensive hippocampal networks are used for spatial memory. On the other hand, when the hippocampus is damaged, networks as little as those that can be included in the first dorsal quarter can do.

Now the question was whether also the ventral hippocampus had a role in spatial learning. It was initially shown that, in proportion, the ventral hippocampus has much fewer space cells than the dorsal part, and that ventral place cells exhibit lower spatial selectivity (Jung et al., 1994). Nevertheless, it was later discovered that the relative size of place fields in the CA3 area increases linearly when moving from the dorsal to the ventral hippocampus (from 1 to 10 meter-place fields) (Kjelstrup et al., 2008). Therefore, the whole hippocampus has a role in spatial memory with a functional gradient with respect to the spatial scale, that increases along its major axis in a dorsoventral direction. This design presents the advantage of encompassing both spatial resolution and contiguity. Very interestingly, when rodents explore a constant environment, the place cells firing are determined by time (MacDonald et al., 2011) (Kraus et al., 2013) and not by the location. It is not known yet whether these “time fields” expand from dorsal to ventral position as place fields do.

Hippocampal place cells are involved in multiple, independent, spatial representations (Muller & Kubie, 1987). On the other hand, there exist the so called “grid cells” in the entorhinal cortex (EC) that encode a universal metric of the spatial map, directionally oriented and topographically organized (Hafting et al., 2005). The environment locations of grid-cell firing define a triangular or hexagonal periodic array representing the whole environment of the animal. These locations are set on external cues, but maintained when cues are removed or when changes in the animal speed and direction occur (Hafting et al., 2005). Therefore, grid-cells might drive the spatial selectivity of place cells (O’Keefe & Burgess, 2005) (Solstad et al., 2006) (S. J. Zhang et al., 2013). Strikingly, the increasing size of place fields along the dorsoventral axis of the hippocampus is mimicked by an increased spacing between grid-cell firing loci when moving from the dorsomedial to the ventrolateral medial EC (Hafting et al., 2005) (Fyhn et al., 2004) (Brun et al., 2008). Unlike the place field size, whose gradient is continuous, the spatial gradient of the EC exhibits a discretized, step-like pattern (Stensola et al., 2012). Anyways, the existence of discrete transitions also in the place fields cannot be excluded. Moreover, they could coincide with transitions between genetic domains.

Furthermore, assuming that place fields actually vary in a continuous way, this could be the result of different combinations (S. J. Zhang et al., 2013) (Fyhn et al., 2007) of the inputs coming from the discretized EC. Indeed, modules of increasing spatial scale exhibit great anatomical overlapping in the dorsoventral axis of the EC (Stensola et al., 2012), meaning that inputs to the hippocampus may be coming from different modules, even if they come from the same EC level. Upon environment compression/deformation, the rescaling of grid fields occurs in modules with large grid scales, but not in those with small ones. It is not known whether a similar dichotomy is present between large and small place fields along the dorsoventral hippocampal axis. Anyways, although a systematic investigation was not performed yet, recordings from the dorsal hippocampus revealed that compression can occur at small scales, hinting for an independence between place and grid cells. Importantly, the spatial orientation capacity of an animal does not only rely on place and grid cells. Indeed, another type of neurons referred to as “head-direction cells” is pivotal for spatial learning. Head-direction cells increase their firing rates only when an animal is pointing its head in a specific direction (Taube et al., 1990) (Yoder & Taube, 2009). Therefore, the combination of the activities of place, grid and head-direction cells, allows proper spatial cognition and conscious movement in the environment (Mullet-L et al., 1996). All in all, these findings showed that, despite a gradient of place fields and molecular and anatomical domains having different boundaries, a combination of diverse afferent signals to the hippocampus can give rise to gradual changes in functional properties.

### *Spatial learning in primates*

Magnetic resonance imaging (MRI) studies in humans found a relationship between activation (Hartley et al., 2003) (Suthana et al., 2009) and structural changes (Maguire et al., 2000) in the posterior hippocampus during navigation, which matches the dependence of spatial learning on the dorsal hippocampus in rodents. Even though most of the studies observed responses in either the anterior or posterior hippocampus, some studies reported functional double dissociation between loci along the dorsoventral axis (Strange et al., 1999) (Nadel et al., 2013). In support of a prominent role for the posterior hippocampus in spatial processing is the double dissociation observation that the posterior hippocampus is pivotal for accurate way-finding, whereas the anterior allows the formation of the representation of a new virtual-reality environment (Hartley et al., 2003). Moreover, a study reported a dorsoventral dissociation with respect to spatial size and complexity: in virtual settings consisting of mazes of different sizes, numbers of corridors and landmark images,

the activation of the anterior hippocampus increases with the number of corridors (*i.e.*, complexity), whereas the activation of the posterior part increases with the size of the mazes (Baumann & Mattingley, 2013).

In primates, the electrophysiological evidence for the involvement of the posterior hippocampus in spatial processing is limited. Nevertheless, one non-human primate study based on a spatial delayed match-to-sample task (the animal is presented with a sample stimulus; after a short delay, the sample stimulus is shown again along with a novel alternative and the subject must choose between the two), observed a higher activity in the posterior portion of the hippocampus during the delay period (Colombo et al., 1998).

Intracranial recordings in humans hint for the existence of place-cell-like responses during navigation (Ekstrom et al., 2003), but their distribution along the anteroposterior axis and how their response vary with respect to the environmental scale still needs elucidation.

Electrophysiology studies on non-human primates observed grid-cell-like properties in the posterior EC: the spatial scale varies according to the distance from the rhinal sulcus (Killian et al., 2012) (analogous to the dorsomedial-to-ventrolateral axis in the rodent medial EC), hinting for spatial scale variation also along the primate anteroposterior axis.

### *The anatomy of emotions: Fear*

Anatomical studies showed that the connectivity between the amygdala, on the one hand, and the CA1/subiculum, on the other hand, is limited to the ventral two-thirds of the hippocampus (Pitkänen et al., 2002) (Petrovich et al., 2001). According to some studies, only the dorsal extremity of the hippocampus does not project to the amygdala (Kishi et al., 2006). This connectivity is anatomically arranged along the major axis of the hippocampus so that the ventro-dorsal efferent projections from the CA1/subiculum correspond to medio-lateral afferent projections to the amygdala (Pitkänen et al., 2000). This pattern might be at the basis of the emotion-related functions of the hippocampus. Indeed, a significant portion of the dorsoventral hippocampal axis projects to the basolateral amygdala, which has a pivotal function in fear learning (Kishi et al., 2006) (Pitkänen et al., 2000). This might in fact explain why some studies observed an involvement of the ventral part of the hippocampus in fear-conditioning, whereas some others, on the contrary, found a role for the dorsal part (Bast et al., 2001) (Maren et al., 1997) (Carvalho et al., 2008). Noteworthy, the origin of the hippocampus-to-amygdala projections in primates is narrower, meaning that the neurons projecting to the amygdala are focally restricted to the most anterior CA1 and prosubiculum zones (Fudge,

2012). This might explain why emotional memory stimulation in humans activates anterior hippocampal regions (Dolcos et al., 2004) (Murty et al., 2010).

In contrast to those studies showing an involvement of either the ventral or the dorsal hippocampus in fear-conditioning, other works observed that neither of them are involved in conditioned fear responses. On the other hand, it was observed that only ventral lesions reduce defensive unconditioned fear responses (Kjelstrup et al., 2002). Further studies showed that the effect of ventral hippocampal lesions on fear tests extend to non-spatial tasks such as latency to eat novel food (Bannerman et al., 2002), and the social interaction extent in a familiar environment (Bannerman et al., 2004). Importantly, the fact that amygdala lesions do not impair defensive responses (Kjelstrup et al., 2002) means that the ventral hippocampus might be able to affect fear responses in an amygdala-independent way; *i.e.*, via its direct projections to the hypothalamus, a brain region endowed with endocrinal and behavioral control systems (Risold and Swanson, 1996). Going back to what concerns the longitudinal axis topographical organization, as mentioned above, only lesions in the ventral one-third of the hippocampus affect unconditioned emotional responses (Kjelstrup et al., 2002), suggesting a segregation to the ventral hippocampus for the emotional topography. Very interestingly, on the other hand, contextual fear conditioning appears to be dependent on the dorsal portion of the hippocampus (Anagnostaras et al., 2001).

It is interesting to consider that the same ventral portion of the hippocampus that has a prominent role in unconditioned emotional responses, has also a major role in big scale spatial processing. Moreover, in non-spatial fear conditioning tests, place cells respond to non-spatial stimuli (*e.g.*, the sound in the *tone-shock fear conditioning* test) only when the animal is in those cells' place field (Moita et al., 2003). Therefore, it is tempting to think that larger place fields in the ventral hippocampus, which is linked to defensive behavior-related circuits in the hypothalamus (Petrovich et al., 2001), might be an evolutionary expedient: the life-saving capability to detect a danger when it is still far away.

### *Episodic memory*

Episodic memory is the capability of recalling that an everyday-life episode occurred in a relatively precise space and time. Together with semantic memory (general knowledge about facts of the world), episodic memory is part of declarative memories, *i.e.*, memories that can be brought to conscious awareness and verbalized. Early studies suggested a partition for episodic memory encoding and retrieval between anterior and posterior hippocampus, respectively (Lepage et al.,

1998). Moreover, the activation of the anterior hippocampus upon novel stimuli has been frequently reported (Köhler et al., 2002) (Düzel et al., 2003). Furthermore, a number of studies observed a double dissociation of anterior hippocampus activation in response to novel stimuli and posterior activation in response to already-experienced ones (Strange et al., 1999) (Daselaar et al., 2006). These data suggest that encoding (novel memory experience) and retrieval (familiarity detection) are dissociated processes in the hippocampus (Lepage et al., 1998). Nonetheless, other extensive research suggests that the processes of memory encoding and retrieval are executed by the same cells, because memory is given by the reactivation of the same neuronal network that was formed when the event was encoded (J. F. Miller et al., 2013) (McNaughton & Morris, 1987) (Paulsen & Moser, 1998). Overall, it seems plausible that, with respect to resolution, episodic memory might follow a gradient similar to that of spatial memory along the hippocampal long axis; *i.e.*, the more detailed and specific the memory, the more posterior the hippocampal area activated (Hirshhorn et al., 2012) (Addis et al., 2004), and vice versa (Poppenk et al., 2013). Anyways, this hypothesis still needs to be experimentally proven.

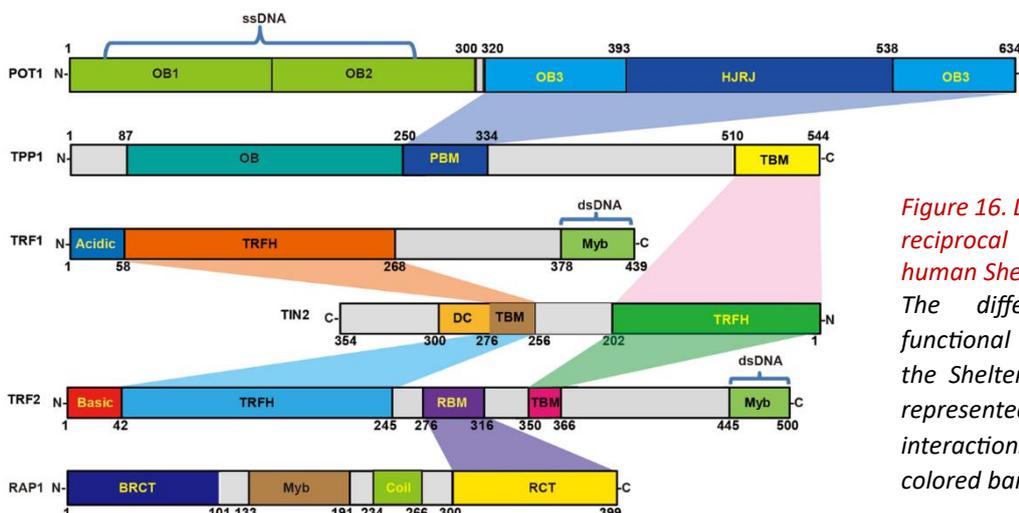
*After displaying the beauty and frailty in the realm of the brain  
and the landscape of its disputed hippocampal reign  
it is time to describe the hero of this tale*

### 3. Telomeric repeat-binding factor 2

Telomeres are the nucleoprotein structures located at the extremities of eukaryotic chromosomes. They consist of hexanucleotide DNA repeats bound by a protective protein complex named Shelterin. Due to the linear structure of eukaryotic chromosomes, their extremities (the telomeres, indeed) would be mistakenly recognized as double strand breaks (DSBs) by the DNA damage response (DDR) machinery if it was not for the presence of the Shelterin complex that binds and hides telomeres from DDR (De Lange, 2009). Interestingly, among the Shelterin subunits, Telomeric repeat-binding factor 2 (TRF2), which was discovered by our group in 1997 (Bilaud et al., 1997), appears to be of crucial importance.

#### *Telomeres and genome stability*

Human telomeric DNA consists of 10-15 kilobase[kb]-long (30-150 kb-long in mice) 5'-TTAGGG-3'/3'-AATCCC-5' double strand repeats that culminate with a 30-100 nucleotide-long single strand TTAGGG, 3' G-rich overhang (Makarov et al., 1997) (W. E. Wright et al., 1997) (Stewart et al., 2003) (Dai et al., 2010). The Shelterin complex binds the telomeric DNA (De Lange, 2009) via two homeodomain-containing dsDNA-binding factors, namely Telomeric repeat-binding factor 1 (TRF1) and TRF2. These two proteins share significant sequence homology, apart from the N-terminal region that is basic in TRF2 and acidic in TRF1. Despite their similarity, TRF1 and TRF2 have different functions. Only TRF2 can directly bind human Repressor activator protein 1 (hRAP1), but both TRF2 and TRF1 bind to TRF1-interacting nuclear factor 2 (TIN2). TIN2 acts as a bridge between TRF1-TRF2 and TIN2-interacting protein 2 (TPP1), which in turn binds to the ssDNA-binding protein Protection



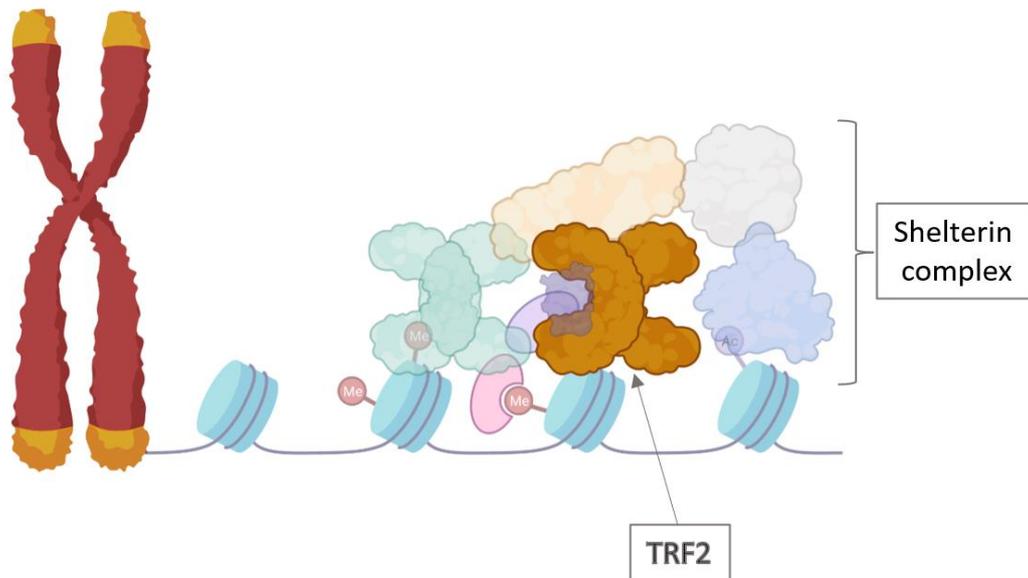
*Figure 16. Domain organization and reciprocal interactions of the human Shelterin subunits*

*The different structural and functional domains of each one of the Shelterin subunits are linearly represented. The reciprocal physical interactions are highlighted by the colored bands (Hu et al., 2017).*

of telomeres 1 (POT1) (De Lange, 2009) (Sfeir et al., 2009) (Baumann, 2001). TRF2 binding to RAP1 is not the only functional difference between TRF1 and TRF2 since the phenotype caused by RAP1 deletion is not comparable to that caused by either TRF2 or TRF1 deletion (Kabir et al., 2014) (Hu et al., 2017).

When telomeres are functional, the telomeric DNA is protected by Shelterin, nucleolytic processes are prevented and the DDR is blunted, which avoids check point-mediated cell cycle arrest and unnecessary repair events. In any case, during aging, the cell division-mediated telomere erosion causes telomere deprotection. When telomeres shorten over a critical threshold (Hayflick and Moorhead, 1961) or are damaged by environmental insults (*e.g.*, oxidative stress and radiations), the DDR is elicited and the cell either dies via apoptosis or stops permanently into a non-dividing, but metabolically active, state referred to as replicative senescence (D'Adda Di Fagagna et al., 2003). Importantly, DNA damage-induced senescence often correlates with a persistent DDR at the telomere level, leading to the activation of DNA damage signalling kinases (*e.g.*, ATM – Ataxia telangiectasia mutated) and p53-dependent cell cycle arrest. Therefore, damaged and shortened telomeres are considered a dangerous source of irreparable DNA damage (Fumagalli et al., 2012) (Hewitt et al., 2012).

The above-mentioned replicative senescence occurs because the expression of the enzyme telomerase is turned off in all somatic cells of an individual (while still being expressed only in germ and stem cells). Telomerase activity consists of adding 5'-TTAGGG-3' nucleotide repeats to the 3'-end of the telomere thanks to a template RNA containing the 3'-AAUCCC-5' sequence, thereby counteracting the relentless shortening of telomeres that takes place at each cell cycle due to the very nature of the DNA replication mechanism: the use of RNA-based priming for DNA synthesis and the unidirectionality of DNA polymerases. Interestingly, telomerase expression is under the control of TRF2. Via an extratelomeric activity, TRF2 binds to the G-quadruplex structures that are present in the promoter of human Telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase. In this context, TRF2 also binds REST (described later) which in turn recruits the Polycomb repressor complex PRC2, which causes H3K27 trimethylation, chromatin compaction and repression of the hTERT gene (Sharma et al., 2021).



**Figure 17. Telomere structure**

3D representation of telomeres. Telomeres consist of nucleoprotein structures located at the extremities of eukaryotic linear chromosomes. The multimeric protein complex Shelterin, with TRF2 localized at its core, is pivotal for telomere protection and stability.

### **Telomere integrity**

TRF2 depletion leads to telomere deprotection and activation of the ATM Kinase (Karlseder et al., 1999) (Celli & de Lange, 2005) (Denchi & De Lange, 2007). In turn, ATM activation causes the phosphorylation of the histone H2AX ( $\gamma$ H2AX) and the recruitment of Tumor suppressor p53-binding protein 1 (53BP1) to telomeres, which results in the formation of Telomere dysfunction-induced foci (TIFs) (Takai et al., 2003). Noteworthy, upon DNA damage, ATM can directly phosphorylate TRF2 at the Thr188 residue. This transient phosphorylation causes TRF2 not to bind to telomeres and to localize to the DNA lesion sites (H. Tanaka et al., 2005). Moreover, the loss of TRF2 leads to chromosome end-to-end fusion via classical non-homologous end-joining (cNHEJ) mediated by the concerted activity of Ku70/80 (Celli et al., 2006) and DNA ligase IV (Smogorzewska et al., 2002). Chromosomal fusions finally lead to genomic instability and proliferative impairment. A cooperative interaction of TRF2 with the other Shelterin subunits is necessary for preventing ATM activation. Particularly, the TIN2 subunit is necessary for the optimal binding of TRF2 to telomeres. Indeed, a TRF2 mutant lacking the TIN2-binding domain is partially defective in preventing ATM activation (Takai et al., 2011). Interestingly, the induced telomere localization of a TIN2 mutant deficient in TRF1 binding rescues telomere protection after TIN2 depletion, meaning that the TIN2-TRF1 interaction is dispensable for the telomere protective role of TRF2 (Frescas & de Lange, 2014).

### *The different states of telomere protection*

TRF2 presents four different functional domains (Figure 15). i) An N-terminal glycine- and arginine-rich (GAR) basic domain; ii) a Telomeric repeat factors homology (TRFH) domain pivotal for the formation of homodimers and higher order oligomers; iii) a flexible hinge domain required for protein-protein interactions; and iv) a C-terminal Myb/homeodomain-like DNA-binding domain that specifically binds to telomeric T<sub>2</sub>AG<sub>3</sub> repeats (Bilaud et al., 1996) (Broccoli et al., 1997) (Court et al., 2005). Noteworthy, other than specific DNA repeats, TRF2 can also bind, independently of the sequence, diverse DNA conformational structures such as double strand(ds)-single strand(ss) DNA junctions (Stansel et al., 2012), Holliday junctions (Fouché et al., 2006) (Poulet et al., 2009), and positively supercoiled DNA (Amiard et al., 2007) (Ye et al., 2010). To date, the ultimate telomeric signal that elicits senescence remains unknown, but an ever-increasing amount of data points at TRF2 as a key factor. Indeed, TRF2 overexpression (Karlseder et al., 2002) or stabilization (Fujita et al., 2010) can postpone replicative senescence. The more telomeres shorten, the less binding sites TRF2 has, thus different TRF2 levels at telomeres can correspond to different stages of senescence. The spontaneous telomere deprotection that takes place in immortalized cancer cells and the one that occurs in replicative senescence lead to three different telomeric structural states that correspond to the gradual decline of TRF2 (Cesare et al., 2013). Namely, i) Closed, ii) Intermediate, and iii) Uncapped (Cesare et al., 2009) (Kaul et al., 2012) (Cesare & Karlseder, 2012). In non-senescent cells, telomeres are long and allow for abundant binding of TRF2, promoting a telomeric “Closed State”, which prevents DDR activation and aberrant chromosomal fusions thanks to the sequestration of telomeres in higher order secondary structures (Karlseder et al., 2002). The “Intermediate State” is characterized by limited telomere shortening resulting in reduced TRF2 binding. Noteworthy, an experimentally-induced TRF2 downregulation can reproduce the Intermediate State (Cesare et al., 2013). Cells with Intermediate State telomeres undergo telomeric DDR characterized by histone H2AX phosphorylation (γH2AX). Despite this, the quantity of TRF2 molecules that can still bind is sufficient to avoid chromosomal fusion via the cNHEJ pathway (Cesare et al., 2013). Alike internal chromosome DSBs, Intermediate State telomeres activate the ATM kinase, but not in a “full blown” manner: the phosphorylation of Checkpoint kinase 2 (CHK2) is not detectable (Cesare et al., 2013). Moreover, these Intermediate State telomeres do not trigger a cell cycle arrest in the G<sub>2</sub>/M phase; instead, the cell goes through mitosis and the daughter cells undergo a p53-dependent cell cycle arrest in the G<sub>1</sub> phase (Kaul et al., 2012) (Cesare et al., 2013). Another phenomenon, unrelated to senescence, that could induce the Intermediate State is the Aurora B

kinase-mediated repression of Shelterin which occurs upon aberrant or prolonged mitosis (Orthwein et al., 2014). In fact, a prolonged mitotic arrest causes partial TRF2 dissociation from telomeres, which in turn leads to an ATM-dependent DDR, but without prominent chromosomal fusions (M. T. Hayashi et al., 2012). Indeed, DNA repair during mitosis is prevented at two levels, impeding i) the recruitment of ubiquitin-ligase RNF8, and ii) the binding of 53BP1 to Ubiquitinated H2A (being H2A the target of RNF8). This is due to the activities of mitotic kinases that phosphorylate both RNF8 and 53BP1, thereby inhibiting their recruitment to the DSB-flanking chromatin. Experimentally restoring RNF8 and 53BP1 binding to DSBs during aberrant/prolonged mitosis paves the way for an Aurora B kinase-dependent Shelterin repression leading to telomere fusions and genome instability (Orthwein et al., 2014) (Cesare, 2014).

With aging, excessive telomere shortening deprives TRF2 of binding sites to the point that telomeres get unprotected and fusogenic. Interestingly, this “Uncapped State” of telomeres can also be induced by genotoxic stress during mitosis (Orthwein et al., 2014). Further than increased levels of chromosomal fusions, this context is characterized by enhanced DDR signalling and CHK2 phosphorylation. Importantly, the extent of CHK2 phosphorylation is still inferior to that elicited by genomic DSBs following ionizing radiations (IR) (Cesare et al., 2013). This fact matches with the hypothesis that telomeres have intrinsic anti-check point properties. Noteworthy, upon DNA damage, CHK2 can phosphorylate TRF2 at Ser20, thereby decreasing TRF2 binding affinity to telomeres and permitting its localization to genomewide-localized DSB sites (Buscemi et al., 2009). Different TRF2 domains can suppress diverse stages of the telomeric DDR (Okamoto et al., 2013). *E.g.*, the TRFH (dimerization) domain is necessary to inhibit ATM, most likely because it is pivotal for the strand invasion phase that leads to T-loop formation (described in the next paragraph) (Amiard et al., 2007). Moreover, The C-terminus (aa 407-431) of the Hinge domain inhibits a later stage of the DDR (after H2AX phosphorylation and upstream of 53BP1 recruitment), where TRF2 inhibits the recruitment of the E3 ubiquitin ligase RNF168 with the involvement of the deubiquitinating enzyme BRCC3 and the ubiquitin ligase UBR5, thereby avoiding end-to-end chromosomal fusions (Okamoto et al., 2013).

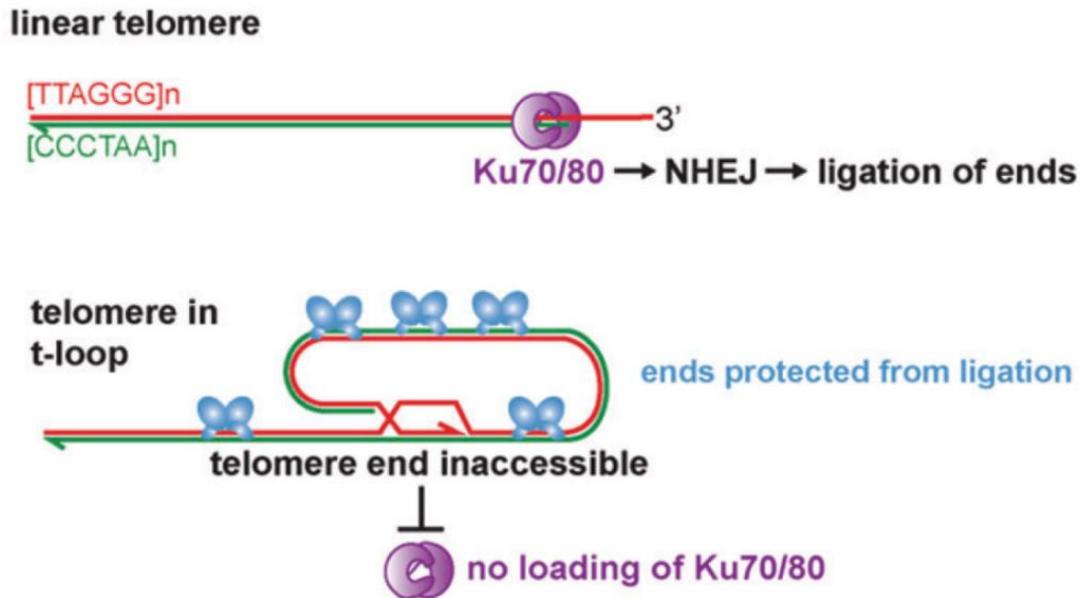
### *The telomeric (T)-loop*

TRF2 restricts the accessibility of DDR factors to telomeres by remodeling the linear chromosome extremities into loops that are stabilized when the 3'G-overhang folds back and inserts into the ds telomere repeats, whereby forming a structure referred to as telomeric (T)-loop. T-loops were first

observed via electron microscopy (EM) in telomere-enriched, psoralen-cross-linked, deproteinized DNA of murine and human cells (Griffith et al., 1999). TRF2 can also elicit the formation of T-loops *in vitro* on bare telomeric DNA (Fouché et al., 2006) (Griffith et al., 1999), as long as its TRFH domain is intact (Amiard et al., 2007). Moreover, TRF2 stabilizes T-loops *in vitro* by promoting the formation of recombination intermediates and preventing resolvase-mediated cleavage (Poulet et al., 2009). Until a decade ago, though, there was no *in vivo* evidence for T-loop existence and for the TRF2 role in maintaining this structure. Super-resolution fluorescence imaging allowed researchers to observe in unperturbed conditions a subset of telomeres with a TRF2-dependent loop structure of variable size (Doksani et al., 2013). Importantly, the decrease in T-loop frequency upon TRF2 depletion is not dependent on ATM activation and telomere fusions, since the concomitant ATM depletion does not rescue T-loop occurrence (Doksani et al., 2013). This result, added to the fact that downregulation or depletion of the other Shelterin subunits does not impair T-loop formation, proves that TRF2 is the pivotal major factor responsible for the formation and maintenance of T-loops at functional telomeres.

T-loop formation most likely accounts for TRF2 ability to suppress the ATM signaling cascade which starts with the association of the MRN complex (MRE11-NBS1-RAD50) to exposed DNA ends. In the same way, the T-loop configuration avoids binding of the ring-shaped Ku70/80 to telomere ends, thereby impeding cNHEJ (De Lange, 2015).

Very importantly, TRF2 can also directly bind ATM in correspondence of its FAT domain, which contains the Ser1981 residue, the critical target for ATM pro-activity autophosphorylation, thereby inhibiting its activation (Karlseder et al., 2004).

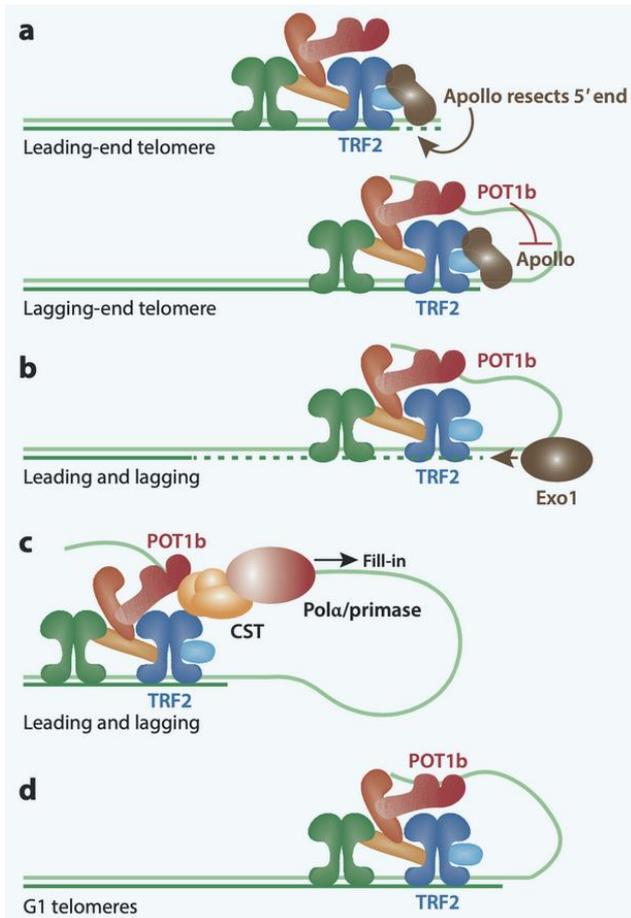


**Figure 18. The T-loop**

The foldback structure of the T-loop, whose formation is orchestrated by TRF2, allows the protection of telomeric ends from Ku70/80 binding, avoiding the ensuing NHEJ which would lead to aberrant chromosomal fusions (De Lange, 2015).

### **The synthesis of 3'G-overhang**

The formation of T-loops requires 3'G-overhangs to invade the duplex telomeric DNA (Stansel et al., 2012) (Griffith et al., 1999). Importantly, TRF2 cooperates with POT1 for post-replicative telomere end-processing leading to the formation of the 3'G-overhang. In this setting, TRF2 recruits Apollo (van Overbeek & de Lange, 2006) (Lenain et al., 2006), an Artemis-related exonuclease (Moshous et al., 2001), to elicit the resection of the leading-end of telomeres (P. Wu et al., 2010). Single strand formation due to Apollo nuclease activity allows for POT1 binding to telomeric ssDNA (P. Wu et al., 2012), which may protect telomeres from unwanted DDR activation until the T-loop is formed. While TRF2 recruits Apollo to telomeres, POT1 is a negative regulator that prevents excessive resection (P. Wu et al., 2012). At the telomere lagging end, instead, the resection mediated by Apollo is not necessary because the incomplete synthesis of the lagging strand already provides a ss 3'G-overhang for POT1 binding (P. Wu et al., 2012). Exonuclease I (ExoI) further processes both leading- and lagging-end telomeres via its 5'-3' exonuclease activity to transiently extend the ss 3'G-overhangs (P. Wu et al., 2012). Finally, POT1 recruits the CST complex (CTC1, STN1, and TEN1) whose activity consists in filling in the extended overhangs generated by ExoI (P. Wu et al., 2012). Most likely, other factors are involved in telomere processing.

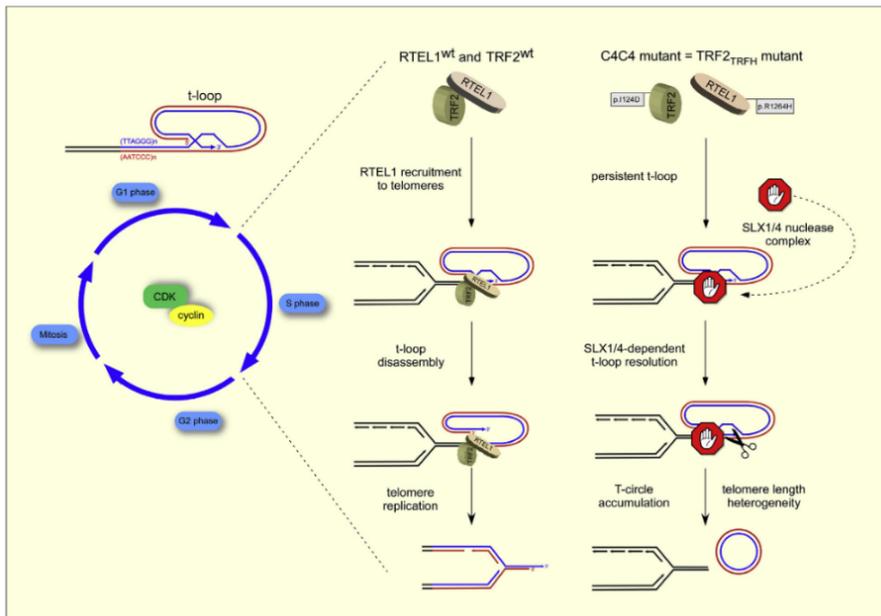


**Figure 19. TRF2–Apollo interaction-mediated synthesis of the 3′G-overhang**

a) TRF2 recruits the exonuclease Apollo to the leading-end telomere, which resects the 5′ end of it. b) The exonuclease Exo1 further resects the 5′ end of both leading- and lagging-end telomeres. c) POT1b recruits the CST complex including Polα/primase, which fills in the extended gap resulted from Exo1 activity. d) The 3′G-overhang is now of the proper length to form a T-loop and allow protection of telomeres from an unnecessary DDR (<https://delangelab.org/generation-and-maintenance-of-telomere-ends>).

### **T-loop unwinding**

TRF2 activity during telomere replication is crucial for the stability of mammalian telomeres. Indeed, TRF2 is a hub for several enzymes and non-Shelterin factors necessary for telomere protection. For example, RTEL1, a helicase characterized by intrinsic T-loop dismantling activities, is recruited by TRF2 via its TRFH domain during S phase (Sarek et al., 2015). At the telomere level, RTEL1 can both unwind T-loops (Uringa et al., 2012) and impede the formation of telomeric guanine quadruplex (G4) (Vannier et al., 2012), a major cause of telomere fragility during replication. When TRF2 recruitment of RTEL1 during S phase is impaired, a mild DDR is elicited, but a catastrophic loss of telomeres takes place. This latter event is likely due to the inappropriate resolution of the t-loops by the SLX1-SLX4 nuclease complex (described in the second next paragraph). Noteworthy, the TRF2-RTEL1 interaction is controlled by a post-translational modification (PTM). Indeed, the protein kinase CDK phosphorylates TRF2 at Ser365, which disrupts TRF2-RTEL1 interaction and impedes RTEL1 recruitment to telomeres. During S phase, the phosphatase PP6R3 dephosphorylates TRF2, thereby allowing RTEL1 recruitment, T-loop unwinding and telomere replication (Sarek et al., 2019).



**Figure 20. RTEL1-mediated T-loop unwinding**

RTEL1 is recruited to T-loops by the TRF2 TRFH domain. RTEL1 helicase activity allows for T-loop unwinding and proper replication of telomeric DNA. Impaired RTEL1 recruitment due to a TRFH mutation causes telomere fragility and improper T-loop resolution by the SLX1-SLX4 nuclease complex with the ensuing T-circle formation and telomere shortening (Sarek et al., 2015).

### Other TRF2 PTMs

Interestingly, TRF2 can also undergo SUMOylation (the covalent link of Small ubiquitin-like modifier [SUMO] proteins to specific lysine residues), which is essential for TRF2 recruitment to telomeres (Potts & Yu, 2007) (Yalçın et al., 2017). Moreover, in mammalian cell lines, TRF2 SUMOylation by SIAP1, leads to TRF2 ubiquitination by ubiquitin ligase RNF4 and proteasomal degradation (J. Her et al., 2015). Furthermore, TRF2 can be acetylated at Lys293 by the histone acetyltransferase P300, which stabilizes TRF2 by inhibiting its ubiquitin-dependent proteolysis, and ensures its binding to telomeres (Y. R. Her & Chung, 2013). Finally, Poly[ADP-ribose] polymerase 1(PARP1)-mediated Poly[ADP-ribose](PAR)ylation of TRF2 also stabilizes TRF2 binding to telomeres in primary murine cells, and PARP1 deficiency leads to increased rates of chromosomal fusion and genomic instability (Gomez et al., 2006).

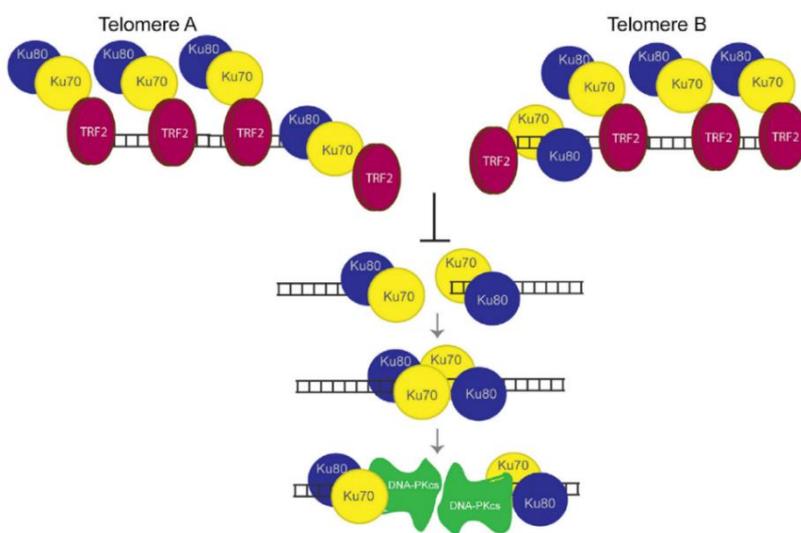
### Telomere trimming

Other than mediating T-loop formation and unwinding, when telomeres are long, TRF2 can process T-loops to yield circular, extrachromosomal DNA, referred to as telomeric(T)-circles (Wan et al., 2013). T-circles are a product of T-loop resolution by the SLX4 resolvase (a structure-specific endonuclease subunit), recruited by the TRFH domain of TRF2 (Wan et al., 2013). SLX4 is a Fanconi anemia (FA)-associated protein whose role is to bring together the structure-specific endonucleases Xeroderma pigmentosum complementation group F (XPF)-Excision repair cross-complementation group 1 (ERCC1), MUS81 structure-specific endonuclease subunit (MUS81)-Essential meiotic

structure-specific endonuclease 1 (EME1), and SLX1 structure-specific endonuclease subunit homolog A (SLX1) (Fekairi et al., 2009) (Y. Kim et al., 2013) (I. M. Muñoz et al., 2009) (Svendsen et al., 2009). It was suggested that the interaction between SLX4 and TRF2 forms a scaffold capable of recruiting the above-mentioned nucleases with the aim to avoid telomere overlengthening, resulting in the resolution of T-loops into T-circles, a process referred to as “telomere trimming” (Pickett et al., 2009). If, on the one hand, the TRFH domain of TRF2 recruits SLX4 and its associated endonucleases, on the other hand, the TRF2 N-terminal domain inhibits their activity (Saint-Léger et al., 2014). Indeed, the expression of a TRF2 mutant lacking its N-terminal domain (TRF2 $\Delta$ B) leads to the disproportionate yield of T-circles and telomere loss in human cells (R. C. Wang et al., 2004). Noteworthy, the N-terminal domain of TRF2 is dispensable for inhibiting telomeric DDR. Indeed, TRF2 $\Delta$ B expression causes neither TIF occurrence nor chromosomal fusions (Okamoto et al., 2013). It might be that the loss of T-loops after T-circle excision be so transient that DDR is not activated. Alternatively, the remaining TRF2 $\Delta$ B might inhibit ATM in a T-loop-independent manner (*e.g.*, the TRF2 direct inhibition of ATM). At any rate, T-loop resolution by itself is not enough to elicit DDR and chromosomal fusions.

Moreover, TRF2 has a direct inhibitory effect on Ku70/80, which might explain why the DDR is not triggered in the absence of T-loops (Ribes-Zamora et al., 2013). Indeed, TRF2 directly interacts with the N-terminus of Ku70, thereby impairing the multimerization of Ku and therefore its ability to bring DNA ends close to each other for NHEJ to happen (Ribes-Zamora et al., 2013).

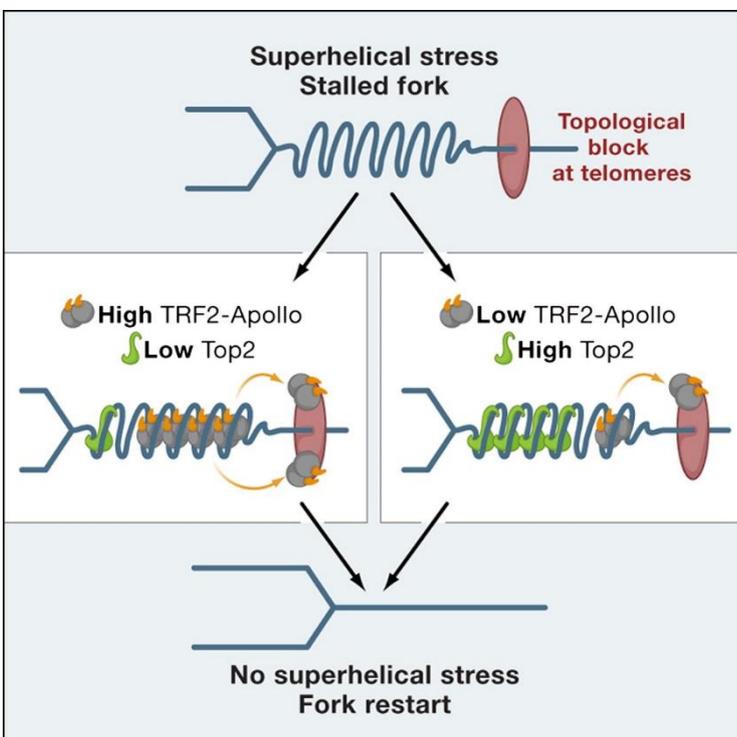
All in all, TRF2 controls multiple mechanisms involved in the formation and resolution of the T-loop thereby ensuring a stable telomere structure.



*Figure 21. TRF2 direct inhibition of Ku70*  
 TRF2 interaction with Ku70 inhibits Ku heterotetramerization at telomeres and the synapsis of telomere ends, thereby impeding the NHEJ leading to aberrant chromosomal fusions (Ribes-Zamora et al., 2013).

### *TRF2 protects from replicative telomeric damage*

During S phase, TRF2 modulates the exonuclease activity of Apollo thereby guaranteeing telomere integrity (Ye et al., 2010). Indeed, TRF2 can localize close to the replicative fork with Apollo and prevent the formation of topological obstacles, with no overt need for DNA topoisomerase II $\alpha$  (TOP2 $\alpha$ ). Indeed, TRF2 negatively regulates the recruitment of TOP2 $\alpha$  at telomeres (Ye et al., 2010). Another important aspect of telomere stability is the repression of homologous recombination (HR) between sister chromatids. When Telomeric sister chromatid exchanges (T-SCEs) happen, telomere lengths can vary if the exchanged segments are not equal. Furthermore, HR at telomeres can lead to the deletion of T-loop-sized fragments (R. C. Wang et al., 2004). During S phase, TRF2 must interact with RTEL1 to avoid T-SCEs (Sarek et al., 2015). Moreover, the interaction between TRF2 and Ku70 can repress HR at telomeres (Celli et al., 2006). Indeed, Ku70/80 can bind to mammalian telomeric DNA ends *in vitro* (Bianchi & De Lange, 1999). Furthermore, the TRF2–RAP1 interaction is pivotal for inhibiting NHEJ at human telomeres (Bae & Baumann, 2007). Altogether, TRF2 avoids replication-derived telomere damage by controlling several different processes.



*Figure 22. Relief of topological stress by TRF2-Apollo*

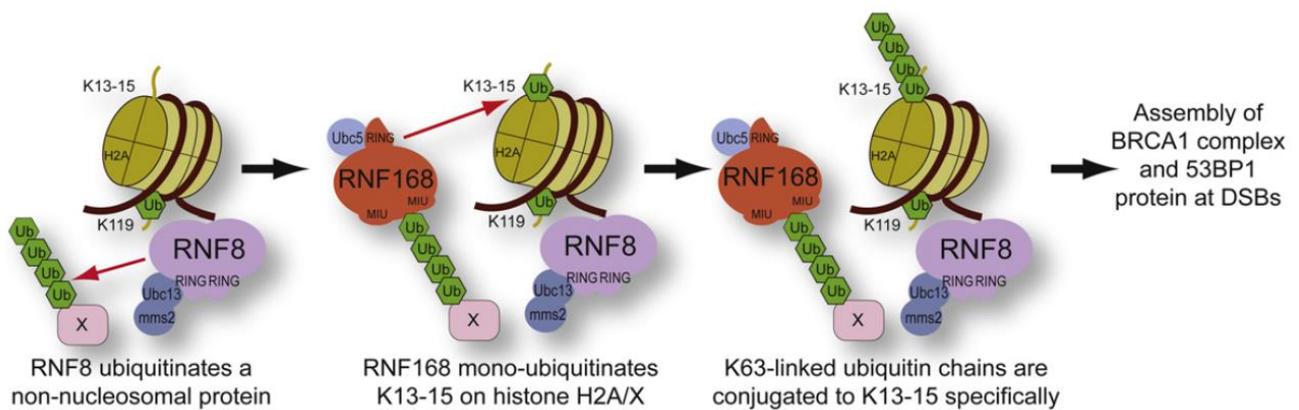
*TRF2 regulates the 5' nuclease activity of its binding partner Apollo. Binding of TRF2-Apollo to positively supercoiled DNA antagonizes TOP2 binding to telomeric DNA. Both TRF2-Apollo and TOP2 act in the same pathway of telomere protection. The result is the relief of topological stress during telomere replication and the maintenance of telomere integrity (Ye et al., 2010).*

### *TRF2 and the telomeric chromatin environment*

Telomeres are structured into tightly packed nucleosomes characterized by the presence of histones H3 and H4 respectively trimethylated at lysine 9 (H3K9me3) and 20 (H4K20me3), and low levels of histone acetylation (Schoeftner & Blasco, 2009). Even though TRF2 might be able to control chromatin structure by altering the intranucleosomal distance in a cell cycle-dependent manner [increase of intranucleosomal spacing during the late or post-replicative phase] (Galati et al., 2012), telomere uncapping does not seem to affect nucleosome structure (P. Wu & de Lange, 2008). Nonetheless, telomere deprotection due to TRF2 depletion may affect the chromatin environment via modification of telomeric histones. Indeed, TRF2 loss unleashes ATM kinase activity leading to phosphorylation of histone H2AX on Serine 139, yielding histone  $\gamma$ H2AX.  $\gamma$ H2AX recruits Mediator of DNA damage checkpoint 1 (MDC1) and the E3 ubiquitin-protein ligase Ring finger protein 8 (RNF8), that in turn ubiquitinates histones H2A and H2AX (Jacobs, 2012) (Peuscher & Jacobs, 2011). When TRF2 is depleted, yet another E3 ubiquitin-protein ligase named Ring finger protein 168 (RNF168) localizes at telomeres in a RNF8-dependent manner (Peuscher & Jacobs, 2011) amplifying the ubiquitin signal via the formation of K63-linked polyubiquitin chains (Jacobs, 2012) (Mattioli et al., 2012). When telomeres are deprotected, the RNF8/RNF168 ubiquitinating activity is necessary to recruit Tumor suppressor p53-binding protein 1 (53BP1), a pivotal cNHEJ factor that further mobilizes telomeric chromatin (Peuscher & Jacobs, 2011) (Dimitrova et al., 2008), increasing the chance for telomeres to find free fusogenic DNA extremities. 53BP1 directly interacts with chromatin by binding to H4K20me2 with its tudor domain, as well as with ubiquitylated H2A with its C-terminal ubiquitination-dependent recruitment (UDR) motif (Dimitrova et al., 2008) (Fradet-Turcotte et al., 2013).

A portion of the TRF2 hinge domain, named iDDR, was suggested to have a role in preventing RNF168 recruitment and the ensuing 53BP1 localization at telomeres (Okamoto et al., 2013). Indeed, the TRF2 iDDR domain recruits the MRN complex, which (via NBS1) interacts with the deubiquitinating (DUB) enzyme BRCA1/BRCA2-containing complex subunit 3 (BRCC3), thereby providing a physical connection between TRF2 and BRCC3 (Okamoto et al., 2013). BRCC3 might counteract RNF8 activity and avoid 53BP1 binding, thereby impeding cNHEJ. Moreover, the TRF2 iDDR domain can also interact with the Ubiquitin protein ligase E3 component N-recognin 5 (UBR5), which targets RNF168 for proteasomal degradation, ultimately preventing H4K20me2 unmasking, the ensuing 53BP1 accumulation and chromosomal fusions (Okamoto et al., 2013).

Ring1B and its interacting factor Bmi are also necessary for cNHEJ to occur at TRF2-depleted telomeres (Bartocci et al., 2014). Ring1B is a RING finger E3 ubiquitin ligase that can ubiquitinate histone H2A on K119 (H2AK119ub), a histone modification usually associated to gene silencing and chromosomal compaction (R. Cao et al., 2005) (Stock et al., 2007). The fact that an enzymatically inactive mutant for Ring1B still rescues the fusogenic phenotype (Bartocci et al., 2014) hints for a role of Ring1B that in this context is independent from its ubiquitinating activity and that involves the activity of an as yet unidentified interactor.



**Figure 23. Ring finger proteins (RNFs) ubiquitinase activity in DDR**

*RNF8 ubiquitinase activity recruits RNF168 to telomeres, which in turn ubiquitinates histone H2A, a step that is required for 53BP1 (NHEJ) or BRCA1 (HR) recruitment and the ensuing DDR progression (Mattioli et al., 2012).*

### **Telomeric repeat-containing RNA (TERRA)**

TRF2 can affect chromatin structure by controlling the levels of the long non-coding Telomeric repeat-containing RNA (TERRA), which is transcribed from subtelomeric regions and is a structural component of telomeres (Azzalin et al., 2007) (Schoeftner & Blasco, 2008) (Luke & Lingner, 2009) (Z. Deng et al., 2009) (Porro et al., 2010). Via its TRFH (homodimerization) domain, TRF2 directly represses the transcription of TERRA (Porro et al., 2014b). Noteworthy, when TRF2 is depleted, TERRA transcription is induced in a DDR-independent fashion (Porro et al., 2014b), hinting for an upstream or ATM-parallel role for TERRA in the telomeric DDR.

If, on the one hand, TERRA transcription depends on chromatin structure, on the other hand, TERRA itself can affect telomeric chromatin boosting H3K9me3 formation (Z. Deng et al., 2009) (Arnoult et al., 2012). Indeed, TERRA interacts with and activates the histone methyltransferase Suppressor of variegation 3-9 homolog 1 (SUV39H1) that in turn can methylate H3K9 (Porro et al., 2014b), giving rise to a marker of heterochromatin. Interfering with H3K9me3 levels via the inhibition of either

SUV39H1 (Bartocci et al., 2014) (Porro et al., 2014b) or Prdm3 (Bartocci et al., 2014) lysine methyl transferases (KMTs) impairs cNHEJ at dysfunctional telomeres (Bartocci et al., 2014) (Porro et al., 2014b) with no effect on DDR late factors recruitment, such as 53BP1 and Replication timing regulatory factor 1 (RIF1) (Bartocci et al., 2014). Being H3K9me3 a hallmark of mammalian heterochromatin, its accumulation at TRF2-deprived telomeres may compact telomeric chromatin and favor cNHEJ, as already observed for other genomic regions (Chiolo et al., 2011) (K. M. Miller et al., 2010). On the other hand, H3K9me3 can also promote the decondensation of damaged heterochromatin regions, functioning as a docking site for the acetyltransferase Lysine acetyltransferase 5 (Tip60/KAT5) that is involved in chromatin acetylation and initiation of the ATM-dependent DDR (Sun et al., 2007) (Sun et al., 2009). Indeed, the chromatin decompaction of damaged heterochromatin regions is necessary for the recruitment of DDR factors and lesion repair (Cann et Dellaire, 2011).

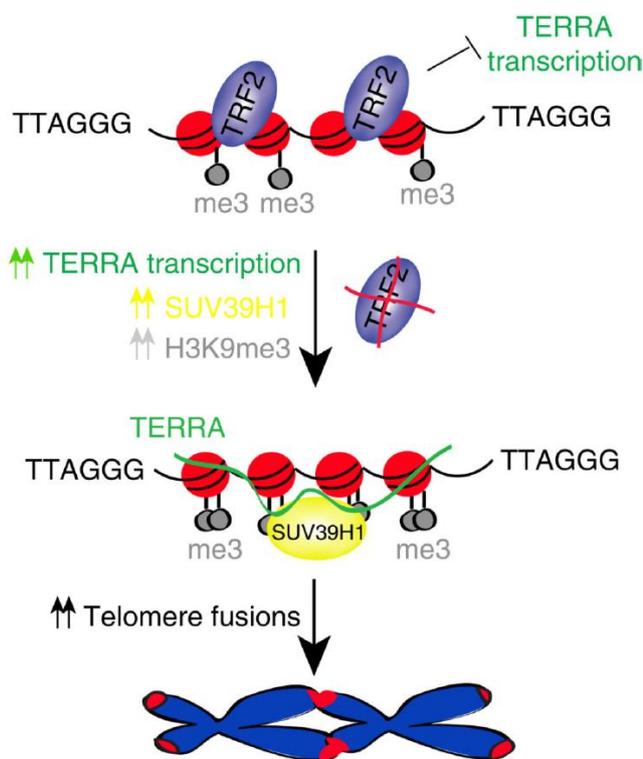
Other than its function in modelling the epigenetic environment of dysfunctional telomeres, TERRA itself is part of the telomeric chromatin and may guide other telomere-associated factors involved in the DDR elicited by the loss of TRF2. Indeed, TERRA associates with Lysine-specific demethylase 1 (LSD1) at dysfunctional telomeres (Porro et al., 2014a) and it has probably a role in its recruitment and stabilization at chromosome ends. LSD1 removes mono- and dimethyl groups from histone H3 lysin 4 (H3K4) and 9 (H3K9) (Y. Shi et al., 2004) (Metzger et al., 2005) (Lan et al., 2008), but also has non-histone targets such as p53, E2F1, and DNA methyltransferase 1 (DNMT1) (R. C. Wang et al., 2004) (J. Huang et al., 2007) (Cho et al., 2011) (Kontaki & Talianidis, 2010).

In an uncapped telomere-context, LSD1 has no overt effect on histone methylation, transcription, or DDR activation, but it is instead pivotal for the removal of telomeric 3'G-overhang (Porro et al., 2014a), a stage that is just upstream telomere fusion via cNHEJ (Celli & de Lange, 2005) (Attwooll et al., 2009) (Y. Deng et al., 2009). Meiotic recombination 11 homolog A (MRE11), a nuclease whose activity is pivotal for the removal of the telomeric 3'G-overhang, is likely involved in this process (Porro et al., 2014b) (Attwooll et al., 2009) (Y. Deng et al., 2009). MRE11 promotes the recruitment of LSD1 and binds it via the same LSD1 domain implicated in TERRA binding. This interaction elicits MRE11 nuclease activity and thus telomere processing.

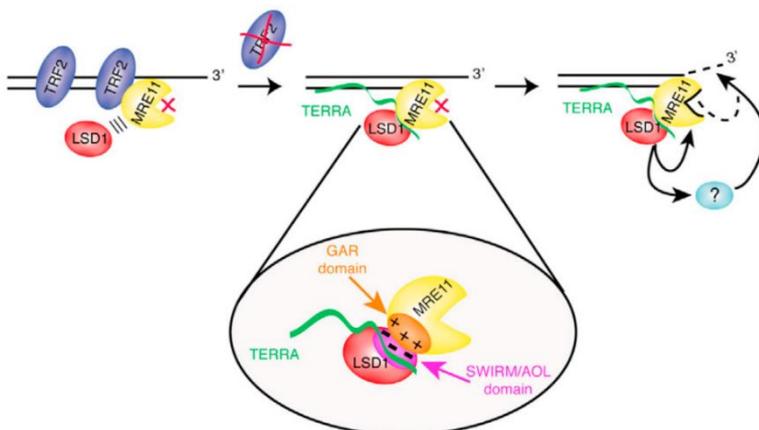
All in all, TERRA can affect telomere chromatin structure and telomere-end processing in a TRF2-depleted context via the recruitment of distinct factors.

Very interestingly, *in vitro* experiments with mouse and human cell lines also revealed that telomere deprotection induced by TRF2 depletion leads to the transcription of both strands of telomeric DNA.

Furthermore, the ensuing production of the so-called DNA damage response RNAs (DDRNs) by the riboendonuclease enzymes DICER and DROSHA is necessary for NHEJ-mediated DNA repair to happen. Remarkably, antisense oligonucleotides (ASOs) against telomeric (t)DDRNs repress DDR and NHEJ. Importantly, these results can be recapitulated *in vivo*. Indeed, upon TRF2 downregulation in an inducible system followed by ASOs administration, 53BP1 and pKAP1 (being KAP1 an ATM target) foci intensity is strongly reduced ( $\gamma$ H2AX foci, as previously reported, are not affected) (Rossiello et al., 2017).



**Figure 24. TERRA and chromatin structure**  
 TRF2 loss increases TERRA transcription at subtelomeric regions. TERRA, in turn, can recruit the histone methyltransferase SUV39H1 to telomeres causing Histone H3K9 trimethylation (H3K9me3), which facilitates chromatin compaction and NHEJ, leading to aberrant chromosomal fusions (Porro et al., 2014b).



**Figure 25. TERRA and 3'G-overhang processing**  
 Decreased TRF2 levels allow TERRA transcription and binding to LSD1 that, in turn, binds the MRE11 nuclease via the same TERRA-binding domain. MRE11 activity at telomeres is pivotal for 3'G-overhang processing, a likely feature of Intermediate State telomeres (Porro et al., 2014a).

### 3.1. Extratelomeric roles of TRF2

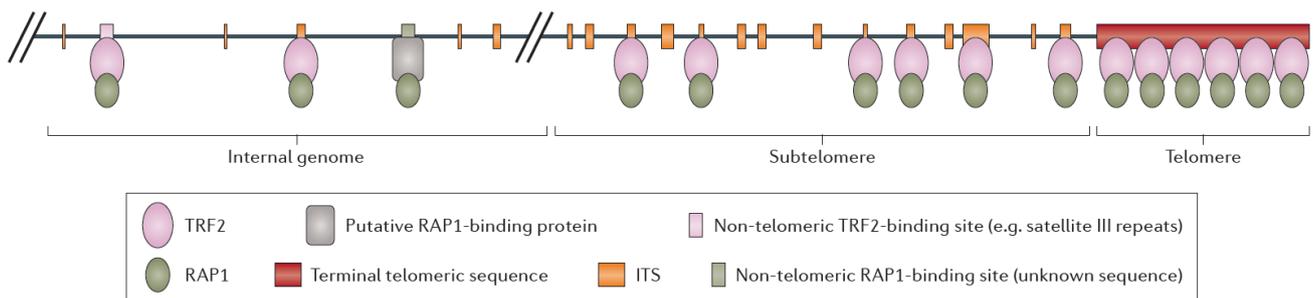
Other than its well-known roles in telomere protection which requires its localization to telomeres, TRF2 exhibits several non-canonical extratelomeric activities that will be hereafter discussed.

i) ChIP-seq and ChIP-qPCR analyses in human cancer cell lines respectively found and confirmed that TRF2 (with its binding partner RAP1) is bound to non-telomeric DNA sequences throughout the whole genome in the proximity of gene promoters or within introns.

Interestingly, while the vast majority of these sequences contains Interstitial telomeric sequences (ITSs) characterized by repetitions of the TRF2 DNA binding sequence TTAGGG (several repeats in subtelomeric regions, and few repeats in internal chromosomal sites), TRF2 can also bind to centromeric and pericentromeric satellite 2/3 regions characterized by the pentameric repeat sequence CCATT, LINE1 and alphoid satellite sequences. Therefore, TRF2 can bind to DNA sequences different from the canonical TTAGGG hexamer.

Moreover, since some of the binding site hits were not enriched in all the cell lines tested, the cell line-specific state of chromatin compaction could also play an important part in TRF2 capacity to bind DNA.

From a neuronal perspective, it is important to consider that TRF2 binds in the proximity or to introns of genes involved in vesicular transport (*Snap25*, *Arf-Gap3*, *Pacsin2*), ion transport (*Cacna1b*, *Cln6*, *Lcn2*) and axonal growth (*Plxnb2*, *Ehd4*, *Vcan*), which might influence their expression. (Simonet et al., 2011).



**Figure 26. Interstitial telomeric sequences**

TRF2 can bind interstitial telomeric sequences (ITS) and non-telomeric sequences dispersed throughout the whole genome, including subtelomeric regions and internal genome sites (Modified from: Ye et al., 2014).

ii) TRF2 has also a role in the immune response to cancer. It is established knowledge that tumor cells express high levels of TRF2 in both mice and humans. Indeed, tumor cell lines downregulated for TRF2 (via RNA interference or via the expression of a dominant negative protein [TRF2<sup>dn</sup>]) have a decreased proliferative capacity, but do not show increased levels of DDR and senescence, despite

expressing high levels of IL-6, which appears to be necessary to avoid DDR in such a context. Accordingly, the downregulation of TRF2 in murine tumor xenografts impairs the tumorigenicity of the implanted tumors without impacting DDR and telomere length or uncapping.

Impressively enough, the *in vivo* injection of tumor cells downregulated for TRF2 markedly increased the number of tumor-infiltrating NK cells, their activation with IFN- $\gamma$  production, and decreased the tumor neovascularization, when compared to their controls expressing TRF2.

Importantly, ATM inhibition showed no effect on TRF2<sup>dn</sup> cells capacity to recruit and activate NK cells, thereby proving that TRF2 controls NK cells independently of its role in DDR and telomere protection. Furthermore, ChIP-seq data analysis revealed that TRF2 can bind an intron of the gene *HS3ST4*, encoding for the enzyme Heparan sulfate-glucosamine 3-O-sulfotransferase 4, whose activity consists in the sulfation of heparan sulfate proteoglycans. TRF2 downregulation in tumor cells causes the decreased expression of this gene. *HS3ST4* downregulation in injected tumoral cells recapitulates the results observed with the downregulation of TRF2 with respect to NK cells recruitment. Noteworthy, the simultaneous downregulation of TRF2 and *HS3ST4* does not show an additive effect, suggesting that they act in the same pathway in this context. Importantly, in early-stage human colon carcinomas the expression of TRF2 is progressively increased and inversely correlates with NK cells tumoral infiltration.

Thus, TRF2 regulates the tumor microenvironment in a DDR-independent manner via the regulation of the sulphation of heparan sulfate proteoglycan molecules. Therefore, it is tempting to speculate that TRF2 modulates the sulphation of molecules expressed on the surface of tumor cells via *HS3ST4* activity, thereby impairing tumor cell recognition and elimination by NK cells (Biroccio et al., 2013).

**iii)** TRF2 roles further expand to tumor angiogenesis. *In vitro* assays with Human umbilical vein endothelial cells (HUVECs) showed that TRF2-compromised cells exhibit impaired proliferation, migration and endothelial vessel formation.

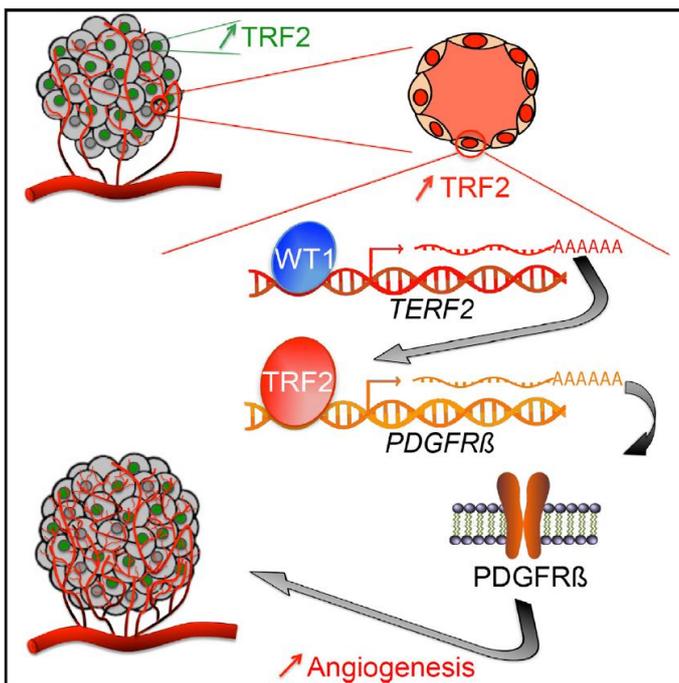
Moreover, endothelial cells isolated from lung tumors express higher levels of TRF2 when compared to cells isolated from tumor-less lungs. Importantly, the latter exhibit impaired proliferation, migration and vessel formation capacity when compared to the endothelial cells isolated from lung tumors.

Furthermore, the expression of TRF2 depends on the transcription factor Wilms tumor protein 1 (WT1), which is notoriously overexpressed in endothelial cells of a broad variety of solid human

cancers, promoting proliferation, migration and vessel formation. Indeed, WT1 directly binds to the TRF2 promoter eliciting its gene expression.

Importantly, the TRF2 modulations experimented with HUVECs do not correlate with telomeric damage and DDR, despite ATM inhibition does impair HUVECs growth and migration capacities.

Finally, TRF2 binds to the promoter and drive the expression of the well-established pro-angiogenic factor Platelet-derived growth factor receptor  $\beta$  (PDGFR $\beta$ ), whose activity is pivotal for endothelial cell proliferation, migration and vessel formation (El Maï et al., 2014).



**Figure 27. TRF2 and tumor angiogenesis**

TRF2 levels are elevated in tumor endothelial cells when compared to cells isolated from tumor-less controls. TRF2 expression is under the positive regulation of the transcription factor WT1. In turn, TRF2 positively regulates the expression of the proangiogenic endothelial receptor PDGFR $\beta$  (El Maï et al., 2014).

**iv)** Ribonucleoprotein [RNP] immunoprecipitation (RIP) assays revealed that a shorter, cytoplasm-localized isoform of TRF2 (TRF2-S[Small]) isolated from cultured hippocampal neurons (DIV9) binds to about 140 transcripts (identified by Illumina microarray analysis). Gene Ontology classification revealed that most of them encodes for proteins involved in neuronal structure and function. Noteworthy, 34 out of the 140 transcripts stood out for being involved in axonogenesis.

Very often, RNA-binding proteins (RBP) bind their own coding mRNA, which is also the case for TRF2-S. Nonetheless, pull-down experiments with rat brain extracts revealed that *Trf2-S* mRNA also pulls down the Fragile x mental retardation protein (FMRP), another known RBP.

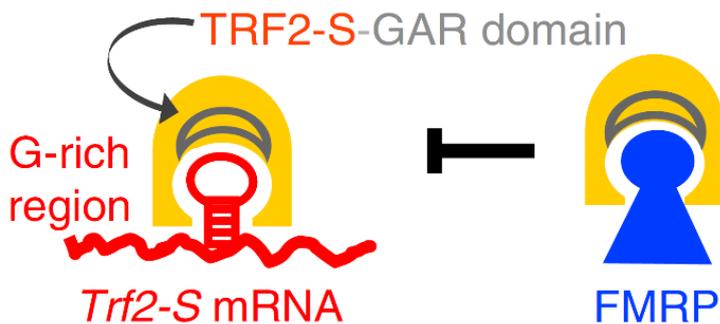
Interestingly, coimmunoprecipitation assays with deletion mutants of both TRF2-S and FMRP showed that TRF2-S can also bind FMRP, and FMRP and *Trf2-S* mRNA binding sites in TRF2-S overlap. Indeed, Co-IP experiments revealed that the presence of mRNA affects the binding between TRF2-S and FMRP. Analysis of protein extracts from homogenized brain tissue of *Fmr1* KO mice (mice KO for

FMRP) showed that TRF2-S expression is increased when compared to WT mice. Moreover, TRF2-S pull downs with *Fmr1* KO samples are enriched in TRF2-S target mRNAs when compared to the WT condition. Furthermore, TRF2-S can pull down FMRP in WT lysates, but not in *Fmr1* KO lysates. Importantly, biotinylated mRNAs encoding for proteins involved in presynaptic vesicle trafficking and neurotransmitter release (*i.e.*, *Rab3a* and *Aplp1*) can pull down both TRF2-S and FMRP, and FMRP competes for TRF2-S binding to mRNAs by occupying the TRF2-S GAR (glycine- and arginine-rich) domain.

Staining of rat brain tissues and neurons revealed that TRF2-S and FMRP puncta colocalize in the neuron cell body. Interestingly, in the axons there is much less FMRP and less TRF2-S–FMRP colocalization. Fluorescence *in situ* hybridization (FISH) assays with cultured neurons revealed that TRF2-S knock-down causes decreased localization of *Rab3a* and *Aplp1* mRNAs in the axons, whereas FMRP knock-down causes their increase. Moreover, microfluidic chamber experiments with cortical neurons (DIV9) showed that TRF2-S is pivotal for *Rab3a* and *Aplp1* mRNAs transport along the axons, which is impaired if TRF2-S lacks its GAR domain.

Interestingly, over the course of maturation of primary cultured neurons, the expression of TRF2-S gradually increases, together with the expression of the synaptic markers Synaptophysin and PSD95, whereas the expression of FMRP declines. Moreover, the shRNA-mediated downregulation of TRF2-S causes decreased levels of *Rab3a* and *Aplp1* mRNAs in isolated axons and whole-cell extracts. On the other hand, the downregulation of FMRP leads to the increased expression of the above-mentioned mRNAs. Importantly, TRF2-S overexpression leads to increased axonal growth of cultured neurons, whereas the expression of the TRF2-S mutant lacking the GAR domain fails to do so. On the contrary, TRF2-S knock-down impairs axonal growth, while FMRP knock-down increases it, when compared to controls.

Finally, experiments with cultured hippocampal neurons revealed that TRF2-S overexpression causes increased in mean Miniature excitatory post-synaptic currents (mEPSCs) frequency, but not in their amplitude. On the same line, the shRNA-mediated downregulation of TRF2-S significantly decreases the mean mEPSCs frequency, whereas FMRP knock-down significantly increases it. The amplitude of the mEPSCs is not affected by TRF2-S or FMRP modulation. Therefore, TRF2-S and FMRP activities respectively increase and decrease glutamate release from presynaptic terminals (P. Zhang et al., 2015).



**Figure 28. TRF2-S-mRNA/FMRP interaction**  
 TRF2-S can bind several transcripts, including its own coding mRNA, via the GAR domain. The RNA binding protein FMRP competes with mRNAs for binding to TRF2-S at the level of the same GAR domain (Zhang et al., 2015).

v) TRF2 also has roles in heterochromatic DNA stability outside telomeric regions. Indeed, TRF2 binds to pericentromeric DNA in a sequence-independent manner, and the *in vitro* TRF2 downregulation triggers an ATM-dependent DDR pathway at pericentromeres. Noteworthy, the N-terminal and TRFH domains of TRF2 seem to be required for binding to pericentromeres, while the Myb/Telobox DNA-binding domain, pivotal for telomere binding, appears to be dispensable.

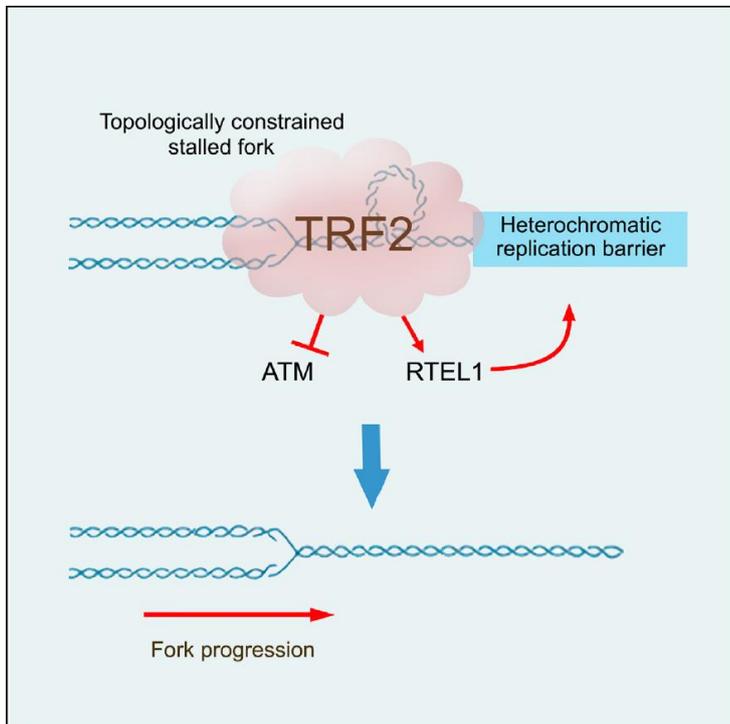
Interestingly, the downregulation of the histone methyl transferases SUV39H1 and G9a, that can methylate Histone H3 at the Lys9 residue (H3K9me3), promotes TRF2 dissociation from pericentromeres but not from telomeres. The same results are recapitulated by inhibiting histone deacetylases I and II, which reduce H3K9me3 levels.

TRF2 recruitment at pericentromeres is increased during the S phase of the cell cycle, suggesting a role for TRF2 in pericentromeric DNA replication. Noteworthy, the overexpression of TRF2 decreases the occurrence of hydroxyurea-induced Pericentromeric dysfunction-induced foci (PIFs) during S phase.

TRF2 downregulation causes a slowing down of DNA replication specifically at pericentromeric regions. Moreover, the levels of fork asymmetry (reflecting the levels of fork stalling) are increased specifically at pericentromeres upon TRF2 downregulation. Furthermore, ATM inhibition concomitant to TRF2 downregulation only partially rescues the speed of pericentromeric replication, meaning that different TRF2 functions, other than ATM inhibition, are necessary for fork replication progression through pericentromeric chromatin.

ChIP experiments revealed that, RTEL1 helicase binding to pericentromeres is TRF2-dependent. Moreover, pericentromeric damage is similarly increased when either TRF2 or RTEL1, or both, are downregulated, and the downregulation of either or both of them decreases fork replication speed and increases fork asymmetry to the same extent. Mechanistically, the concerted activity of TRF2 and RTEL1 allows the dissolution of G-quadruplexes (G4) and G4-like structures, thereby removing a physical obstacle to the replication fork progression at pericentromeres. Furthermore, ChIP

experiments with human cell lines showed that, upon TRF2 downregulation,  $\gamma$ H2AX is enriched at both pericentromeric (SatII repeats) and telomeric regions. Therefore, TRF2 is necessary not only for successful telomeric DNA replication, but for broader range genome-wide heterochromatin replication (Mendez-bermudez et al., 2018).



**Figure 29. TRF2 safeguards DNA replication at pericentromeres**

TRF2 can bind in a sequence-independent manner to pericentromeres where it recruits RTEL1 helicase, thereby resolving topological barriers and favoring DNA replication of these heterochromatic regions (Mendez-Bermudez et al., 2018).

**vi)** TRF2 affects the redox balance in postmitotic muscle cells. Importantly, analyses of human muscle biopsies revealed that, similarly to telomere length, TRF2 protein levels (but not those of the other Shelterin subunits) decrease with age.

Interestingly, whereas the *in vitro* TRF2 downregulation in mitotic myoblasts triggers telomeric DNA damage, knocking down TRF2 in postmitotic differentiated myotubes does not elicit increased levels of Telomeric dysfunction-induced foci (TIFs). Nevertheless, myotubes exhibit higher levels of basal DNA damage when compared with myoblasts.

Moreover, TRF2 downregulation in myotubes induces increased ROS production and boosts the expression and activity of the pro-longevity factor Forkhead box O3 (FOXO3a). Furthermore, TRF2 downregulation causes increased levels of mitochondrial DNA, decreased complex I and increased complex IV activity of the mitochondrial electron transport chain (ETC), and punctuated appearance of the mitochondrial network, whose structure is restored by antioxidant treatment.

ChIP-seq analysis of transduced myotubes revealed that TRF2 binds to Interstitial telomeric sequences (ITSs) inside the locus and the promoter of the *SIRT3* gene, and TRF2 downregulation

decreases TRF2 binding to these ITs. Noteworthy, SIRT3 is the only sirtuin whose expression levels and activity are affected by TRF2 modulation. Remarkably, the ectopic expression of SIRT3 upon TRF2 downregulation restores SIRT3 activity and mitochondrial activity (ETC), while simultaneously decreasing ROS production and mitochondrial DNA content.

Chromatin conformation capture and three-dimensional fluorescence in situ hybridization (3D-FISH) techniques allowed the observation of a TRF2-dependent chromatin loop encompassing the 11p telomere and the *SIRT3* locus that is less frequent in TRF2-compromised myotubes. Noteworthy, the same loop is conserved also in mitotically-arrested mouse embryonic fibroblasts, and less frequent upon TRF2 downregulation.

Finally, mice where TRF2 expression is specifically downregulated in mature muscle fibers exhibit normal development, weight and behavior up to ten months of age. Nevertheless, the skeletal muscles (*e.g.*, *Gastrocnemius* and *Soleus*) of transgenic mice do not exhibit an increased occurrence of TIFs, but an increased proportion of oxidative fibers in the *Soleus*. Moreover, TRF2 downregulation in the muscles of transgenic mice causes increased levels of mitochondrial DNA and a more fragmented mitochondrial structure. Furthermore, as in cultured myotubes, TRF2 downregulation causes decreased complex I and increased complex IV activity, decreased SIRT3 levels and strong FOXO3a activation (Robin et al., 2020).

**vii)** TRF2 is also necessary for Origin recognition complex subunit 2 (ORC2) recruitment to pericentromeric SatIII regions, thereby favoring the initiation of DNA replication at the pericentromeric heterochromatin (PCH) level. Indeed, TRF2 downregulation in HeLa cells specifically decreases the occurrence of origins of replication (origin density) at pericentromeres, leaving unaffected centromeric and global DNA regions. Noteworthy, this TRF2 activity depends on its N-terminal domain, whereas the C-terminal Myb/Telobox DNA-binding domain is dispensable (Bauwens et al., 2021).

**viii)** Pericentromeric heterochromatic (PCH) DNA is selectively damaged during senescence. *In vitro* experiments with MRC5 cells showed that, upon hypoxia-induced senescence, telomeres are the first chromosomal regions to undergo deprotection and suffer DNA damage. Following, five population doublings (PDs) after telomere uncapping, the pericentromeres are the regions getting compromised next (SatII/III sequences). Importantly, other chromosomal regions, such as the centromere, were unaffected and ChIP-seq analyses could not detect any  $\gamma$ H2AX enrichment at centromeric regions (alphoid, rDNA and LINE1 sequences) when compared to control cells.

Upon senescence, pericentromeric damage slightly precedes heterochromatin decondensation as revealed by measuring the diameter of the signal given by SatIII-targeting fluorescent probes. Interestingly, the DNA damage induced by the senescence-inducing stressor bleomycin is repaired after bleomycin removal at a global DNA level, but the telomeric and pericentromeric DNA damage persists also after the stress inducer is removed. Therefore, telomeres and pericentromeres are deemed regions of irreparable DNA damage. On the other hand, centromeric DNA damage levels remain low all the way through the treatment. The same holds true when using cells that age normally due to replicative senescence.

Comet assay coupled with probes specific for the different chromosomal regions (*i.e.*, centromeric, pericentromeric and telomeric) revealed that at the onset of senescence, telomeres and pericentromeres, but not centromeres, undergo DNA damage characterized by DSBs formation. In turn, DSBs formation results in increased occurrence of illegitimate recombination events and loss of DNA sequences.

Interestingly, DNA consisting of SatIII sequences could be observed in the cytoplasm of pre-senescent cells. Impressively, in this pre-senescence state, the appearance of Pericentromeric dysfunction-induced foci (PIFs) coincides with the decreased expression of TRF2 (known to bind and protect PCH). Importantly, the decreased expression of TRF2 causes an almost complete loss of TRF2 binding to pericentromeric regions, a 50% loss of its binding to telomeres, and increased DNA damage rates at both regions, thereby suggesting that TRF2 downregulation at the onset of replicative senescence undermines PCH integrity.

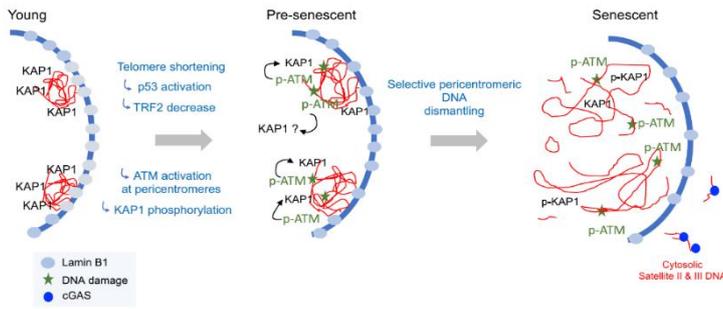
Downregulation of TRF2 in pre-senescent cells was already shown to be dependent on the p53-mediated Siah1 E3 ubiquitin-protein ligase upregulation and ensuing TRF2 degradation via the proteasome (Fujita et al., 2010). Indeed, inhibition of p53 expression in pre-senescent (60 PDs) and senescent cells (67 PDs) counteracts TRF2 downregulation and leads to decreased PCH DNA damage, diminished PCH cytosolic DNA levels, and no PCH decondensation. Noteworthy, the same results can be recapitulated in MRC5 cells treated with bleomycin that did not undergo telomere shortening and replicative senescence. Remarkably, the ectopic overexpression of TRF2 from PD50 until senescence delays senescence onset by 5 PDs. At this stage, cells exhibit a senescence characterized by the expression of p16, contrary to canonical replicative senescence which is characterized by p21 expression.

Furthermore, Proximity ligation assays (PLA) targeting the Proliferating cell nuclear antigen (PCNA) and SatIII sequences revealed that the pre-senescence decline of TRF2 leads to defective DNA

replication at PCH level. Moreover, restoring TRF2 levels in bleomycin-arrested cells leads to decreased PCH damage levels and less decondensation, suggesting that TRF2 decrease is also responsible for replication-independent PCH damages.

Importantly, ATM phosphorylation is increased at PCH in senescent cells, and the inhibition of its phosphorylation avoids PCH decondensation. Notably, senescence-dependent TRF2 downregulation triggers ATM activation, which in turn elicits KRAB-associated protein 1 (KAP1) phosphorylation and the release of P-KAP1 and Lamin B1 (LMNB1) from PCH, leading to PCH decondensation, all of which is reversed by the ectopic expression of TRF2. The ensuing cytoplasmic PCH DNA is bound by the cyclic GMP-AMP synthase (cGAS – an innate immunity Pattern recognition receptor [PRR]) resulting in the expression of Interferon-stimulated genes (ISGs). Therefore, the programmed p53-dependent TRF2 downregulation during senescence triggers a specific PCH decondensation leading to PCH DNA leakage into the cytosol, which in turn is bound by and activates cGAS and an ensuing interferon response.

Interestingly, the infection of senescent cells with a lentivirus bearing an shRNA construct against p21 mRNA leads to the restart of the cell cycle seven days post infection (DPI). Cells co-transfected with an empty vector enter back into senescence at 9DPI, whereas cells co-transfected with a full-length TRF2 reenter senescence at 12.5DPI and show markedly decreased PCH DNA damage, despite no variation in global DDR, when compared to control cells (Empty vector). Telomere length keeps decreasing with the ensuing increase in TIFs occurrence in both the conditions. Co-transfection with hTERT causes restored telomere length, TRF2 expression and PCH stability (condensation and content). Noteworthy, in p21-downregulated cells a partial PCH recondensation takes place, while TRF2 levels remain low, meaning that factors other than TRF2 play a role in pericentromeres integrity. Notably, post-senescent cells downregulated for p21 exhibit chromosomal breakpoints and rearrangements preferentially located in heterochromatic regions, which are significantly reduced upon TRF2 co-transfection. Therefore, upon senescence evasion, heterochromatic regions undergo breaks and rearrangements (counteracted by TRF2 restored levels), the same that are commonly observed in cancer cells (Mendez-bermudez et al., 2022).



**Figure 30. The role of TRF2 in pericentromeric stability**

Age-dependent telomere shortening elicits p53 activation, which in turn leads to TRF2 degradation via Siah1 ubiquitin-protein ligase activation and proteasome-mediated degradation.

TRF2 depletion unleashes ATM activation which leads to KAP1 phosphorylation, featuring a pre-senescence state. At senescence, PCH is dismantled and Satellite II and III pericentromeric DNA is released into the cytosol where it is bound by the innate immunity receptor cGAS, thereby triggering an ISG immune response (Mendez-Bermudez et al., 2022).

**ix)** Very interestingly, in another animal model such as Zebrafish, the orthologue of human TRF2, zfTRF2 (encoded by the *terfa* gene) does not seem to act as a specific telomeric factor. Experiments in Zebrafish cell lines showed that only a small fraction of zfTRF2 (<10%) colocalizes with telomeres, and, in turn, only 10% of telomeres colocalizes with zfTRF2; a percentage that is very close to the colocalization with TRF2 of other chromosomic regions, such as centromeres (<5%) and pericentromeres (10-30%). Also, during S phase, zfTRF2 localization increases at pericentromeres, but not at telomeres or centromeres. Moreover, zfTRF2 downregulation via RNA interference markedly increases a global (non-telomere-specific) DDR. Interestingly, when zfTRF2 is expressed in a human cell line, >70% of telomeres colocalizes with it. Downregulation of human(h)TRF2 only partially reduces zfTRF2 colocalization with telomeres in this context, meaning that hTRF2-zfTRF2 dimerization is not the event driving zfTRF2 localization at human telomeres. All in all, being zfTRF2 proficient in telomere binding, it might be that other factors compete with zfTRF2 for telomere binding in Zebrafish cell lines. The TRF2 depletion *in vivo* (obtained via the generation of a *terfa* frameshift mutation) leads to embryonic lethality in the homozygous mutant (*terfa*<sup>-/-</sup>), and to premature aging characterized by spinal curvature, skin depigmentation, cardiac dysfunction and impaired brain development, in the heterozygous mutant (*terfa*<sup>+/-</sup>), with a 50% rate of survival to the adult stage. Brain edema is the first prominent phenotype appearing in *terfa*-compromised Zebrafish. Brain development failure is corroborated by the decreased expression of the neurodevelopmental marker *neurogenin 1*, at two days post fertilization (dpf). At this stage, other organs developmental markers do not change, meaning that the brain is the first organ to face impaired development upon zfTRF2 depletion. Importantly, the injection of *terfa* mRNA (but not of a *terfa* lacking the sequence encoding for the telobox) fully rescues the brain edema phenotype.

zfTRF2 depletion also results in increased brain rates of apoptosis and senescence, increased DDR, decreased cell proliferation and fewer numbers of Glial fibrillary acid protein(*Gfap*)-positive astrocytes, *Huc*-positive neurons, and *Sox2*-positive pluripotent stem cells. Interestingly,  $\gamma$ H2AX positivity and apoptosis are most prominent in GFAP-positive cells. Like in the cell lines tested, the DDR due to the lack of zfTRF2 is not telomere-specific but globally distributed, and telomere length and integrity are not affected. Importantly, ATM (but not p53) depletion in *terfa*-compromised Zebrafish completely blunted the DDR. At any rate, even though the inhibition of either *Atm* or *p53* rescues almost completely heart and belly edema, it only partially reduces brain edema, neuronal loss and embryonic lethality. Therefore, ATM-dependent DDR is only partially responsible for the observed neurodevelopmental phenotype of *Terfa*-compromised Zebrafish. These results suggest a DDR-independent role for TRF2 in this context. Indeed, RNA-seq analyses of 3dpf WT, *Terfa*<sup>+/-</sup> and *Terfa*<sup>-/-</sup> zebrafish embryos revealed that *Terfa*-compromised zebrafish exhibit higher expression levels of genes involved in cell cycle control (*Cdkn1a*, *Gadd45*), inflammation (e.g., *Il6* and *Isg15*) and neuronal dysfunction (e.g., genes involved in calcium signaling and ligand-receptor interaction). Noteworthy, in *Terfa*<sup>-/-</sup> zebrafish embryos the expression levels of neuronal genes such as *Ppp2r2c* and *Snap25* were downregulated. Importantly, mRNA injection of *Ppp2r2c* (but not *Snap25*) into *Terfa*-compromised zygotes decreases the rate of brain edema. Noteworthy, mRNA injection of *Ppp2r2c* and *Snap25* combined has an additive rescue effect in *Terfa*<sup>+/-</sup> fish. Therefore, zfTRF2 controls the expression of genes involved in neuronal development. This transcriptional effect of zfTRF2 might involve its interaction with Repressor element 1 silencing transcription factor (REST) (reported in mammalian cells), which is a master transcriptional repressor of neuronal genes. Nevertheless, although *Snap25* is a known target of REST and TRF2, the overexpression of *Rest* mRNA in *Terfa*-compromised Zebrafish does not rescue the phenotype. Overall, zfTRF2 depletion impairs neurodevelopment in a DDR-independent fashion and via the downregulation of a specific set of neuronal genes, notably *Ppp2r2c* and *Snap25*. Very interestingly, the selective restored expression of zfTRF2, in *Terfa*-compromised Zebrafish, in *Gfap*-positive glial cells alone, but not in either *Huc*-positive neurons or *Nestin*-positive progenitors, was sufficient to rescue the neurodevelopment (reduction of brain edema and increased numbers of neurons and glial cells), abolish the increased DDR and the transcriptional dysregulation. Therefore, zfTRF2 levels in glial cells have a specific role in neuronal homeostasis, probably because during development GFAP-positive radial glial cells can give rise to diverse neural cell types, including neurons. Importantly, the restoration of zfTRF2 levels in brain GFAP-positive cells has extra-brain effects rescuing other organs

such as heart and belly from edema, on the one hand, and eyes and intestine from DDR, on the other hand. Therefore, either *Terfa* plays a role in GFAP-positive cells outside the nervous system, or the restoration of a normal neurodevelopment positively affects the development of other organs (Ying et al., 2022).

**x)** RNA-seq analysis of young (PD30) vs senescent (PD71) human lung primary fibroblasts (MRC5 cells) identified 1051 differentially expressed genes (DEGs), of which those within 2Mb distance from telomeres (the range of that chromosomal region referred to as subtelomere) were positively enriched upon senescence. Noteworthy, this fold enrichment is a feature of a subset of seven chromosome ends (8q, 9q, 11p, 16p, 16q, 19p, 22q).

The lentivirus-mediated overexpression of TRF2 in pre-senescent cells (PD60) delays senescence onset to PD75, whereas control cells (empty vector) still senesce at PD71. RNA-seq of young (PD30) and pre-senescent (PD60) MRC5 overexpressing TRF2, and young and pre-senescent MRC5 transduced with an empty vector found 1366 and 1051 DEGs, respectively. The intersection of these two data sets (856 genes) represents those genes whose expression varies upon senescence in a TRF2-independent manner. On the other hand, 510 is the number of genes whose expression is modulated upon senescence only when TRF2 is overexpressed (TRF2<sup>HIGH</sup>-dependent), and 195 is the number of genes whose expression is modulated only when TRF2 levels are low (TRF2<sup>LOW</sup>-dependent). As for the whole transcriptome analysis of young vs senescent cells (PD30 vs PD71), also in this context, enrichment of upregulated genes at the subtelomeric regions is found among the TRF2-independent and TRF2<sup>LOW</sup>-dependent DEGs, but not in the TRF2<sup>HIGH</sup>-dependent DEGs. Therefore, TRF2 downregulation upon telomere shortening is responsible for the expression of part of the subtelomeric genes. Noteworthy, 27 out of the 195 TRF2<sup>LOW</sup>-dependent DEGs are subtelomeric, and 26 of these are upregulated. Interestingly, the sole downregulation of TRF2 in non-senescent MRC5 cells is sufficient to recapitulate the upregulation of the genes belonging to the TRF2<sup>LOW</sup>-dependent group. Unsurprisingly, these genes are involved in pathways related to cell cycle arrest and senescence.

The senescence-associated TRF2 downregulation decreases the levels of the nuclear lamina protein Lamin B1 (LMNB1), which is a regulator of chromatin architecture. Importantly, LMNB1 downregulation yields 2607 DEGs, of which 104 are upregulated subtelomeric genes. These results mirror those obtained with replicative senescence or TRF2 overexpression experiments (TRF2<sup>LOW</sup>-dependent group), with no enrichment in downregulated subtelomeric genes. Noteworthy, the two subtelomeres enriched upon LMNB1 downregulation (8q and 22q) are included in the seven

identified upon replicative senescence. Among the 2607 DEGs given by LMNB1 downregulation, 460 are also DEGs in the replicative senescence context, and a big portion of the TRF2<sup>LOW</sup>-dependent and TRF2-independent DEGs overlaps with the LMNB1 downregulation-dependent upregulated subtelomeric genes.

A comparison of RNA-seq dataset revealed that oncogene-induced senescence (OIS) also induces a subtelomeric enrichment of upregulated genes, as replicative senescence does, in chromosome ends 3q, 8q, 9q, 16q, 20q, of which 3q, 8q, and 9q overlap with the seven found in the young vs senescent RNA-seq analysis of MRC5 cells. Noteworthy, five out of seven of the chromosome ends (but not necessarily the same genes) were enriched also in aged human samples. Overall, combining all the previously-mentioned data, nine subtelomeres are significantly enriched in senescence-related upregulated genes: 1p, 8q, 9q, 11p, 16p, 16q, 19p, 20q, 22q.

Very interestingly, the enrichment of upregulated DEGs in these nine subtelomeric regions positively correlates with the presence of CCCTC-binding factor (CTCF) and the Myc-associated Zinc-finger protein (MAZ), factors that are notoriously associated with insulation and chromatin looping. Moreover, these DEGs correlate with histone markers such as H3K27me3 and H3K9Ac. On the other hand, a negative correlation exists with H3K9me2 and H3K9me3, suggesting that positively enriched DEGs are associated with facultative rather than constitutive heterochromatin. Nevertheless, the combination of several data obtained via ChIP-seq, ATAC(Assay for transposase-accessible chromatin using sequencing)-seq and Hi-C (Chromosome conformation capture technology) of primary fibroblasts and OIS cells allowed the creation of heatmaps that revealed that, despite most of the subtelomeric regions enriched in upregulated DEGs be characterized by markers of open chromatin (CTCF, MAZ, H3K27me3), on the contrary, some of them are characterized by markers of closed chromatin (H3K9me3), which suggests complex gene expression regulation at senescence (Rey-Millet et al., 2023).

**xi)** As previously mentioned, TRF2 knock-down in cultured human myotubes does not trigger DDR at telomeres, but mitochondrial dysfunction and increased ROS production (Robin et al., 2020). Surprisingly, antioxidant treatment concomitant to TRF2 downregulation induces telomeric DNA damage, suggesting that an oxidative stress-induced mechanism protects telomeres when TRF2 is absent.

Interestingly, TRF2 downregulation in myotubes causes the activation of the transcription factor Forkhead box O3 (FOXO3a – pivotal longevity factor) and its ensuing translocation into the nucleus. Moreover, downregulation of either TRF2 or FOXO3a does not elicit telomeric DDR, but the

simultaneous knock-down of both of them does it, importantly, in an ATM-dependent fashion. Noteworthy, FOXO3a knock-down upon TRF2 downregulation does not further increase the ROS levels due to the lack of TRF2. Moreover, antioxidant genes (*e.g.*, *CAT* and *SOD2*) whose expression is downregulated upon FOXO3a knock-down, are not further downregulated with the simultaneous downregulation of TRF2, suggesting that the telomere deprotection caused by the double TRF2-FOXO3a knock-down cannot be due to increased ROS levels or decreased antioxidant activities.

Confocal microscopy experiments performed with human myotubes revealed that 12% of telomeres colocalizes with FOXO3a, and upon TRF2 downregulation this percentage rises to 21%. Moreover, Co-IP assays with human muscle biopsies revealed that the binding rate of either TRF2 or FOXO3a to telomeres respectively decreases or increases with aging.

In a replicative context, such as that of BJ-HELT cells, FOXO3a downregulation does not lead to increased telomeric DNA damage, whereas TRF2 downregulation does. Noteworthy, the simultaneous downregulation of both exacerbates telomere damage. Therefore, reminiscently of myotubes, the telomere-protective function of FOXO3a is triggered by TRF2 downregulation, but TRF2 knock-down in BJ-HELT cells elicits neither an increase of FOXO3a expression nor its nuclear translocation. Importantly, the downregulation of none of the other Shelterin subunits can recapitulate the results obtained with TRF2 downregulation, when also FOXO3a is knocked down.

Interestingly, among different DNA stressors, only bleomycin (which causes ss and dsDNA damage, as well as base damage) increases the rate of telomeric DNA damage upon FOXO3a downregulation. Indeed, upon bleomycin treatment, FOXO3a translocates into the nucleus and binds to telomeres. Noteworthy, also starvation stress causes nuclear translocation of FOXO3a, but not to telomeres, differently from bleomycin treatment. Importantly, the downregulation of none of the other FOXOs existing in mammals (FOXO1, FOXO4, FOXO6) in BJ-HELT cells treated with bleomycin leads to increased telomeric DNA damage as FOXO3a downregulation does. Therefore, FOXO3a specifically protects telomeres upon ATM-eliciting DNA damage (*i.e.*, bleomycin treatment or TRF2 depletion).

Finally, mutant complementation assays revealed that the DNA binding domain of FOXO3a is dispensable for its telomere-protection role, whereas the Conserved region 2C (CR2C) is indispensable. Importantly, the CR2C domain of FOXO3a is also indispensable for telomere protection in postmitotic human myotubes downregulated for TRF2, as a double knock-down of TRF2 and FOXO3a is not rescued by the FOXO3a mutant lacking CR2C (Jacome Burbano et al., 2023).

All in all, TRF2 presents several extratelomeric roles that affect gene expression, chromatin structure and chromosome stability in both mitotic and postmitotic contexts.

### 3.2. Repressor element 1 silencing transcription factor (REST)

One of the most studied interactors of TRF2 in an extra-telomeric, neuronal-related context is the already-mentioned neuronal transcription repressor REST. Therefore, its relationship with TRF2, as elucidated by several studies, will be described hereafter.

i) More than 95% of the pre-mRNAs of human multiexon genes undergo alternative splicing, a process that allows the exclusion of one or more exons (or portions of exons) to yield an alternative mRNA product, thereby coding for a different protein isoform. Therefore, thanks to alternative splicing, one single gene can give rise to several protein products. Noteworthy, this is particularly frequent for genes involved in neuronal functions and development. Interestingly, in the murine cerebellum, TRF2 (whose pre-mRNA has ten exons) exhibits a splice variant derived by the excision of a portion of exon 7 (the bc segment) which causes a frameshift that gives rise to an earlier stop codon and premature interruption of translation. The resulting protein product is a shorter isoform lacking the DNA-binding Myb domain and the nuclear localization signal (NLS), while containing a new nuclear exclusion signal (NES). Due to its smaller size, this protein isoform is referred to as TRF2-S (Small).

Interestingly, qPCR analyses showed that the TRF2-S/TRF2 ratio is particularly high in murine brain regions such as cerebellum and cortex (10-fold and 7-fold, respectively) when compared to other tissues. Developmentally speaking, the brain protein levels of TRF2 (60 kDa) are high until embryonic day 14 (E14); thereafter, TRF2 levels decrease and TRF2-S (32 kDa) becomes the predominant isoform. Moreover, cultured cortical neurons express no TRF2, whereas they exhibit higher and higher levels of TRF2-S as long as they mature. On the contrary, cultured primary glial cells only express full-length TRF2 and no TRF2-S. Noteworthy, retinoic acid (RA) and BDNF treatment of neuroblastoma cells to induce their terminal differentiation shifts the expression of TRF2 to the TRF2-S isoform. Moreover, treatment of primary cortical neural progenitor cells (NPCs) to either maintain their self-renewal state (+FGF2, +EGF) or induce their differentiation (+BDNF, -FGF2, -EGF) showed that, while NPCs only express TRF2, the differentiated neurons mainly express TRF2-S.

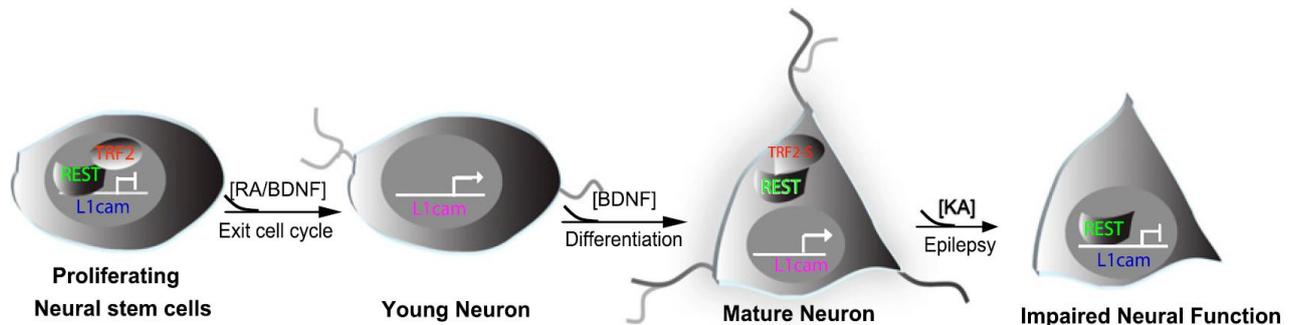
Repressor element 1 silencing transcription factor (REST) is the most notorious transcriptional repressor of neuronal genes, which is degraded via the ubiquitin-proteasome system upon NPCs differentiation. Indeed, at day-in-vitro (DIV)5 of young neurons, REST is barely detectable, as are TRF2 and TRF2-S. Quite surprisingly, REST levels increase as long as neurons mature. Interestingly, REST increase is accompanied by TRF2-S increase, together with the augmented expression of other markers of mature neurons (*e.g.*, Synaptophysin and Postsynaptic density protein 95). Cell

fractionation analysis showed increased REST and TRF2-S cytoplasmic levels between DIV7 and DIV13, whereas nuclear REST levels decreased and stayed low during neuronal maturation. Importantly, bimolecular fluorescence complementation (BiFC) analysis of primary cortical neurons showed cytoplasmic colocalization of REST and TRF2-S, whereas TRF2–REST foci were concentrated in the nuclei. Moreover, co-transfection of HeLa cells with TRF2-S mutants allowed to discover that the TRFH dimerization domain of TRF2-S is pivotal for the TRF2-S–REST interaction, and the NES sequence is fundamental for their cytoplasmic localization.

Microscopy analysis of murine cortical coronal sections showed that newly generated neurons expressing high levels of young neuronal makers such as TUJ1 and Microtubule-associated protein 2 (MAP2), but no markers of mature neurons such as L1 cell adhesion molecule (L1cam) and NeuN, exhibit no evident TRF2-S–REST colocalization. On the other hand, mature neurons (E14) express also high levels of L1cam and NeuN, which correlate with strong TRF2-S–REST coimmunoreactivity in the cytoplasm. On the same line, proliferative Ki67<sup>+</sup> neuronal progenitors also exhibit little TRF2-S–REST immunoreactivity, whereas young neurons express more and more colocalized TRF2-S and REST in the cytoplasm as long as they mature. Therefore, in cortex development, TRF2-S and REST expression patterns coincide in space and time.

The expression of the Glutamate receptor subunit 2 (GluR2), a subunit of the major excitatory glutamate receptor, is under the control of REST. In excitotoxic conditions, REST accumulates in the nucleus and inhibits GluR2 expression. Indeed, the ectopic overexpression of TRF2 and REST in cultured neurons decreases GluR2 levels, whereas overexpression of TRF2-S and REST enhances GluR2 immunoreactivity in soma and neurites. Moreover, the seizure-inducing excitotoxin Kainic acid (KA) drives REST overexpression and TRF2-S downregulation, which correlates with the decreased expression of REST target genes, including L1cam and GluR2. Remarkably, the adeno-associated viruses(AAV)-mediated expression of TRF2-S counteracts REST nuclear accumulation and gene repressor activity. Noteworthy, the increase in REST levels upon KA treatment derives from protein stabilization rather than increased mRNA transcription. Moreover, CHIP analysis revealed that KA induces increased REST occupancy in eleven out of the twenty-six RE1 DNA binding-sites of REST, which accordingly causes the decreased expression of the corresponding genes (*e.g.*, L1cam and GluR2) as measured via Western blotting. Furthermore, ectopic TRF2-S overexpression upon KA treatment decreases REST binding to RE1 sites, as corroborated by the increased mRNA expression of the REST-repressed genes.

Overall, the switch from nuclear TRF2 to cytoplasmic TRF2-S expression, due to alternative splicing of TRF2 pre-mRNA, controls the induction of neuronal maturation and the maintenance of neuronal traits via cytoplasmic retention of TRF2-S-bound REST and the ensuing expression of REST-repressed neuronal genes (P. Zhang et al., 2011).



**Figure 31. Kinetics of REST and TRF2/TRF2-S expression with respect to neuronal maturation**

REST and TRF2 levels, initially high and localized into the nucleus in primary murine neural stem cells (NSCs) from the cortex, decrease upon NSCs differentiation into young neurons. Over time, the maturing neurons restart to express REST and the alternative splicing-dependent TRF2-S isoform (but not TRF2), whose complex is retained in the cytoplasm, thereby allowing for neuronal gene expression. Upon excitotoxic stress (KA treatment), TRF2-S levels decrease while “free” REST reenters the nucleus suppressing neuronal gene expression and, therefore, impairing neuronal function (Zhang et al., 2011).

ii) Several cancer cell lines exhibit abundant levels of REST. Colon carcinoma and hepatocellular carcinoma cell lines show significantly increased levels of REST compared to non-tumoral cognate tissues.

siRNA-mediated downregulation of REST in the HCT116 (human colon cancer) cell line drastically reduces cell viability due to increased apoptosis, as revealed by Annexin V expression. Interestingly, REST knock-down correlates with increased levels of p53, phospho-p53,  $\gamma$ H2AX, and phospho-CHK2, but decreased levels of TRF2, all of which suggests increased DDR. Noteworthy, in the same context, telomere length is also significantly affected by REST downregulation.

Among several genotoxic stresses,  $H_2O_2$  causes a time- and concentration-dependent decrease in REST levels with a concomitant downregulation of TRF2 in several cell lines, suggesting that oxidative stress has a specific role in REST downregulation. Unsurprisingly, treatment with the ROS scavenger N-acetylcysteine (NAC) restores REST levels and TRF2 levels to a similar extent. Interestingly, qPCR analysis revealed that the regulation of the expression of REST and TRF2 upon oxidative stress is at the post-transcriptional and transcriptional level, respectively.

The decreased protein levels of REST upon  $H_2O_2$  treatment of HCT116 cells depend on proteasome activation as the inhibition of the proteasome by the specific inhibitor MG132 rescues REST protein levels. Indeed, the polyubiquitination of REST is dramatically increased upon  $H_2O_2$  treatment.

The overexpression of REST in both HCT116 and HeLa cells restores the TRF2 levels suppressed by H<sub>2</sub>O<sub>2</sub> treatment. Interestingly, TRF2 mRNA expression mirrors its protein levels, suggesting that REST regulates TRF2 expression at the transcriptional level. Importantly, the overexpression of either REST or TRF2 inhibits the phosphorylation of CHK2 and H2AX upon oxidative stress, but the concomitant REST overexpression and TRF2 downregulation leads to DDR activation following H<sub>2</sub>O<sub>2</sub> treatment. On the same line, either REST or TRF2 overexpression in HCT116 cells exposed to H<sub>2</sub>O<sub>2</sub> lead to decreased cell death, which is instead increased upon TRF2 downregulation via siRNA. Therefore, the REST-induced TRF2 expression is pivotal to protect cancer cells from DDR activation and the ensuing cell death upon oxidative stress.

REST downregulation in HCT116 and HeLa cells leads to both decreased TRF2 mRNA and protein levels, suggesting that REST is responsible for TRF2 downregulation upon oxidative stress. Moreover, REST downregulation increases phospho-CHK2 and  $\gamma$ H2AX levels which are brought back to basal levels by the concomitant overexpression of either REST or TRF2. Importantly, the apoptosis induced by REST downregulation is avoided by TRF2 overexpression, supporting the idea that the REST decrease upon oxidative stress boosts the DDR by downregulating TRF2 expression (Kwon et al., 2013).

**iii)** Further research revealed that the mRNA expression of TRF2 does not change upon human embryonic stem cell (hESCs) differentiation into neural progenitor cells (NPCs). Instead, the protein levels of TRF2 increase dramatically upon neural differentiation of H1 and H7 hESC lines and are maintained high thereafter. Therefore, upon differentiation of hESCs into NPCs, TRF2 is regulated at the post-transcriptional level. Interestingly, TRF2 localization in NPCs is no more solely restricted to telomeres but spread across the nucleus.

The differentiation of hESCs into neural, hepatic or fibroblast lineages revealed that TRF2 upregulation only occurs in NPCs, but not in hepatocyte progenitors or fibroblasts, suggesting that TRF2 plays a specific role in neural commitment. Moreover, upon further differentiation of NPCs into postmitotic neurons or glial cells, TRF2 levels decrease again.

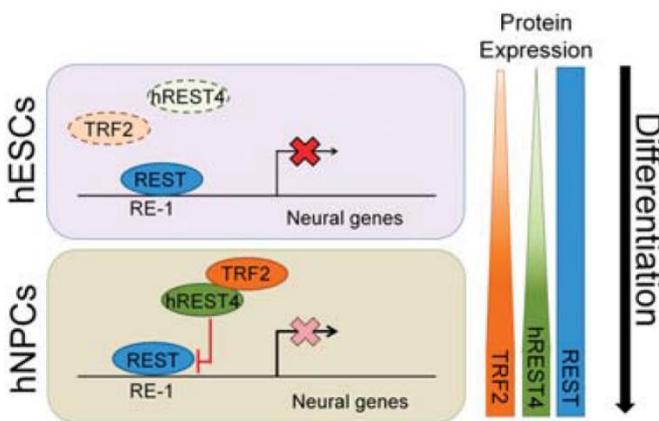
Unsurprisingly, in hESCs telomeres are long and stable due to high expression of telomerase. Upon differentiation into NPCs, telomerase expression is downregulated and telomeres shorten as long as cells divide. Nevertheless, at later stages telomerase expression is rescued and telomeres are lengthened, according to a dynamic which does not correlate with that of TRF2 expression. Therefore, telomere length and telomerase expression are independent of TRF2 levels.

In line with the increased levels of TRF2 in NPCs as compared to hESCs, the lentivirus-mediated TRF2 overexpression in hESCs leads to their differentiation into NPCs and formation of colonies with neural rosette-like structures with bipolar neural progenitors. qPCR analysis revealed a concomitant downregulation of pluripotent markers such as *Oct4* and *Nanog*, and an upregulation of neural markers such as *Sox1*, *Mash1* and *Snap25*. Noteworthy, part of the TRF2-overexpressing cells can express neural lineage markers despite being cultured in hESC culture conditions. Moreover, when conditions are switched to NPC culture, TRF2-overexpressing cells can differentiate faster than control cells, expressing the NPC marker Nestin, but not the hESC marker Oct4. Importantly, the fact that telomere shortening occurs later than neural differentiation, plus the fact that  $\gamma$ H2AX levels are not increased, rules out a role for telomeres and DDR in hESCs differentiation into NPCs.

Importantly, TRF2 downregulation upon hESCs differentiation into neurons yields much less TUJ1(neuronal marker)-positive neurons than control cells with normal levels of TRF2. Moreover, neural differentiation of hESCs downregulated for TRF2 leads to lower numbers of NPCs, which in turn results in less neurons upon growth factor withdrawal. Furthermore, TRF2 downregulation in NPCs leads to decreased mRNA expression and protein levels of the NPC marker Sox2. Morphologically speaking, the downregulation of TRF2 in NPCs that further differentiate into either neurons or glia causes a flatter shape characterized by fewer outgrowths, as well as a decreased expression of TUJ1 and GFAP (glial marker), respectively. Importantly, even in this context, TRF2 downregulation has no effect on telomere length and chromosome stability. Therefore, TRF2 expression is pivotal for neural gene expression and its downregulation further impairs NPCs differentiation into neurons and glia, with no overt effect on telomeres and chromosome stability. Surprisingly (given its role in neural gene repression), REST expression does not change upon hESCs differentiation into NPCs. Moreover, REST levels do not change neither upon TRF2 overexpression in hESCs and HEK293T cells or upon its downregulation in NPCs. Interestingly, differently from hESCs, m(mouse)ESCs differentiation into NPCs is accompanied by a decrease in REST levels, raising the question as to how REST-mediated repression of neural genes is alleviated during hESCs differentiation into NPCs. As a matter of fact, a variant of REST referred to as human(h)REST4 was discovered. This variant was named after the rodent ortholog REST4, which is known to counteract the repressive role of REST. Remarkably, the ectopic expression of hREST4 in hESCs increases the rate of cell differentiation into NPCs even under hESC culture conditions. Moreover, upon differentiation culture conditions, hREST4 overexpression leads to quicker conversion into NPCs, suggesting an antagonism with REST. Interestingly, TRF2 overexpression in hESCs increases hREST4 protein levels,

whereas they are decreased upon TRF2 downregulation in NPCs. Furthermore, the overexpression of hREST4 in NPCs downregulated for TRF2 rescues the impaired neural gene expression and boosts the differentiation into either neurons or astrocytes.

Co-IP experiments revealed that hREST4, but not REST, interacts with TRF2 via the direct binding of TRF2 with the TRF2-core-binding-motif [Y/F]xL of hREST4 (included in 13 amino acids at the C-terminus of the protein), which is hREST4-specific and not present in REST. Moreover, high levels of TRF2 inhibit the ubiquitination of hREST4 preventing its proteasome-mediated degradation. Overall, increased levels of TRF2 in hESCs stabilize hREST4 impeding its degradation and thereby promoting the differentiation into NPCs and paving the way for their further differentiation into either neurons or glial cells (Ovando-Roche et al., 2014).



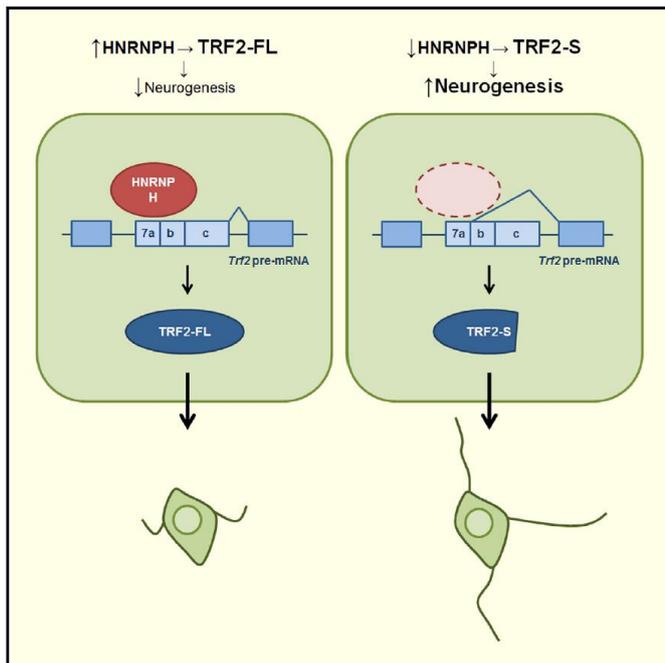
**Figure 32. Expression kinetics and interactions of TRF2 and REST/hREST4 upon neural differentiation of hESCs**  
Upon differentiation of hESCs into hNPCs, REST levels are unchanged. Instead, the levels of TRF2 and hREST4 similarly increase as long as cells mature into neural progenitors. Notably, TRF2 stabilizes hREST4 which competes with REST for binding to neural genes promoters, thereby leading to neural differentiation (Ovando-Roche et al., 2014).

**iv)** Pull-down experiments with the biotinylated TRF2-S pre-mRNA exon 7 plus flanking regions revealed a number of RNA-binding proteins (RBPs) that selectively bind to it. Experiments with rat pheochromocytoma PC12 cells showed that, among these RBPs, the knock-down of two of them, namely Heterogeneous nuclear ribonucleoprotein H1 and H2 (HNRNPH1 and HNRNPH2), increases the expression of TRF2-S.

RBP immuno-precipitation (RIP) followed by mRNA amplification analysis starting from PC12 cells extracts reveals enrichment of TRF2-S mRNA in HNRNPH1 and HNRNPH2 pull-downs. Moreover, western blotting shows that upon downregulation of either HNRNPH1 or HNRNPH2, TRF2-S and TRF2 full-length (-FL) levels are respectively increased and decreased.

Interestingly, the *in vitro* differentiation of rat cortical neurons is accompanied by a gradual decrease in HNRNPH1/2 protein levels, but no change in their mRNA expression. Concomitantly, TRF2-S and TRF2-FL protein levels respectively increase and decrease. Corroborating these results, the Nerve growth factor(NGF)-mediated differentiation of PC12 cells causes a decrease in HNRNPH1/2 protein levels, suggesting an inhibitory role for HNRNPH proteins with respect to neuronal differentiation.

The western blot analysis of PC12 cells expressing Cas9 and a guide RNA targeting *Hnrnp2*, confirms the downregulation of HNRNPH2 levels and shows an increased TRF2-S/TRF2-FL ratio. Importantly, *Hnrnp2*-crisped-out cells exhibit faster differentiation when compared to control cells at DIV6 (differentiation implying the presence of at least one neurite twice as long as the diameter of the cell body), confirming the idea that HNRNPH activity inhibits neuronal differentiation (Grammatikakis et al., 2016).



**Figure 33. Alternative splicing of *Trf2* pre-mRNA and neuronal differentiation**

The RNA-binding protein HNRNPH impedes the expression of *Trf2-S* pre-mRNA, which would skip part of exon 7, yielding a shorter transcript coding for a shorter isoform of TRF2 (*TRF2-S*). Increased *TRF2-S* protein levels correlate with rat NPCs neuronal differentiation and neurogenesis (Grammatikakis et al., 2016).

v) ChIP-qPCR experiments with human cancer cell lines (HT1080 and MDA-MB-231) revealed that TRF2 binds the promoter of *p21* (between position -1029 and -1178 from the transcription start site). Moreover, the downregulation or upregulation of TRF2 respectively increases or decreases *p21* promoter activity. Importantly, when either the basic or the Myb domain of TRF2 (or both) are absent, TRF2 cannot repress *p21* expression. Noteworthy, the same results can be recapitulated in MRC5 primary lung fibroblasts.

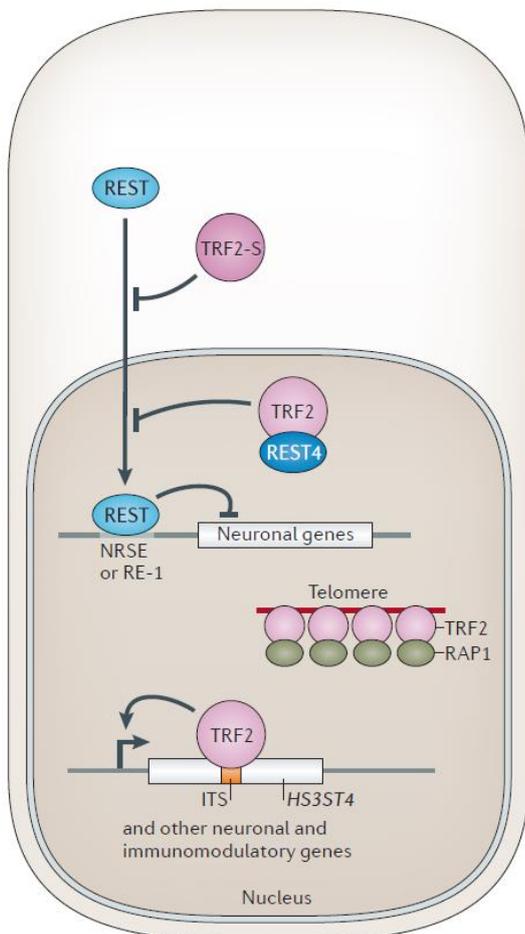
Further ChIP experiments showed that HT1080 cells overexpressing TRF2 have much less transcriptional activation markers, such as H3K4me and H3K4me2, bound to the *p21* promoter. Indeed, TRF2 overexpression causes the increased recruitment of Lysine-specific demethylase 1 (LSD1) to the *p21* promoter, which demethylates H3K4me and H3K4me2. Moreover, Co-IP experiments revealed that TRF2 interacts with LSD1. As expected, since LSD1 is a component of the transcriptional repression complex REST-coREST-LSD1, TRF2 downregulation decreased the localization of all the three subunits to the *p21* promoter.

Importantly, the *p21* promoter locus forms G-quadruplex (G4) motifs, whose integer structure is pivotal for TRF2 binding.

Interestingly, though the expression of *p21* is notoriously dependent on p53 activation, TRF2 downregulation in HT1080 cells causes increased *p21* expression (both mRNA and protein) even when p53 is simultaneously downregulated.

Moreover, drug-induced DNA damage in the same cell lines cannot induce *p21* expression when TRF2 is ectopically overexpressed. Importantly, also in this case, TRF2 requires its basic and Myc domains for repressing the expression of *p21*.

Furthermore, the simultaneous use of a competitor ligand (360A) for TRF2 binding to G4 structures rescues *p21* expression in these TRF2-overexpressing cells that undergo drug-induced DNA damage. On the other hand, vector-transformed control cells increasingly stop at G2/S phase upon drug-induced DNA damage. Moreover, the combined treatment with the DNA damage-inducing drug (doxorubicin) and the G4 competitor ligand 360A increased the rate of G2/S arrest even for cells overexpressing TRF2. Noteworthy, thirty-eight (including 360A) out of fifty well-known G4 binding ligands can recapitulate the above-mentioned results (Hussain et al., 2017).



*Figure 34. Resume of the main extratelomeric roles of TRF2 in a neuronal context*

*In neural progenitor cells (NPCs), TRF2 can bind to and stabilize hREST4 in the nucleus, which inhibits REST activity and unleashes neuronal gene expression, leading to neuronal differentiation. Alternatively, the short isoform of TRF2, TRF2-S, can bind and sequester REST in the cytoplasm, achieving the same results. Moreover, TRF2 can bind to interstitial telomeric sequences (ITS) located next to promoters or inside gene introns, potentially affecting the expression of these genes (Ye et al., 2014).*

*The description of our champion is finally complete  
it is now time to portray its feared enemy  
may our hero cause its defeat*

## 4. Ataxia telangiectasia mutated

As already anticipated above, Ataxia telangiectasia mutated (ATM) is a pivotal target of TRF2 with respect to DDR control (Denchi & De Lange, 2007). Therefore, the roles of this kinase endowed with a plethora of different targets and functions will be discussed hereafter.

Ataxia telangiectasia pathology (from now on referred to as A-T) is a rare multifaceted autosomal recessive disease characterized by cerebellar degeneration, ataxia (impaired motor functions), thymus and gonads atrophy, immunodeficiency, radiation sensitivity and increased susceptibility to cancer development (Rothblum-Oviatt et al., 2016). The defective gene responsible for A-T was identified in 1995 by an international consortium led by Yosef Shiloh (Savitsky et al., 1995). In A-T, the activity of the protein kinase Ataxia telangiectasia mutated (ATM) is lost due to missense or nonsense mutations in its gene *Atm* (Shiloh & Ziv, 2013) (Concannon & Gatti, 1997). ATM belongs to the Phosphatidylinositol 3-kinase-related kinases (PIKKs) protein family, whose C-terminal domain is structurally related to Phosphatidylinositol 3-kinases (Lovejoy & Cortez, 2009) (Paull, 2015). Many of these enzymes, such as ATM, Ataxia telangiectasia and Rad3-related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), participate to the DNA damage response (Jackson & Bartek, 2009). ATM has several hundreds of identified substrates and its activity consists in the phosphorylation of serine and threonine residues next to glutamine (SQ-TQ motif) (Matsuoka et al., 2007) (Bensimon et al., 2010) (Schlam-Babayov et al., 2021). ATM is activated by the formation of double strand DNA breaks (DSBs), which leads to the phosphorylation of several proteins involved in DNA repair, cell cycle check points and apoptosis (Lavin, 2008). Despite for many years ATM was believed to have a role restricted to DSBs response, recent works unveiled a broader activity for this protein ranging from DNA damage response to lesions other than DSBs, to responses to endogenous sources of reactive oxygen species (ROS) (Paull, 2015) (Zhang et al., 2018) (J. Lee et al., 2018) (Z. Guo et al., 2010) (Cosentino et al., 2011) (J. H. Lee & Paull, 2020). Importantly, these pathways have major effects on mitochondrial and protein homeostasis, and on the regulation of transcription and translation (J. Lee et al., 2018) (Sharma et al., 2014) (D'Souza et al., 2013) (Tresini et al., 2016) (Sarkar et al., 2021).

### *ATM activation*

The DNA damage-related ATM activation can occur via two different pathways:

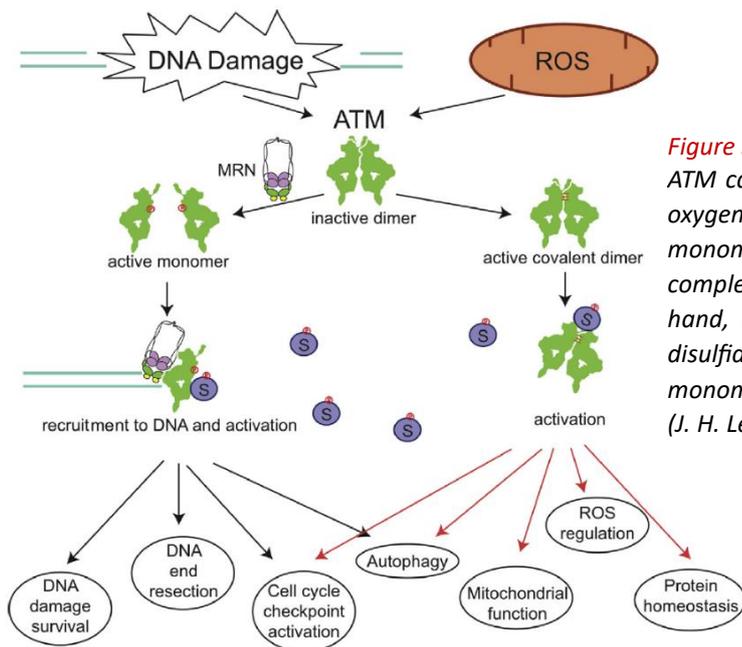
i) The MRN complex (composed of MRE11, RAD50 and NBS1) binds to DSBs, recruits the ATM homodimer and induces its monomerization which immediately follows ATM autophosphorylation. The monomer kinase can then interact with the DNA ends and its substrates (Paull, 2015) (Lee et al., 2005) (Uziel et al., 2003). Mutations in ATM impair the physical interaction with MRN (J. Lee et al., 2018) (Zhou et al., 2017), and post-translational modifications modulate its activity. For example, the acetylation of Lys3016 by the acetyltransferase TIP60 elicits ATM autophosphorylation at Ser1981, which is considered a hallmark of ATM activation in response to DSBs (Sun et al., 2005) (Sun et al., 2007). Indeed, Ser1981 is immediately phosphorylated upon DSBs formation and is necessary for ATM monomerization in human cells (Kastan & Bakkenist, 2003). Nevertheless, other ATM residues are also autophosphorylated upon DNA damage; namely Ser367, Ser1893, Ser2996, and Thr1885 (Kozlov et al., 2011) (Kozlov et al., 2006).

ii) Alternatively, ATM can be activated by single strand DNA breaks (SSBs) (Khoronenkova & Dianov, 2015), Topoisomerase cleavage complexes (TOPcc) (C. P. Lin et al., 2009), splicing intermediates (Tresini et al., 2016) and R-loops (Sordet et al., 2009) (Sordet et al., 2010). Noteworthy, postmitotic neurons were used in several of these studies, suggesting that the conversion of these lesions into DSBs, which occurs during the S phase of the cell cycle of mitotic cells, is not necessary to elicit ATM activation.

Importantly, ATM can also be directly activated by oxidative stress which causes the formation of disulfide bonds between its two monomers, independently of DSBs formation and recruitment by MRN (Z. Guo et al., 2010). Disulfide bonds were mapped at Cys2991 in the PIKK Regulatory Domain (PRD) of the two monomers, which regulate ATM activation by oxidative stress. Indeed, mutations in this domain [*e.g.*, C2991L (Z. Guo et al., 2010)] impair the H<sub>2</sub>O<sub>2</sub>-induced ATM activation in human cells *in vitro* (J. Lee et al., 2018). After ATM activation, the disulfide bonds are finally reduced, leading to an ATM inactive state, by a Thioredoxin I-dependent reaction (Zhang et al., 2018).

Interestingly, independently of DNA damage, ATM can also be activated in an Aurora B kinase-dependent fashion during normal mitosis: ATM phosphorylation at residue Ser1403, and ATM-mediated phosphorylation of the kinetochore protein Bub1 at Ser314 are both necessary for proper mitosis to take place (Yang et al., 2011). Moreover, recent research revealed that ATM is activated upon metabolic stress due to glycolysis inhibition and its depletion leads to impaired mitochondrial Ca<sup>2+</sup> homeostasis, increased ROS production and defective mitochondrial dynamics (Yeo et al., 2021). Furthermore, ATM is activated by ATP depletion via a mechanism involving ROS, leading to

the ATM-mediated activation of the transcription factor NRF1 and the ensuing expression of nuclear-encoded mitochondrial genes with protective outcomes (Chow et al., 2019).



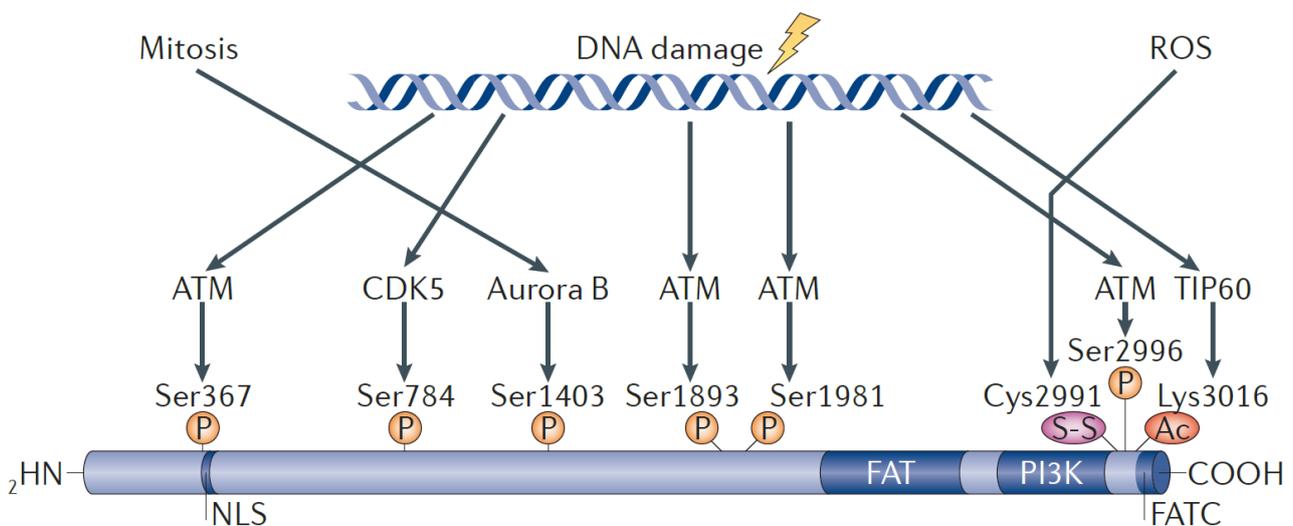
**Figure 35. ATM activation**

ATM can be activated by either DNA damage or reactive oxygen species (ROS). Upon DNA damage, the ATM dimer monomerizes following the interaction with the MRN complex and autophosphorylation at Ser1981. On the other hand, ROS activate ATM via the formation of covalent disulfide bonds at the Cys2991 residues of the two monomers, which activates ATM in its homodimeric form (J. H. Lee & Paull, 2020).

### ATM structure

Cryo-electron microscopy recently allowed to determine the structure of ATM, which revealed that the dimer contains Huntingtin, elongation factor 3, A subunit of protein phosphatase 2A, TOR1 (HEAT – antiparallel helices linked by a flexible loop) repeats that hold the two auto-inhibited monomers in a butterfly-like shape with an extensive dimerization interface (Yates et al., 2020) (Baretic et al., 2017)(X. Wang et al., 2016) (Lau et al., 2016) (Xin et al., 2019). The interaction surface can be considered as split into two parts: upper and lower. In the upper part, the PIKK regulatory domain (PRD) and LST8-binding element (LBE) of the FATC LBE activation loop and PRD (FLAP) region in the kinase domain of one monomer interact with the FLAP-binding element (FLAP-BE) region of the other monomer causing the PRD helices to obstruct the access to the active site. On the other hand, the lower part is where the interaction between the Middle-FRAPP-ATM-TTRAP (M-FAT) domains of the two monomers takes place (Yates et al., 2020). Therefore, these extensive surface of interaction allows for the inhibition of the kinase activities of the two monomers. Human ATM was reported to have two diverse dimeric conformations. On the one hand, a “closed” symmetrical dimer and, on the other hand, an “open” asymmetrical one (Baretic et al., 2017). Noteworthy, in the open dimer, the upper part interaction between the homodimers is partially lost, suggesting that this state might be ready for eventual interactions with putative substrates. It is evident from the localization of the

known regulatory sites of ATM (in or near the PRD domain) that post-translational modifications could actually affect the kinase-activating rearrangement of the PRD domain. Indeed, the autophosphorylation sites of ATM located at Ser1981 and Ser2996, and the disulfide bond formation site located at Cys2991 (pivotal for ATM activation by ROS) are located in disordered loops nearby PRD (Baretic et al., 2017). Moreover, the residues Arg2579 and Arg2580, that are pivotal for ATM binding to MRE11-RAD50, are also situated in a disordered loop of the HEAT repeats domain (HRD) in proximity of the disordered loops adjacent to PRD. As previously mentioned, ROS lead to the formation of multiple disulfide bonds between the two monomers of ATM, which culminates in its activation (Z. Guo et al., 2010). Therefore, a speculation is that the oxidation of Cys2991 could cause the upper part interaction of the monomers to get lost, thereby freeing the active site of ATM and allowing its kinase activity to take place. Interestingly, in this context, ATM kinase activity is independent of the phosphorylation state of S1981 and the interaction with MRN (Pizzamiglio et al., 2020).



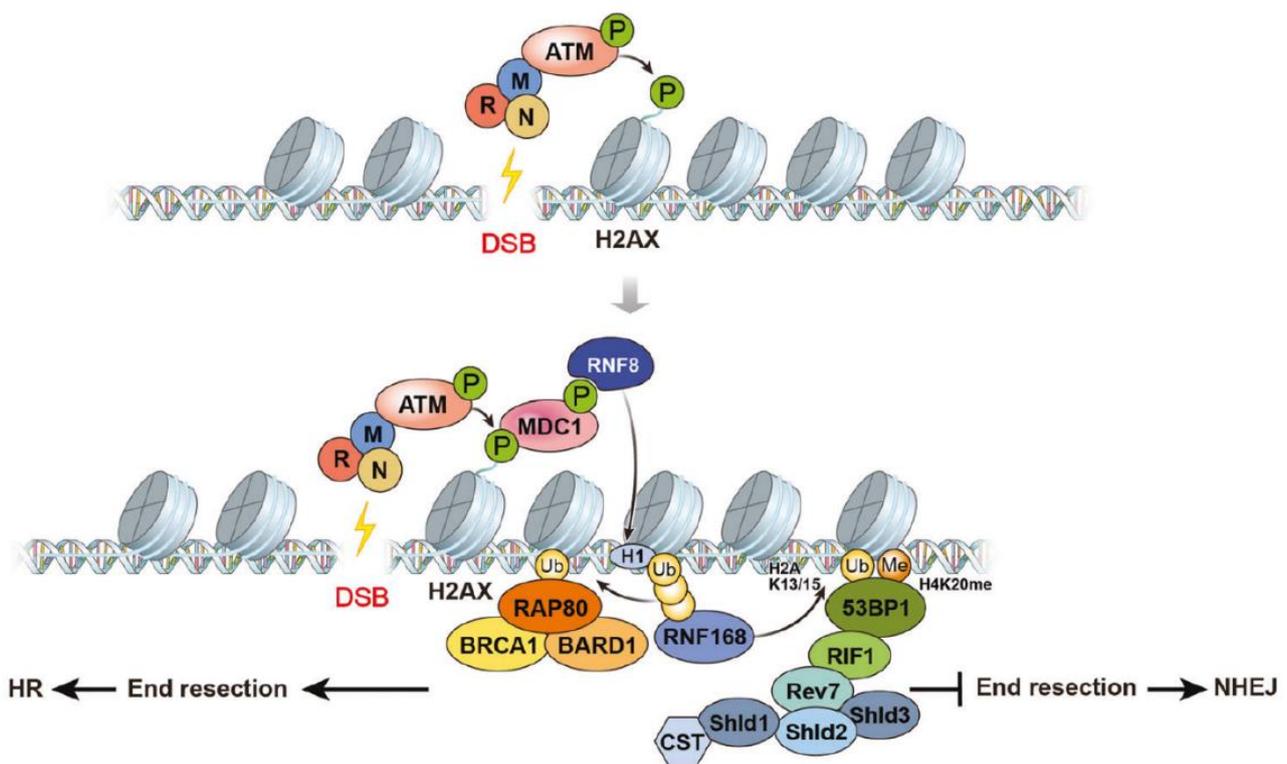
**Figure 36. ATM structure**

Linear representation of ATM. Highlighted are the major domains, the residues undergoing post-translational modifications and their upstream inducers. Among them, the pivotal pro-activity autophosphorylation target Ser1981, and the Cys2991 residues, whose ROS-mediated oxidation causes disulfide bond formation between the two ATM homodimers and protein activation [Ac, acetylation; FATC, FAT carboxy-terminal; NLS, nuclear localization sequence; P, phosphorylation; ROS, reactive oxygen species; S-S, disulfide bridge] (Shiloh and Ziv, 2013).

### ATM and DNA repair

Genotoxic stress is a constant challenge for our genome. This includes replication-dependent lesions (Tubbs et al., 2017), ROS (Aditi & McKinnon, 2022), and exogenous insults like ionizing radiations and

radiomimetic agents, resulting in the formation of SSBs and DSBs (Santivasi & Xia, 2014) (Chatterjee & Walker, 2017). Interestingly, cells derived from A-T individuals are hypersensitive to ionizing radiations and radiomimetic agents, but not to other kinds of genotoxic agents such as UV radiations, replication inhibitors and alkylating agents (the latter ones mainly causing SSBs), meaning that ATM primarily acts to repair DSBs (Shiloh & Ziv, 2013) (Paull, 2015). In eukaryotic cells, ATM is also the major catalyzer of the phosphorylation of H2AX on Ser139 (resulting in  $\gamma$ H2AX), a pivotal event in the sequence of biochemical reactions that characterize DDR (Stiff et al., 2004) (Caron et al., 2015) (Burma et al., 2001). Upon H2AX phosphorylation by ATM, Mediator of DNA damage checkpoint 1 (MDC1) binds to  $\gamma$ H2AX providing a docking platform for other DDR factors (Coster & Goldberg, 2010), including the MRN complex itself, eliciting a positive feedback loop (L. Wu et al., 2008) (Melander et al., 2008) (Spycher et al., 2008) (Min et al., 2022).



**Figure 37. ATM-driven DSBs repair**

Upon DNA damage, DSBs are detected by the MRN complex which is in turn bound by the ATM homodimer. The interaction with MRN induces ATM monomerization due to its autophosphorylation at Ser1981. The now active ATM monomer phosphorylates histone H2AX at Ser139. Phosphorylated H2AX ( $\gamma$ H2AX) recruits MDC1 and further propagates  $\gamma$ H2AX signaling at DSB sites. MDC1 is in turn phosphorylated by ATM, and its phosphorylated residue recruits the ubiquitin ligase RNF8. The RNF8-UBC13 complex promotes Lys63 polyubiquitination of H1, which is recognized by ubiquitin ligase RNF168. RNF168 initially monoubiquitinates H2A at Lys13/15 (K13/15) and amplifies polyubiquitin chains. K13/15-monoubiquitinated H2A and H4K20me are recognized and bound by 53BP1, which further recruits RIF1 and the Shielding complex to promote NHEJ. Alternatively, during replication, the RNF168-mediated polyubiquitin chains recruit RAP80 and the BRCA1/BARD1 complex, which promotes HR (Min et al., 2022).

### *ATM-mediated double strand break repair*

i) When a double strand break (DSB) is repaired via homologous recombination (HR – the DNA repair process that makes use of the homologue sequence of the sister chromatid as a template to faithfully repair the DNA lesion), the DNA ends are resected to form 3' single-stranded DNA overhangs that are necessary for the strand invasion of the integer chromatid mediated by RAD51 (Kowalczykowski, 2015). C-terminal-binding protein 1(CtBP)-Interacting Protein (CtIP – a 5' endonuclease), in concert with the MRN complex, is pivotal for the initiation of the resection process in mammalian cells (Kowalczykowski, 2015) (Sartori et al., 2007). CtIP is recruited to DSB following damage sensing by NBS1 (Subunit of the MRN complex) and ATM (You et al., 2010) that phosphorylates CtIP on Thr859 to elicit its resection activity (H. Wang et al., 2013). Afterwards, the 5' exonucleases Exonuclease 1 (EXO1) and DNA2 extend the 3' overhangs into much longer ssDNA portions that are needed for the recruitment of Replication protein A (RPA – major ssDNA binder in eukaryotic cells) and the formation of the RAD51 filament (*i.e.*, a ssDNA coated by RAD51 proteins that catalyze the homologous pairing and DNA strand exchange) (Symington, 2016). ATM phosphorylates several of the proteins involved in this process; *e.g.*, EXO1 (Bolderson et al., 2010), Bloom syndrome protein (BLM – a RecQ family helicase) (Ababou et al., 2000), Breast cancer type 1 susceptibility protein (BRCA1 – a tumor suppressor protein) (Cortez et al., 1999) (Xu et al., 2002) (Gatei et al., 2002), and Partner and localizer of BRCA2 (PALB2) (Ahlskog et al., 2016). Interestingly, in a negative feedback fashion, ATM also phosphorylates MRE11 (Subunit of the MRN complex) leading to reduced MRE11 binding to DNA and decreased EXO1-dependent resection (Kijas et al., 2015) (Di Virgilio et al., 2009). Some of the targets of ATM, including MRE11, CtIP and EXO1 are also phosphorylated by Cell cycle-dependent kinases (CDKs). The DNA end resection occurs properly only in the S and G2 phases of the cell cycle [the cell cycle phases to which HR is restricted] (X. Zhao et al., 2017) (Aylon et al., 2004) (Ferretti et al., 2013), and in fact CDKs control on MRE11 and NBS1 promotes resection (Simoneau et al., 2014) (Falck et al., 2012) (Buis et al., 2012). Moreover, also EXO1 resection activity is boosted when it is phosphorylated by CDK1 and CDK2 (Tomimatsu et al., 2014).

ii) On the other hand, the non-homologous end-Joining (NHEJ) is also under the control of ATM. The main factors involved in NHEJ, from DNA damage recognition to DNA processing and ligation are several: Ku70/80, DNA-PKcs, DNA ligase 4, XRCC4, XRCC4-like factor (XLF), PAXX, Cell cycle regulator of non-homologous end-joining (CYREN), Artemis, DNA Pol $\mu$  and DNA Pol $\lambda$  (Chang et al., 2017). In mammalian cells, one of the first complexes to localize to DSB is Ku70/80, which in turn recruits DNA-PKcs (G. Yang et al., 2018) (Britton et al., 2013) (Jette et al., 2015). Among the above-mentioned

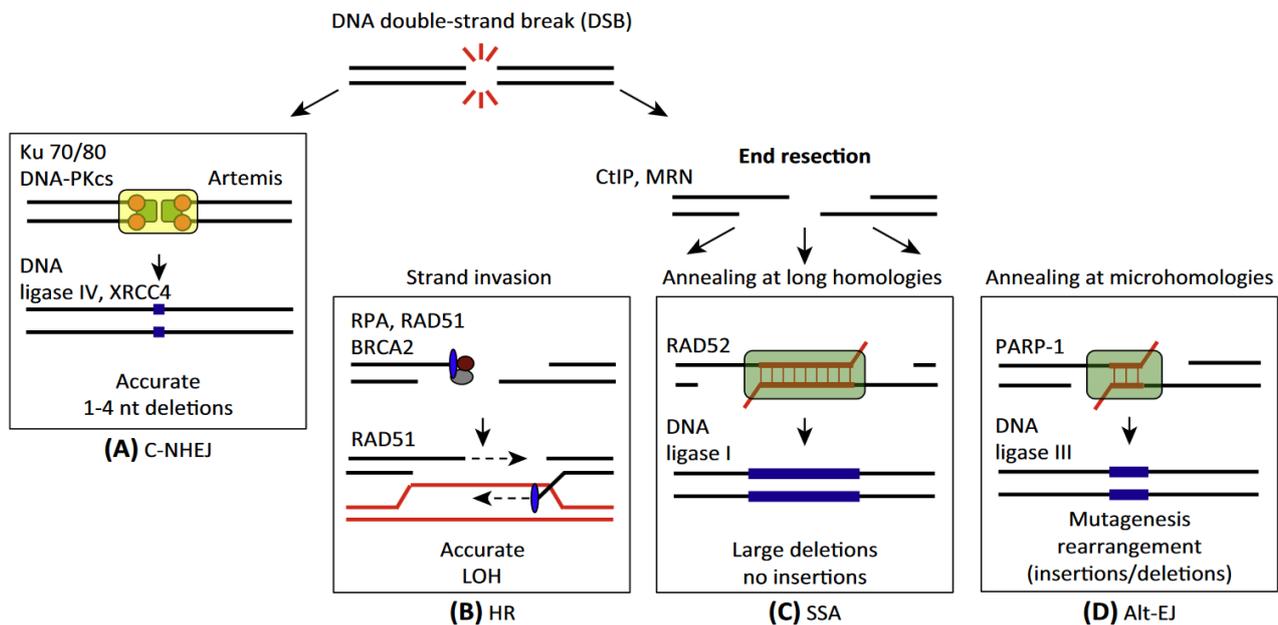
proteins, DNA-PKcs (B. P. C. Chen et al., 2007), Artemis (Riballo et al., 2004) (Goodarzi et al., 2006), XRCC4, XLF (Imamichi et al., 2014) (Yu et al., 2008), and Pol  $\lambda$  (Sastre-Moreno et al., 2017) are all phosphorylated by ATM, which elicits NHEJ. DNA repair is promoted by the ATM-mediated phosphorylation of DNA-PKcs (B. P. C. Chen et al., 2007). On the contrary, DNA-PK-mediated phosphorylation of ATM inhibits the interaction between ATM and MRE11-RAD50, therefore providing ATM with a negative feedback with respect to the DNA damage response (Zhou et al., 2017).

NHEJ is also promoted by the large scaffold protein TP53-binding protein 1 (53BP1), which is recruited to chromatin following ubiquitination and methylation of proteins at DSB sites (Mirman & de Lange, 2020). The N-terminal domain of 53BP1 has more than two dozen phosphorylation sites that are target of ATM activity, which results in attenuated DNA end resection (Bothmer et al., 2011). Moreover, the interaction of 53BP1 with proteins such as RAP1-interacting factor 1 homologue (RIF1) and PAX-interacting protein 1 (PTIP), especially in the absence of pivotal HR factors such as BRCA1 (Bunting et al., 2010), further promotes NHEJ (Bothmer et al., 2011) (J. R. Chapman et al., 2013) (Feng et al., 2013) (Zimmermann et al., 2013) (Virgilio et al., 2013) (Callen et al., 2013).

Interestingly, replication-induced DSBs are inappropriately joined by NHEJ in ATM-deficient cells (Balmus et al., 2019). Noteworthy, the depletion of NHEJ factors (*e.g.*, LIG4, XLF or XRCC4) in the absence of ATM avoids toxic NHEJ and chromatid fusions leading to cell death. In accordance to these findings, proteomics analysis revealed that several NHEJ factors inappropriately associate with chromatin upon TOP1 inhibitory treatment (which gives rise to replication-induced DSBs) when ATM is simultaneously inhibited (Nakamura et al., 2021). On the other hand, it is still unclear what are the ATM targets responsible for impeding the recruitment of NHEJ factors to DSBs during DNA replication (Britton et al., 2020). ATM is not pivotal for DNA end resection, but can regulate several factors involved in this process (Katsuki et al., 2020). Therefore, the ATM role during replication seems to be, on the one hand, reducing NHEJ use and, on the other hand, improving resection efficiency (via CtIP activation).

The current knowledge pictures ATM as a positive regulator of both HR and of some crucial steps of NHEJ, which sounds paradoxical seen how these two pathways are historically viewed in competition with one another (Ceccaldi et al., 2016). At any rate, recent works hint for a concerted, stepwise “pathway choice” (Deshpande et al., 2020) (Ingram et al., 2019) (Kakarougkas & Jeggo, 2014). The many roles of ATM in DSBs repair makes it difficult to dissect the mechanisms of the phenotypes in

ATM-deficient contexts. Therefore, a precise analysis of the ATM target residues and the effect of their phosphorylation is needed.



**Figure 38. The destiny of a double-strand break**

(A–D) The destiny of a DSB primarily depends on whether DNA-end resection takes place or not. When resection is not allowed, repair through canonical non-homologous end-joining (C-NHEJ) is favored (a). However, when DNA resection occurs, three alternative pathways can compete for the repair of DSBs. Indeed, there are two levels of competition for DSBs repair. First, at the stage of end-resection, C-NHEJ competes with the resection-dependent pathways. Second, once resection has occurred, (b) homologous recombination [HR], (c) single-strand annealing [SSA] and (d) alternative end-joining [alt-EJ] can compete for the repair. Notably, each of the four repair pathways leads to different genetic outcomes, i.e., loss of heterozygosity (LOH), deletions and insertions. (Ceccaldi et al., 2016).

### ATM and topoisomerase adducts

Covalent bonds between the catalytic tyrosine residue of topoisomerases and the DNA give rise to the most frequent form of blocked DNA ends. This phenomenon usually occurs transiently during the DNA incision-and-ligation cycle of topoisomerases, but can also be caused by oxidative stress and anti-topoisomerase drugs used for cancer therapy (Pommier, 2006) (Pommier, 2013) (Pommier et al., 2022). The DSBs resulting from the formation of cleavage complexes between topoisomerase 1 and the DNA (referred to as TOP1cc) during DNA transcription in postmitotic cells such as neurons and other non-mitotic human cells activate ATM (Sordet et al., 2010). Interestingly, the removal of the R-loops (i.e., the structure resulting from the invasion of the DNA duplex by the newly-transcribed mRNA via the displacement of the non-template DNA strand) that form during transcription by RNase H1 treatment diminishes  $\gamma$ H2AX signal, implying a role for R-loops in DSB formation (Sordet et al., 2009) (Sordet et al., 2010). ATM deficiency increases the levels of TOP1cc in murine astrocytes, consistent with the fact that ATM could contribute to TOP1cc removal (Alagoz

et al., 2013). TOP1cc levels are diminished by inhibition of transcription or by treatment with the ROS scavengers N-acetyl cysteine (NAC) or mannitol, suggesting that endogenous oxidative stress also plays a part in TOP1cc formation.

Elevated TOP1cc levels were measured in the cerebellum of ATM-depleted mice during embryogenesis, postnatal and adult phases (Katyal et al., 2014). Noteworthy, upon induction of DSBs in ATM-depleted cells, the ubiquitin-mediated degradation of TOP1cc by the proteasome is reduced. Interestingly, a study with a murine model of A-T showed that TOP1cc clearance depends on ATM, but not on its kinase activity (Katyal et al., 2014), whereas it relies on the activity of DNA-PKcs (Cristini et al., 2016).

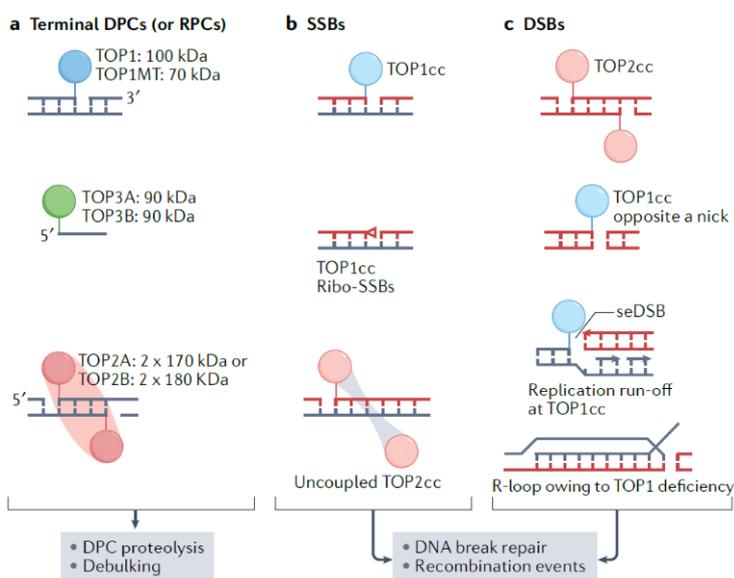
One of the targets of the kinase activity of ATM is Tyrosyl-DNA phosphodiesterase 1 (TDP1 – which is also target of DNA-PKcs), the enzyme that catalyzes the hydrolysis of the phosphodiester bond between TOP1 and DNA. The ATM-mediated phosphorylation of TDP1 elicits its recruitment to TOP1cc sites (Das et al., 2009) (Chiang et al., 2010). Noteworthy, the concomitant depletion of ATM and TDP1 results in a murine lethal phenotype, meaning that the two enzymes have an additive function in clearing TOP1cc, on top of the role that ATM has in promoting TDP1 activity. Interestingly, experiments in mouse embryonic fibroblasts showed that the expression of a kinase-dead ATM leads to a phenotype characterized by higher levels of TOP1cc when compared not only to WT, but even to ATM-null cells, which goes hand in hand with the way more severe, early embryonic phenotype of mice expressing a kinase-deficient ATM when compared to ATM-null mice (Yamamoto et al., 2016) (Yamamoto et al., 2012) (Daniel et al., 2012).

ATM is also involved in the formation and clearance of TOP2cc that are formed by the bond of DNA topoisomerase 2 (TOP2) with the 5' ends of both DNA strands, and are cleared by the proteasome and by Tyrosyl-DNA phosphodiesterase 2 (TDP2) activity (Pommier et al., 2010) (Pommier et al., 2014). Noteworthy, simultaneous TDP2 depletion and topoisomerase inhibition by etoposide cause TOP2cc accumulation when ATM is depleted or inhibited (Álvarez-Quilón et al., 2014). Moreover, removing TDP2 in ATM-null mice increases the rate of thymic cancer (Álvarez-Quilón et al., 2020). Importantly, ATM can phosphorylate DNA topoisomerase 2 $\alpha$  (TOP2 $\alpha$ ) leading to its degradation (Tamaichi et al., 2013). Therefore, ATM can regulate both TOP1 and TOP2 (Katyal et al., 2014).

In this picture, also MRN plays an important role: upon conditional deletion of the *Mre11* gene, TOP2cc spontaneously occur in human cells (Hoa et al., 2016), and MRE11 depletion in mammalian cells leads to a lethal phenotype (Yamaguchi-Iwai et al., 1999) in part due to TOP2cc accumulation, whereas TDP2 overexpression extends lifespan (Hoa et al., 2016). MRN might be directly involved in

the removal of TOP2cc (K. C. Lee et al., 2012) (Aparicio et al., 2016) in a similar way as it does for the removal of SPO11 (Initiator of meiotic double-strand breaks) during the meiotic process, which is initiated by the formation of DSBs (Keeney & Neale, 2006).

When TOP1 is inhibited during DNA replication, Ku and DNA-PKcs gather on single-ended DSBs and the ATM-mediated phosphorylation of CtIP (Matsuoka et al., 2007) is necessary for Ku removal from DNA ends (Chanut et al., 2016). Moreover, DNA-PKcs phosphorylation by ATM is necessary for Ku and DNA-PKcs release from single-ended DSBs (Britton et al., 2020) to prevent toxic NHEJ (Balmus et al., 2019). Therefore, during DNA replication, ATM can inhibit the NHEJ that is elicited by single-ended DSBs formation due to replication fork stalling.



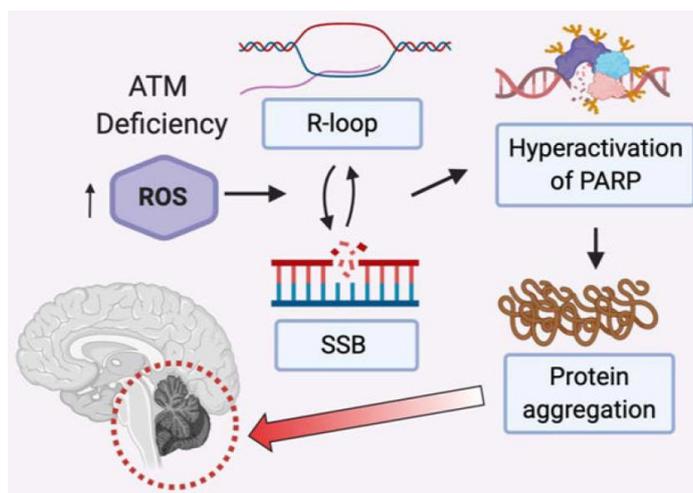
**Figure 39. Topoisomerase-related DNA lesions**

(a) DNA-protein crosslinks (DPCs) can form both at 3' and 5' DNA ends (blue and red circles, respectively formed by TOP1 and TOP2), and RNA-protein cross-links form at 5' RNA ends (green circle). (b) DNA SSBs generated by stalled or irreversible TOP1ccs. TOP1 can also generate SSBs by converting ribonucleotides incorporated by DNA polymerases into nicks with 2',3'-cyclophosphate blocking ends (red triangle). Moreover, DNA cleavage by TOP2 can be asymmetrical, with only one component of the TOP2 homodimer forming a TOP2cc. (c) DSB formation due to TOP2 trapping. TOP1 can also generate DSBs when it generates a nick in front of a pre-existing nick, or by colliding with a replisome, producing a single-ended (se)DSB. Finally, also R-loop formation due to TOP1 deficiency can give rise to DSBs (Pommier et al., 2022).

### ATM-mediated single-strand break repair

Initially, an ATM role in SSB repair was excluded based on the fact that it is not activated by nicked DNA *in vitro* (Lee et al., 2005). Recent works revealed that ATM can be activated in cells with impaired SSB repair and upon exposure to oxidative stress or alkaline treatment that led to SSB formation. Moreover, SSB-mediated ATM activation is followed by a slower G1 phase to allow for SSB repair before DNA is replicated (Khoronenkova & Dianov, 2015). Human cells exhibit high SSBs levels when ATM is depleted, even postmitotic mature neurons, which suggests that ATM can affect the occurrence of SSBs also in a non-replicative scenario. Indeed, ATM deficiency leads to SSB formation (due to ROS, transcriptional stress and R-loops) which causes the recruitment of Poly[ADP-ribose] polymerases (PARPs) at the level of the DNA lesion (described later). In turn, polyADP-

ribos(PAR)ylation drives the aggregation of intrinsically-disordered proteins, which appears to be tissue-specific as post-mortem analysis of human brains of A-T patients revealed protein aggregation in the cerebellum, but not in the neocortex (Lee et al., 2021). In this context, the origin of the SSBs is not clear, but it was shown that ATM promotes the Checkpoint kinase 2 (CHK2)-mediated activating phosphorylation of the SSB repair factor X-ray repair cross-complementing protein 1 (XRCC1), damage recognition and base-excision repair (BER) (Chou et al., 2008). Importantly, the mechanism of SSB repair was proven pivotal for neuronal survival in the context of several neurodegenerative disorders such as Spinocerebellar ataxia with axonal neuropathy 1 (SCAN1), Ataxia with oculomotor apraxia (AOA), Amyotrophic lateral sclerosis (ALS), and A-T-like disorder (ATLD) (McKinnon et al., 2017) (Jiang et al., 2017) (Hoch et al., 2017) (Yoon & Caldecott, 2018). Indeed, human cell lines that express the ATLD alleles of *Mre11* (Lee et al., 2021) exhibit elevated SSBs levels, suggesting that SSBs contribute to the ATLD phenotype.



**Figure 40. ATM deficiency and SSBs outcome**  
 ATM loss leads to SSB occurrence due to increased ROS levels, transcriptional stress and R-loop formation. SSBs are then targeted by PARP enzymes that catalyze the polyADP-ribosylation of proteins at the DNA lesion loci. Finally, ADP-ribose polymers cause the aggregation of intrinsically-disordered proteins, whose toxic accumulation has been reported in the cerebellar neurons of A-T patients (Lee et al., 2021).

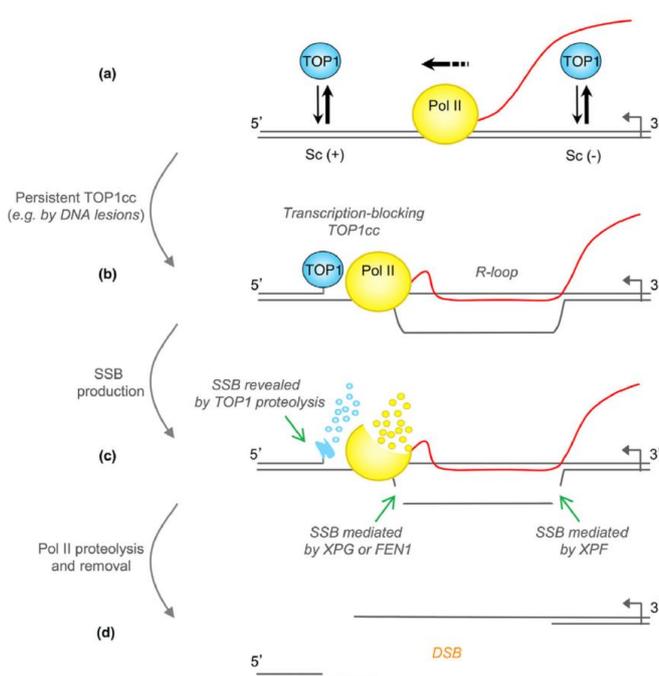
### ATM-mediated regulation of RNA metabolism

Irradiation affects the alternative splicing of pre-mRNA (Katzenberger et al., 2006) (M. J. Muñoz et al., 2009) (Nicholls et al., 2004) involving ATM and ATR. Indeed, in *Drosophila melanogaster*, ATM regulates the activity of the splicing factor U2AF38 in response to ionizing radiations (Katzenberger et al., 2006). ATR and ATM respectively activate CHK1 and CHK2 that in turn phosphorylate splicing factors leading to the increased alternative splicing of the pre-mRNA of TATA-binding protein-associated factor 1 (TAF1) (Katzenberger et al., 2006). A study in *Arabidopsis thaliana* showed that, in response to DNA damage, ATM promotes the alternative splicing of the *Nad2* pre-mRNA which encodes for a subunit of the mitochondrial NADH dehydrogenase (Su et al., 2017). In human cells,

40% of UV-induced splicing changes were shown to depend on the activity of ATM (Tresini et al., 2016). Indeed, the UV-induced stalling of replication fork causes R-loop formation, which in turn leads to ATM activation. Noteworthy, these data refer to a postmitotic context in the absence of DSBs, hinting for a non-canonical role of ATM (Tresini et al., 2016).

R-loops usually occur following UV-induced DNA damage and TOP1 adducts formation (Tresini et al., 2016) (Sordet et al., 2010), but they also accumulate in ATM-deficient mouse testes, accompanied by high DSBs levels and apoptosis (Yeo et al., 2014). Upon depletion of Werner syndrome protein (a RecQ family helicase needed for the resolution of arrested replication forks), ATM is activated by the formation of DSBs dependent on both R-loops and XPG, a nucleotide excision repair(NER)-associated nuclease (Marabitti et al., 2019). XPG is involved in R-loop processing, and, together with other nucleases, elicits H2AX phosphorylation at transcription sites (Sollier et al., 2014) (Yasuhara et al., 2018) (Cristini et al., 2021) (Makharashvili et al., 2018).

In human cells, ATM depletion or inhibition is followed by elevated levels of ROS and R-loop formation (Lee et al., 2021). When ATM is not functioning, SSBs form due to ROS and transcription-dependent R-loops occurrence. Importantly, both in endothelial cells and postmitotic neurons, treatment with N-acetyl cysteine (NAC – ROS inhibitor) or overexpression of Senataxin (Helicase implicated in the resolution of R-loops) inhibit SSB formation. The recessive neurological disorder Ataxia with oculomotor apraxia 2 (AOA2) is caused by mutations in the gene encoding Senataxin, and is characterized by a phenotype similar to A-T (Yüce & West, 2013); therefore, R-loops accumulation upon ATM deficiency might be associated with the onset of A-T.



**Figure 41. RNA-loops and DNA break formation**  
 ROS accumulation (which is accrued upon ATM depletion), UV irradiation and TOP1cc occurrence can all lead to RNA Pol II stalling and R-loop formation. R-loops are then processed by endonucleases like XPG and XPF, leading to SSBs formation. The following TOP1 and Pol II proteolysis and removal exposes DSBs and elicits ATM activation. (Cristini et al., 2021)

### *ATM and ROS regulation*

As previously discussed, ROS are deeply involved in the etiology of neuronal disorders (Singh et al., 2019). Making use of about 20% of our oxygen and making up to only 2% of our body weight, the human brain consumes oxygen at an impressively high rate when compared to other tissues (Watts et al., 2018). Indeed, the disruption of the equilibrium between antioxidants and ROS (produced by mitochondria, but also by other sources) leads to the perturbation of redox homeostasis that is considered to underly A-T and other neurodegenerative diseases (S. Gandhi & Abramov, 2012) (Cenini et al., 2019) (Barzilai et al., 2002).

Primary cells from A-T patients and ATM-null mice have higher levels of ROS, redox-reactive thiols, and antioxidant responses than controls (Barzilai et al., 2002) (Quick & Dugan, 2001) (Pietrucha et al., 2017) (Kamsler et al., 2001) (N. Liu et al., 2005) (Takao et al., 2000) (Reichenbach et al., 2002) (Reichenbach et al., 1999). Moreover, short-term ATM depletion in human cultured cells increases ROS levels, as does the expression of a mutant ATM incapable of activation upon oxidative stress (J. Lee et al., 2018) (Z. Guo et al., 2010). Furthermore, these cells are hypersensitive to oxidative stress (Kamsler et al., 2001) (Semlitsch et al., 2011) (Yeo et al., 2017) (Mosesso et al., 2018) (Ehrenfeld et al., 2020) (J. Kim & Wong, 2009b), suggesting that the ATM anti-ROS activity is pivotal for cellular fitness.

The gravity of the effect of oxidative stress in ATM-deficient mammalian cells is tissue-dependent. For example, the murine brain, but not the liver tissue, undergoes elevated protein oxidation upon ATM loss (Barlow et al., 1999), without exhibiting, though, the typical human cerebellar atrophy, ataxia and neurodegeneration of A-T patients (Kuljis et al., 1997) (Barlow et al., 1996) (Bishop et al., 2000) (Xu et al., 1996). Moreover, the loss of ATM in mice models leads to lymphomas, hematopoietic and NSC death, and shorter lifespan. Importantly, all this can be partially rescued by NAC and Tempol (a reductive agent) treatment, or by mitochondria-targeted catalase, suggesting an important role of ROS in these phenotypes (D'Souza et al., 2013) (Schubert et al., 2004) (Reliene & Schiestl, 2006) (Ito et al., 2004) (J. Kim & Wong, 2009a). From these data it results evident that the ATM function relative to ROS is extremely significant for the survival and fitness of specific cell types and stem cell populations.

Other than the mitochondrion, another organelle that generates ROS, as by-product of fatty acid metabolism, is the peroxisome. The C-terminal FATC domain of ATM is similar to a peroxisome localization signal and experiments with human cells showed that a fraction of ATM colocalizes with Catalase in the peroxisomes (Watters et al., 1999). Moreover, ATM activated by ROS is a regulator of

Peroxisomal biogenesis factor 5 (PEX5) and elicits peroxisome turnover via macroautophagy (Pexophagy) (Zhang et al., 2015). Furthermore, ATM can be found in the cytoplasm (Lim et al., 1998) (Valentin-Vega et al., 2012) (Watters et al., 1997) (Blignaut et al., 2019) of mostly neurons (Li et al., 2009a) (Barlow et al., 2000) (Oka & Takashima, 1998) (Kuljis et al., 1999) (Vail et al., 2016), but its role in this district needs more investigation. Finally, it was shown that in response to genotoxic stress and irradiation ATM exerts a signalling role via Nuclear Factor- $\kappa$ B (NF- $\kappa$ B) (Hadian et al., 2011) (Wu et al., 2006) (Hinz et al., 2010) (Miyamoto, 2011) (G. Wu et al., 2018) (L. Fang et al., 2014).

### *ATM and mitochondrial dysfunction*

The production of ATP via the electron transport chain (ETC) is coupled with the generation of ROS, therefore mitochondria are an important source of oxidative stress (Nolfi-Donagan et al., 2020). The generation of ROS is due to the fact that 0.2/2% of the electrons that are transferred along the ETC leak out of it before reaching oxygen and hydrogen to form water and, instead, convert oxygen into superoxide ( $O_2^-$ ). Once superoxide is formed, the Superoxide dismutase enzymes (SODs) convert it into hydrogen peroxide ( $H_2O_2$ ) which can then diffuse into the cytoplasm and other organelles (R. Z. Zhao et al., 2019). All the major neurodegenerative disorders, such as AD, PD, HD and ALS exhibit mitochondrial dysfunction and cellular damage due to oxidative stress, hinting for a fundamental role of ROS in neurodegenerative pathologies (Singh et al., 2019) (Smith et al., 2019) (Winklhofer & Haass, 2010) (Carmo et al., 2018)

Noteworthy, mitochondrial dysfunction is a characteristic of A-T individuals, ATM-depleted human cell lines and ATM-null mice (Zhang et al., 2018) (J. Lee et al., 2018) (Valentin-Vega et al., 2012) (Eaton et al., 2007) (Ambrose et al., 2007). In A-T cells, the lack of ATM affects mitochondrial DNA levels (Valentin-Vega et al., 2012) and mass (Eaton et al., 2007), mitochondrial respiration (Ambrose et al., 2007) and mitophagy (J. Lee et al., 2018) (Valentin-Vega et al., 2012) (Fang et al., 2016). Indeed, ATM was found to be involved in the expression of DNA ligase III (LIG3) and Ribonucleotide reductase subunits (enzyme that catalyzes the conversion of ribonucleotides into 2'-deoxyribonucleotides), which are all necessary to maintain normal levels of mitochondrial DNA (Sharma et al., 2014) (Eaton et al., 2007). Importantly, upon oxidative stress the transcription factor Nuclear respiratory factor 1 (NRF1) is phosphorylated by ATM, which leads to its homodimerization, consequent nuclear localization and ensuing expression of genes with mitochondrial functions (Chow et al., 2019). Another important canonical primary target of ATM, H2AX, was reported to be pivotal in the context of antioxidant responses via the activation of Nuclear factor erythroid 2-related factor 2 (NRF2)

(Weyemi et al., 2018). Moreover, H2AX loss leads to mitochondrial dysfunction which is rescued by the ectopic expression of PPARG coactivator 1 $\alpha$  (PGC1 $\alpha$ ) (Weyemi et al., 2019), a transcription factor that controls the expression of the ETC subunits. Therefore, ATM may maintain mitochondrial homeostasis via H2AX. Furthermore, when ATM and H2AX are simultaneously deleted, oxidative damage and genomic instability occur (Zha et al., 2008). Unsurprisingly, ATP levels in ATM-depleted cells are significantly lower than control cells (Chow et al., 2019) (Valentin-Vega et al., 2012), which leads to shorter lifespan.

With respect to mitophagy, a restoration of NAD<sup>+</sup> levels that are brought down by ATM deficiency can rescue the mitophagy process (J. Lee et al., 2018) (Valentin-Vega et al., 2012) (Fang et al., 2016). Interestingly, in this context, the kinase activity of ATM is not required. It is instead the physical interaction between ATM and the ubiquitin ligase E3 Parkin to be necessary for rescuing the mitophagy process in human cells (Sarkar et al., 2021) (Sarkar & Gandhi, 2021). Moreover, mitophagy is impaired in cells expressing an ATM mutant (C2991L) that cannot be activated by oxidative stress (J. Lee et al., 2018), meaning that oxidative stress could be the trigger of ATM-induced mitophagy. Among the ATM targets that are involved in the response to oxidative stress and mitochondrial fitness is the already-mentioned CHK2, a cell cycle check point kinase that is phosphorylated (and activated) by ATM in response to ROS and DSBs (Z. Guo et al., 2010) (Lee et al., 2005). The activation of CHK2 leads to p53 phosphorylation and to the ensuing increased translation of the Nitrosylase S-nitrosoglutathione reductase (GSNOR) (Cirotti et al., 2021). Noteworthy, the inhibition of GSNOR expression leads to a phenotype similar to that of ATM depletion or ATM-C2991L expression (Cirotti et al., 2021). Interestingly, the deficiency of GSNOR increases the levels of nitrosative stress (the excessive production of nitric oxide – NO) and the S-nitrosylation of Parkin, thereby impairing the turnover of damaged mitochondria (Rizza et al., 2018). Therefore, a ROS-induced ATM-CHK2-p53-GSNOR axis elicits mitophagy via Parkin. Importantly, following ROS-induced ATM activation, CHK2 can also phosphorylate the autophagy regulator Beclin-1, causing its release from BCL-2 (B-cell lymphoma 2), thereby boosting all forms of autophagy, including mitophagy (Q. Guo et al., 2020).

ROS that activate ATM were reported to be produced by defective mitochondria. Indeed, menadione [a drug that induces mitochondrial ROS production (Criddle et al., 2006)] activates ATM in a ROS-dependent manner (Zhang et al., 2018). The inhibition of ATM increases the levels of mitochondrial ROS in both primary rat neurons and in neuroblastoma cell lines (Fang et al., 2016), and ATM-null murine cells exhibit significantly higher ROS levels (Tassinari et al., 2019) (Agathangelou et al.,

2015). Moreover, experimental evidence shows that, in human cells, ATM is likely activated by H<sub>2</sub>O<sub>2</sub> (Zhang et al., 2018) (J. Lee et al., 2018) (Z. Guo et al., 2010).

Importantly, mitochondrial signalling is affected by mitochondria interaction with the endoplasmic reticulum (Rieusset, 2018). Experiments with ATM-deficient cells point out a correlation between aberrant responses to nutrient deprivation (*e.g.*, glucose deprivation) and impaired physical interaction between mitochondria and endoplasmic reticulum (MAMs – mitochondria-associated membranes) (Yeo et al., 2021). Noteworthy, MAMs are also compromised in a cellular model of Friedreich's Ataxia (FRDA), due to mutations and trinucleotide repeats expansions in the *Fxn* gene, which encodes for the mitochondrial protein Frataxin (Rodríguez et al., 2020).

### *ATM and metabolism*

Other than mitophagy, ATM activation by ROS is pivotal for the production of mitochondrial antioxidants. For example, the expression and the activity of Glucose 6-phosphate dehydrogenase (G6PD) are impaired in ATM-deficient cells (Zhang et al., 2018). The antioxidant function of G6PD consists in yielding NAD(P)H via the Pentose phosphate pathway (PPP), a molecule with great antioxidant capacity able to reduce oxidized glutathione and thioredoxins, which also acts as a cofactor for many antioxidant enzymes (*e.g.*, glutathione reductase), thereby extending the lifespan of model organisms (Zhang et al., 2018) (Fernandez-Marcos & Nóbrega-Pereira, 2016). Indeed, ATM boosts the activity of G6PD via the phosphorylation (and activation) of Heat shock protein 27 (HSPB1) upon which the latter binds and activates G6PD (Cosentino et al., 2011).

Upon oxidative stress, ATM also inhibits mTOR by activating the LKB1/AMPK pathway, thereby reducing mitochondrial activity and ROS levels (Pizzamiglio et al., 2020).

ATM also phosphorylates and activate Akt in response to insulin signalling, guaranteeing glucose uptake, protein translation, cell proliferation and survival (Martelli et al., 2012). Importantly, IGF-1 signalling elicits hippocampal BDNF expression, a key factor for learning and memory (Cotman & Berchtold, 2007).

Noteworthy, it seems that ATM is mostly involved in the DDR in a mitotic cell context, whereas it is mainly involved in Akt signalling and cell survival in postmitotic human neuron-like cells (Li et al., 2012).

Experimental evidence in human cells, mouse models and *C. elegans* showed that ATM deficiency affects the levels of the Tricarboxylic acid (TCA) cycle metabolites (Zhang et al., 2018) (Chow et al., 2019) (Fang et al., 2016). Moreover, in cells lacking ATM, the levels of the rate-limiting enzymes of

glycolysis, Hexokinase 1 and 2, are decreased, which reflects low levels of pyruvate and high levels of lactate (Chow et al., 2019). Therefore, in the absence of ATM, the system becomes dependent on glutamine as a source of carbon for the TCA cycle. Indeed, the supply of glutamine extends the lifespan of ATM-deficient mice (J. Chen et al., 2016). Moreover, the supplementation of NAD<sup>+</sup> can restore part of the metabolic changes related to the TCA cycle in the absence of ATM (Fang et al., 2016). Furthermore, upon hypoxia [an oxygen-deprived condition that still culminates in ATM activation (Bencokova et al., 2009)], ATM-deficient cells exhibit an altered citrate metabolism (Peng et al., 2019). All in all, other than its canonical role in detecting DSBs, ATM results as a pivotal sensor of mitochondria-derived ROS, an activity that in turn can regulate the metabolism of the cell.

### *ATM and oxidative DNA damage*

As ROS are a major cause of genotoxic stress, the DNA damage-induced activation of ATM cannot completely be told apart from its ROS-induced activation. Stunningly, each human cell suffers approximately 70 000 DNA lesions per day, including about 55 000 SSBs and 25 DSBs (Lindahl & Barnes, 2000). Importantly, ROS that are produced during metabolic processes can give rise to DNA lesions and SSBs, which can turn into DSBs when cells replicate (Tubbs et al., 2017).

The rate of spontaneous oxidative lesions (including 8-oxoG – the most common oxidative DNA lesion) in A-T cells and cells from ATM-deficient mice is ten times higher than wild-type cells. On top of this, SSBs levels are also significantly higher in this context (Reichenbach et al., 2002) (Reliene et al., 2004) (Weyemia et al., 2015).

Moreover, ATM-depleted human cells exhibit elevated levels of SSBs, whose occurrence can be avoided by treatment with the antioxidant NAC, indicating that SSBs form via ROS-dependent mechanisms (Lee et al., 2021) (Reliene et al., 2004).

Further experiments showed that both ROS and RNA-dependent lesions have a role in SSBs formation, as blocking transcription or overexpressing RNase H decreases SSBs occurrence (Lee et al., 2021). Importantly, both the excision of damaged bases due to alkylating enzymes, and the depletion of the SSB repair factor XRCC1 activate ATM providing evidence that this kinase can be activated by SSBs (Khoronenkova & Dianov, 2015).

Interestingly, the occurrence of ROS-induced DSBs in murine cortical astrocytes deficient for ATM correlates with high levels of TOP1cc, and antioxidants such as mannitol and NAC decrease its accumulation (Alagoz et al., 2013). Indeed, abasic sites and other ROS-induced lesions can favor the formation of TOP1cc (Pommier et al., 2006).

Moreover, when ATM function is impaired, the oxidation of other cellular components such as low-density lipoproteins in turn facilitates the formation of further ROS which favors DNA damage (DSBs) and genome instability (Semlitsch et al., 2011).

Furthermore, in A-T cells and in cells where ATM is inhibited, the expression of NADPH oxidase 4 (NOX4) is increased, leading to further superoxide production (Weyemia et al., 2015).

Finally, the recombination occurring at DSBs sites is sensitive to the redox balance. *E.g.*, the defective Ig class switch recombination in ATM-null mice is rescued by NAC treatment, meaning that, in mammalian cells, even when the DSB is scheduled it is regulated by ROS, under the control of ATM (Ito et al., 2007).

### *ATM and Poly[ADP-ribose] polymerase (PARP) activation*

Some of the members of the ADP ribosyl transferase family, such as Poly[ADP-ribose] polymerase 1 (PARP1), PARP2 and PARP3, recognize diverse DNA lesions and catalyze the poly[ADP-ribose] (PAR) polymerization on proteins located at these sites, as well as auto-PARylation (Chaudhuri & Nussenzweig, 2017) (Gupte et al., 2017) (H. Liu et al., 2017). Among the PARPs, PARP1 and PARP2 are the ones mainly involved in the DNA damage response being implied in the recruitment of BER enzymes such as XRCC1, LIG3 and DNA polymerase  $\beta$  (Pol $\beta$ ) (Chaudhuri & Nussenzweig, 2017) (El-Khamisy et al., 2003) (Mortusewicz et al., 2007). PARP1 and PARP2 interact with the cofactor Histone PARylation factor 1 (HPF1) to conjugate PAR to serine residues (Bonfiglio et al., 2017) (Gibbs-Seymour et al., 2016) (Suskiewicz et al., 2020) (Larsen et al., 2018), which can be readily removed by the Poly[ADP-ribose] glycohydrolase (PARG) and ADP-ribosyl hydrolase 3 (ARH3) enzymes (Abplanalp et al., 2017) (Slade et al., 2012).

Moreover, BER-deficient cells exhibit PARP1 hyperactivation (Hoch et al., 2017) (Gassman et al., 2012), suggesting that unrepaired SSBs are strong PARP1 activators.

PARP1 hyperactivity is also caused by XRCC1 deficiency, which causes neuronal loss and ataxia in murine *in vivo* models (Hoch et al., 2017). The lack of XRCC1 also causes PARP trapping on the DNA, thereby impeding a functional BER process (Demin et al., 2021).

The accumulation of PAR leads to DNA damage sensitivity and embryonic lethality in PARG-deficient cells and PARG-null mice, respectively (Koh et al., 2004). Therefore, a fine tuning of PAR polymerization and PAR polymers degradation is essential for neuronal survival and function.

Hyper-PARylation causes reduction of NAD<sup>+</sup> levels and depletion of ATP pools leading the cell to apoptosis (Horton et al., 2005) (Alano et al., 2010). According to other findings, the PAR polymer

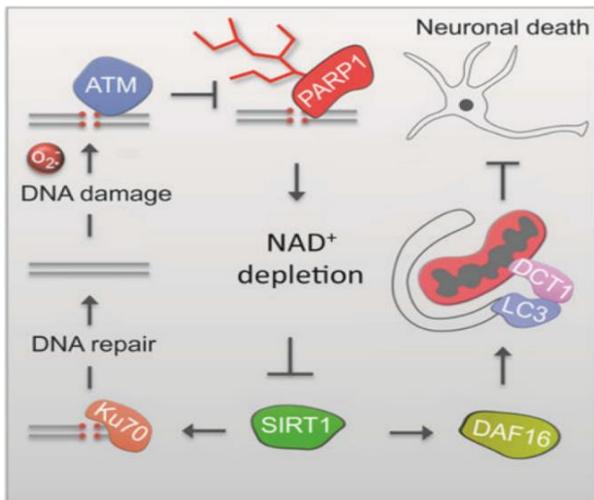
itself can promote a particular form of cell death referred to as parthanatos by interacting with apoptosis-inducing factors (Y. Wang et al., 2012) (David et al., 2009) (Andrabi et al., 2006). Therefore, PARP hyperactivation is reported to be toxic in several organisms and to be involved in many neurodegenerative diseases (Martire et al., 2015). Interestingly, brain tissues from individuals with Alzheimer's disease exhibit high PARylation levels, and PARP inhibitors annihilate the oxidative stress caused by the accumulation of  $\beta$ -amyloid fibrils (Abeti & Duchen, 2012). Analogously, individuals with Parkinson's disease have high PAR levels in the cerebrospinal fluid and brain tissue, which correlates with the formation of  $\alpha$ -synuclein fibrils (Y. Lee et al., 2014) (Kam et al., 2018) (Puentes et al., 2021).

It is hypothesized that the PAR polymers can interact with disease-related forms of proteins containing intrinsically disordered regions (IDRs) facilitating their nucleation into toxic protein aggregates (Leung, 2020). Noteworthy, this process can occur *in vitro* for several neurodegenerative disease-associated proteins (Mastrocola et al., 2013) (Altmeyer et al., 2015) (Singatulina et al., 2019) (Patel et al., 2015) (J. K. Chen et al., 2018) (Y. Duan et al., 2019). Proteins that bind PAR polymers form biomolecular condensates such as DNA repair foci, stress granules, nucleoli, and phase-separated complexes (Leung, 2020) (Banani et al., 2017).

Interestingly, PARP1 has pivotal roles in the transcription of ribosomal DNA and ribosomal biogenesis (D. S. Kim et al., 2020). Moreover, several proteins that form PAR-dependent condensates have RNA-binding domains and bind RNA in the absence of DNA damage (Aarum et al., 2020) (Gitler & Shorter, 2011) (Schwartz et al., 2022). Noteworthy, PARP activation can recruit intrinsically disordered regions (IDRs)-containing proteins, sequestering them away from their RNA-binding partners and leading to the fast relocalization of proteins that takes place upon DNA damage (Leung, 2020) (Altmeyer et al., 2015) (J. K. Chen et al., 2018) (Dutertre et al., 2014).

PARP hyperactivation and NAD<sup>+</sup> depletion are detected in ATM-deficient neurons, while addition of NAD<sup>+</sup> precursors rescues the neuromuscular impairment and increases the lifespan of ATM-deficient mice and *C. elegans*, indicating a likely causal role for PARP hyperactivation in neurodegeneration (Fang et al., 2016). Indeed, the NAD<sup>+</sup>-dependent SIRT1 activity contributes to DNA repair via the deacetylation (and activation) of major DSB repair proteins such as ATM itself and Ku70 (Chalkiadaki & Guarente, 2015) (Dobbin et al., 2013). Importantly, the levels of PARylation in the cerebellar tissue of A-T patients are higher than control subjects (Lee et al., 2021). Moreover, when ATM is knocked down or chemically inhibited in human cultures of epithelial cells and neurons, the SSB rate increases and causes PARP1 hyperactivation. Therefore, it is tempting to speculate that DNA lesions

that activate PARP1 in the absence of ATM include SSBs and may relate to A-T pathology, taken into account the similarity between the cerebellar ataxia of A-T patients and those that are due by other conditions that are still caused by a defective SSB repair (Hoch et al., 2017) (Caldecott, 2003) (El-Khamisy et al., 2005).



**Figure 42. ROS, ATM activation, PARylation and metabolism**  
 Upon ROS-induced DNA damage, ATM activation avoids uncontrolled PARylation at the level of the DNA lesion. When ATM is depleted or mutated (as in A-T patients), excessive PARylation causes exhaustion of the NAD<sup>+</sup> pool of the cell. NAD<sup>+</sup> shortage does not allow for SIRT1 activation and the deacetylation of its targets, including ATM, Ku70 and DAF16 (*Caenorhabditis elegans* ortholog of human FOXO), which would respectively allow DNA repair and the induction of mitophagy to preserve neuronal fitness via mitochondrial turnover (Fang et al., 2016).

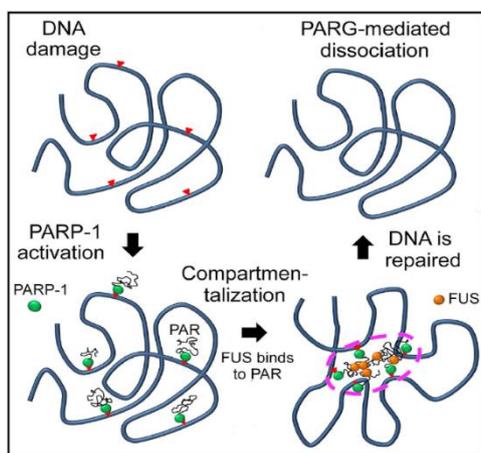
### **ATM and neurodegeneration**

To date, it is not completely clear yet what are the molecular mechanisms driving cerebellar degeneration in A-T patients. Nonetheless, other neurodegenerative diseases are characterized by oxidative stress and loss of protein homeostasis (J. H. Lee & Paull, 2020) (Lévy et al., 2019). Cultured ATM-deficient cells exhibit hypersensitivity upon inhibition of the proteasome, which, in this context, is already dysfunctional despite being upregulated at the nuclear level (Poletto et al., 2017) (Wood et al., 2011). ATM depletion leads to ROS-dependent protein aggregation (J. Lee et al., 2018). Interestingly, the aggregated proteins are rich in intrinsically-disordered regions (IDRs) and are also characterized by high predicted levels of amyloid-like motifs.

A work that studied the link between ATM deficiency and protein aggregation in several cell lines, showed that hundreds of proteins form detergent-resistant aggregates when ATM is lacking, enabling the identification of a group of aggregation-prone factors common to all the cell lines tested (Lee et al., 2021). The addition of ROS further increases the levels of these aggregates. Very importantly, for all the cell lines used and the conditions assessed, the activity of the enzyme PARP was necessary for protein aggregation to occur. Moreover, more than 1100 polypeptides were enriched in the A-T cerebellum fraction but not in other tissues. Furthermore, experiments of immunohistochemistry showed that granule cells and Purkinje neurons have higher PARP levels in

A-T patients (Lee et al., 2021). Therefore, these data depict a scenario whereby the lack of ATM elicits a ROS-dependent chain of events that culminates in PARP hyperactivation and the ensuing accumulation of toxic insoluble protein aggregates (J. Lee et al., 2018) (Lee et al., 2021).

So far, the mechanisms underlying protein aggregation in the absence of ATM are still unknown. Nevertheless, it is shared knowledge that, following DNA damage, specific proteins are recruited to sites of PARylation, and that ATM-dependent phosphorylation is needed for them to be released from these locations. *E.g.*, the physical interaction between the RNA-binding protein Fused in sarcoma (FUS) and PAR polymers forms phase-separated aggregates (Singatulina et al., 2019), but PIKK-mediated phosphorylation of FUS (Gardiner et al., 2008) (Q. Deng et al., 2014) (Rhoads et al., 2018) can resolve these condensates and avoid their cytotoxicity (Monahan et al., 2017).

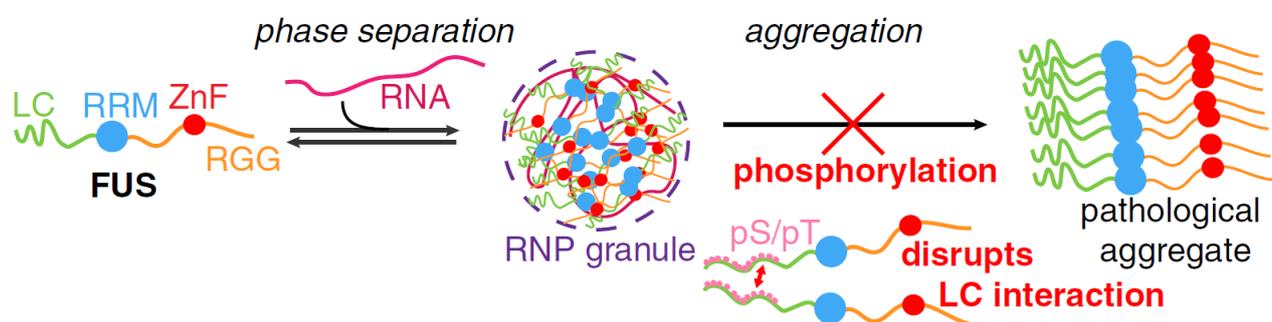


**Figure 43. PARP-1 activation and formation of DNA damage-rich compartments**

PARP-1 activation following DNA damage sensing leads to FUS recruitment to DNA damage loci via its binding to PAR. FUS recruitment causes compartmentalization of DNA damage-rich DNA sites. DNA damage compartments, once DNA is repaired, are dissociated via PARG activity, whereby FUS shuttles from the nucleus to the cytoplasm (Singatulina et al., 2019).

Analogously, PARP1-dependent PARylation recruits Cold-inducible RNA-binding protein (CIRBP) to DNA lesions, which leads to CIRBP polymerization and aggregation. Much alike FUS, CIRBP needs to be phosphorylated by ATM to be released from damage sites (J. K. Chen et al., 2018). Furthermore, ATM-dependent phosphorylation is also necessary for check point proteins, heterochromatin factors and DNA repair enzymes for them to release the DNA (Polo & Jackson, 2011). Most likely, the negative charge of the phosphate groups added to these proteins induces conformational changes that reduce their affinity for the polyPAR which also bears a negative charge. Since ATM has a multitude of direct targets (Matsuoka et al., 2007) (Bensimon et al., 2010) (Schlam-Babayov et al., 2021), it is likely that some of them respond similarly to PARylation. A high rate of endogenous DSBs and other DNA lesions combined with a deficient resolution of PAR-bound condensates may lead to the formation of irreversible protein aggregates. Importantly, the accumulation of misfolded and aggregated proteins that takes place during normal aging, occurs at a higher rate in neurodegenerative diseases (Saez & Vilchez, 2014) (Sweeney et al., 2017). The formation of

aggregates also impairs the very processes of proteostasis and autophagy, which leads to slower translational rates and cell death, especially in the brain (Dasuri et al., 2013). Moreover, the insoluble aggregates can themselves reduce the DNA repair capacity: i) ALS-associated FUS mutants decrease DDR efficiency (Naumann et al., 2018) (W. Wang et al., 2017), and ii) ALS-associated Chromosome 9 open reading frame 72 (C9ORF72) protein mutants increase DNA damage and R-loops levels, and decrease ATM-dependent signaling (Walker et al., 2017). Finally, even low levels of  $\beta$ -amyloid [the toxic protein whose aggregates are bonafide markers of Alzheimer's disease (J. Gandhi et al., 2019) (Olzscha et al., 2011)] can impair the activity of DNA-PKcs and DSBs repair (Cardinale et al., 2012).



**Figure 44. FUS and phase separation/aggregation**

Upon FUS recruitment following PARylation of DNA damage sites, ribonucleoprotein (RNP) granule formation and DNA damage compartmentalization occur. After DNA repair is complete, phosphorylation of FUS by ATM allows for the resolution of RNP granules. If FUS phosphorylation is impeded, RNP granules accumulate leading to pathological consequences [LC, low-complexity domain; RRM, RNA recognition motif; ZnF, Zinc finger motif; RGG, arginine-glycine-glycine-rich domain] (Monahan et al., 2017).

Therefore, the chronological order of the events leading to neurodegeneration in the A-T context include the oxidative stress resulting from elevated levels of ROS, the ensuing DNA damage and hyperactivation of PARP and protein aggregation, which in turn lead to further ROS production, to close the vicious cycle. PARP could be an attractive target for pathologies involving ATM deficiency, but the problems related to targeting a protein that has a pivotal role in SSB repair and in other responses to DNA damage, makes it a challenging task. Noteworthy, the simultaneous depletion of PARP in an ATM-deficient mouse model results in a lethal phenotype (Murcia et al., 2001), which suggests a pivotal role of PARP in embryogenesis when ATM is absent.

Interestingly, *in vitro* studies showed that primary *Atm*<sup>-/-</sup> NPCs exhibit impaired proliferation. In this context, the increased ROS production due to ATM deficiency activates the p38 MAPK pathway, which inhibits the Akt-dependent phosphorylation of polycomb protein Bmi-1, destabilizing the latter. Bmi-1 degradation leads to increased p21 expression and impaired NPC proliferation (J. Kim et al., 2011).

ATM phosphorylation also inhibits Protein phosphatase 2 (PP2A) activity. In A-T patients and ATM-null mice, the unleashed PP2A activity results in decreased HDAC4 phosphorylation and the ensuing raised nuclear localization of the latter, leading to an increase in chromatin compaction that affects the expression of neuronal pro-survival factors such as CREB and BDNF (J. Li et al., 2012).

Interestingly, ATM is known to be preferentially localized in the nucleus of mitotic cells, whilst it is also found in the cytoplasm of postmitotic cerebellar Purkinje cells and granule neurons (Oka & Takashima, 1998). It was reported that ATM can also bind presynaptic vesicle proteins such as VAMP2 and Synapsin-I, whose orchestrated activity mediates vesicle fusion with the presynaptic membrane and neurotransmitter release. Importantly, vesicle transport and neurotransmitter release by primary neurons derived from ATM-deficient mice are impaired (Li et al., 2009b). Indeed, Long term potentiation (LTP) and synaptic plasticity are compromised in *Atm*<sup>-/-</sup> mice (Vail et al., 2019).

Very interestingly, it was shown that in primary cortical neurons, ATM and ATR interact with different classes of vesicles, the excitatory VGLUT1<sup>+</sup> and the inhibitory VGLUT1<sup>-</sup> vesicles, respectively. Also, ATM and ATR “sense” each other’s levels and respond in a compensatory fashion: a decrease in ATM levels causes an increase in ATR levels and vice versa, thereby influencing the excitatory/inhibitory (E/I) balance of neuronal networks (Cheng et al., 2017).

The inhibitory neurotransmitter GABA binds to GABA<sub>A</sub> receptors causing chloride influx and membrane hyperpolarization leading to decreased chances of cell firing. Primary *Atm*<sup>+/-</sup> hippocampal neurons exhibit a higher frequency of miniature inhibitory post-synaptic currents (mIPSCs), increased quantity of inhibitory synapses and GABA-containing synaptic vesicles. Importantly, a proper E/I balance is necessary for brain maturation, including neuronal proliferation, migration and differentiation, which underlie brain plasticity and cognition (Pizzamiglio et al., 2016). In the frontal cortex, cerebellum and hippocampus of AD subjects, ATM levels are reduced in a fraction of the neurons, which correlates with increased HDAC4 nuclear localization, histone H3 trimethylation and increased levels of proteins involved in the cell cycle, such as Cyclin A2, possibly leading to loss of cell cycle control and, ultimately, to cell death (Shen et al., 2016).

Huntington’s disease (HD) is a genetic neurodegenerative disorder characterized by the CAG-repeat expansion in the first exon of the HTT gene. Seemingly contradictory, while in A-T and AD individuals ATM function is absent or reduced, in HD subjects ATM levels are increased. In this context, ATM levels correlate with the progression of the disease. Indeed, reducing ATM dosage rescues behavioral deficits (locomotion, coordination and depressive-like behaviors) and decreases the accumulation

of HTT aggregates and striatal atrophy typical of HD. ATM inhibition might avoid the recruitment of mutant HTT to DNA damage sites, which may obstacle a proper DNA damage repair process (Vail et al., 2019).

ATM also phosphorylates and stabilizes the actin-binding protein Debrin (DBN), which results in maintenance of dendritic spine structure, therefore counteracting the age-induced dendritic spine degeneration, synaptic dysfunction and cognitive decline (Kreis et al., 2019).

Interestingly, ATM is activated by low doses of Chloroquine (CQ), which results in the pro-longevity SIRT6 stabilization, by inhibiting its ubiquitination and proteasomal degradation. CQ systemic administration in murine models ameliorates coordination and increases running endurance during aging. Besides, CQ inhibits glycolysis, decreases serum lactate levels and body weight decline, which results in increased lifespan (Qian et al., 2018).

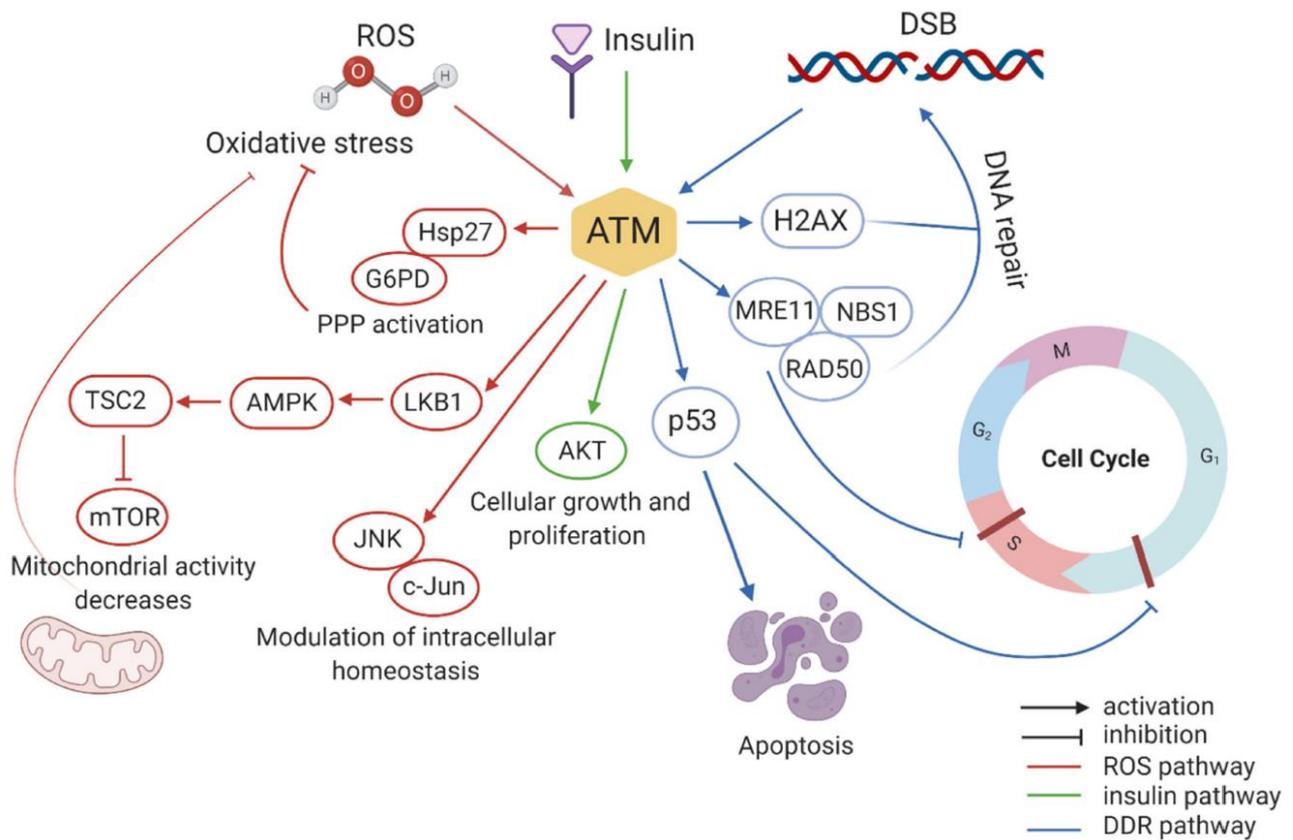
### *ATM and cognition*

Cognition is the mental process that allows the acquisition of knowledge via reasoning about what we experience with our senses, which implies attention, comprehension, judgment, memory and decision making. Interestingly, A-T subjects exhibits impairments in attention, learning and memory that cannot be exclusively related to cerebellar deficits (Hoche et al., 2014). Indeed, *ATM* homozygous and heterozygous individuals display decreased glucose metabolism in different brain regions, including the hippocampus, which is specifically devoted to learning and memory (Volkow et al., 2014). Noteworthy, it seems that the cognitive deficits found in A-T patients start to emerge as long as the individuals age, whereas during youth the disorder is limited to the cerebellar pathology.

Cognitive stimulation elicits DSB formation in the promoters of immediate early genes whose products are necessary for neuronal activity and plasticity, which leads to their expression (Madabhushi et al., 2015). After these genes are expressed, DSBs need to be repaired to avoid genomic aberrations. Indeed, mutations in proteins involved in DDR give rise to devastating neurological disorders. Besides A-T, ATR-Seckel syndrome, LIG4 syndrome, ATLD, and Nijmegen breakage syndrome (Madabhushi et al., 2014). Interestingly, in a murine model of AD, the DSBs induced by exploration persist for longer time than in control mice. Moreover, even at baseline the DSB incidence is higher than controls. This was shown to be due to the decreased expression of the DNA repair protein BRCA1 in both AD patients and murine models. Indeed, knocking down BRCA1 in the DG of WT mice causes neuronal shrinkage and impairs synaptic plasticity, learning and

memory. Furthermore, A $\beta$  accumulation causes BRCA1 neuronal depletion, which might lead to the cognitive impairment observed in AD patients (Suberbielle et al., 2013).

All in all, these findings highlight the importance that some proteins involved in DDR (including ATM) have for cognitive functions.



**Figure 45. ATM signalling network**

Resume of the ATM signalling roles including response to i) ROS, ii) Insulin, and iii) DNA damage, which respectively lead to i) PPP activation, decrease of mitochondrial activity, and modulation of intracellular homeostasis, ii) cell growth and proliferation, and iii) DNA repair, cell cycle arrest and/or apoptosis (Pizzamiglio et al., 2020).

*Time has now come to find out who shall finally prevail  
 n' if princess Memory is going to be saved  
 in the deep hippocampal reign*

## 5. Aim of the Thesis

The physiological modifications that occur in the nervous system during normal aging (*i.e.*, without manifest pathology) are characterized by a progressive decline in neuronal homeostasis reflecting an impairment in cognitive, motor and sensory functions. These facts represent an unsolved medical and societal problem, with serious repercussions on private life and on the whole healthcare system, especially in western world societies where people live longer and longer lives.

One of the most affected regions of the aging brain is the hippocampus, a structure that controls memory and learning.

Aging is a multifaceted phenomenon that includes the progressive variation of several parameters as long as time goes by. These time-dependent alterations might significantly differ inter- and intra-individually according to the organs, tissues or cell types considered.

A well-established driver of mitotic cell aging is cell division-mediated telomere erosion, consisting in the relentless shortening of the telomeres of a cell as it goes through several replication cycles during its lifespan. This mitotic cell-specific process does not occur in postmitotic cells such as neurons. Indeed, it is not known whether telomeric factors play a role in the aging process of postmitotic mature neurons.

Interestingly, during postmitotic myofiber aging the expression of Telomeric repeat-binding factor 2 (TRF2 – pivotal component of the telomere-capping Shelterin complex, encoded by the *Terf2* gene) decreases dramatically, leading to mitochondrial dysfunction and increased ROS production. Therefore, these data suggest that telomeric modifications are also involved in postmitotic cell aging. Furthermore, it is established knowledge that TRF2 can bind DNA outside of telomeres in the proximity or to introns of genes involved in vesicular transport (*Snap25*, *Arf-Gap3*, *Pacsin2*), ion transport (*Cacna1b*, *Cln6*, *Lcn2*) and axonal growth (*Plxnb2*, *Ehd4*, *Vcan*), suggesting that it might influence their expression.

Therefore, based on this knowledge, we decided to investigate the role of the telomeric protein TRF2 in the aging process of the neuron, another type of postmitotic cell.

To understand the role of TRF2 in neuronal aging we decided to set up an *in vivo* model. After discovering that the expression of TRF2 declines in the murine aging hippocampus, we decided to downregulate the expression of TRF2 in the hippocampus of young mice by means of hippocampal stereotaxic injections of adeno-associated viruses (AAV) designed to specifically downregulate TRF2

in hippocampal mature neurons. The aim of this procedure was to assess the effect of *Terf2* downregulation on memory, a hippocampus-dependent phenomenon that is notoriously affected by aging. To test the effect of *Terf2* downregulation on the mnemonic process we performed behavioral experiments to assess three different types of memory, namely: episodic, contextual and spatial memory. We found out that *Terf2* downregulation in the hippocampus of young mice affects episodic and contextual memory, leaving spatial memory unaffected.

With the goal of dissecting the mechanism underlining the TRF2-dependent memory impairment, we performed proteomics and molecular biology analyses, revealing that *Terf2* downregulation affects pathways involved in neuronal activity and synaptic plasticity. On the same line, complementary *in vitro* results unveiled that *Terf2* downregulation also impairs vesicle axonal transport.

With the purpose of understanding whether the mechanism also involved the canonical TRF2 activity consisting in the inhibition of the pivotal DNA damage response (DDR) factor Ataxia telangiectasia mutated protein (ATM), we performed the same behavioral experiments with the simultaneous inhibition of ATM. The results showed that, indeed, TRF2 guarantees proper memory formation by inhibiting ATM activity, which in this context seems to be only partially related to DDR. Once observed the deleterious effects of *Terf2* downregulation on the memory of young mice, we decided to test whether TRF2 overexpression could rejuvenate the memory performance of old mice. Indeed, the overexpression of TRF2 in the hippocampi of old mice completely rescued both episodic and contextual memory.

All in all, we discovered an unheard-of role of a telomeric protein, TRF2, in the mnemonic process controlled by hippocampal mature neurons, which also involves its canonical antagonist, ATM. Therefore, TRF2 appears to be a limiting factor for proper memory formation, and its age-dependent physiological downregulation in the murine hippocampus might be the trigger of memory decline. This discovery highlights TRF2 as a potential target for anti-aging therapies aimed at improving the life quality of millions of people world-wide.

Part of the results that were obtained with this work will be hereafter presented in the format of an article ready to be submitted. Thereafter, other results not included in the article, but that are still relevant to my Thesis, will be discussed.

# Decline of hippocampal TRF2 during aging triggers ATM-dependent memory impairment

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## Abstract

The physiological changes in the brain during normal aging (*i.e.*, without overt pathology) are characterized by a progressive decline in neuronal homeostasis and cognitive functions. Among the regions of the brain most affected by aging is the hippocampus, which controls memory and learning. Our understanding of how the hippocampus ages is still at its infancy. We report that the level of the key telomere-capping protein TRF2 declines in the hippocampi of mice during aging. Next, we modulated the level of TRF2 in hippocampal neurons by stereotaxically injecting AAV9 preparations. In young hippocampi, TRF2 was required for episodic and contextual memory and restoration of the TRF2 level in the hippocampi of old mice rescued their memory capacity to the levels of young mice. In the hippocampus, TRF2 suppressed unwanted ATM activation and modulated the expression of genes involved in synaptic plasticity. Therefore, an age-dependent TRF2-ATM axis controls memory performance. The deleterious effect of TRF2 decline on memory capacity during aging suggests novel strategies for interventions to delay, prevent or even revert age-associated memory loss.

# Introduction

Aging is accompanied by progressive changes in the physiology of organs leading to a decline in their performances and an increased risk of diseases. For instance, brain aging is characterized by a progressive decline in neuronal homeostasis and cognitive functions. Among the regions of the brain most affected by aging is the hippocampus, which controls episodic memory and spatial cognition (Bishop et al., 2010). Age-related hippocampal deficits in neuronal activity and synaptic plasticity lead to learning and memory impairments (S. L. Gray et al., 2015) with repercussions not only for the individual but also for the economy and the healthcare system (Salinas-Rodríguez et al., 2022). Therefore, investigation of the cellular and molecular mechanisms involved in hippocampal aging would promote the development of novel interventions for the cognitive decline associated with age.

The cognitive functions controlled by the hippocampus depend on its neuronal structural reorganization and plasticity. The latter is characterized by continuous rearrangements of dendritic and synaptic complexity, neurotransmission, and the regular generation of adult-born neurons (Leuner & Gould, 2010). The high degree of plasticity of the hippocampus renders neurons therein extremely vulnerable to environmental factors (e.g., malnutrition), systemic factors (e.g., hormonal imbalance), and aging (Levine & Kroemer, 2019) (Obri et al., 2018) (Castellano et al., 2017). The progressive functional decline of hippocampal neurons during aging is associated with mitochondrial dysfunction, impaired autophagy and proteostasis (Glatigny et al., 2019) (De Risi et al., 2020). However, the mechanisms underlying these age-related changes in neurons and cognition are unclear.

If long-lived post-mitotic cells, such as neurons, undergo senescence (Jurk et al., 2012), they progressively lose their function before showing signs of it (Robin et al., 2020) (Jacome Burbano & Gilson, 2020). In addition to their role in the senescence of mitotic cells (Gilson & Géli, 2007), telomeres may behave as an aging clock in long-lived postmitotic cells (Jacome Burbano & Gilson, 2020). First, cardiomyocytes acquire a senescent-like phenotype during aging, which is characterized by persistent DNA damage at telomeres caused by mitochondrial dysfunction, independently of telomere length (Anderson et al., 2019). Second, the level of TRF2, a subunit of the Shelterin complex (encoded by the *Terf2* gene) decreases during aging of skeletal muscle cells. *Terf2* downregulation in myotubes represses *Sirt3* expression and subsequently causes mitochondrial

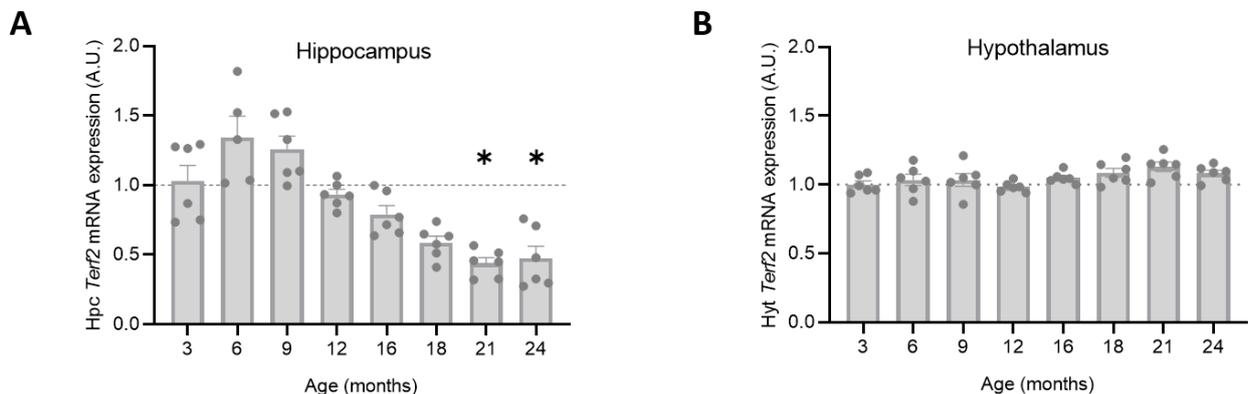
dysfunction and ROS production (Robin et al., 2020). Moreover, TRF2 plays multiple roles in neuronal biology whose significance remains elusive (Ying et al., 2022) (P. Zhang et al., 2011) (P. Zhang et al., 2006) (Ovando-Roche et al., 2014) (P. Zhang et al., 2009) (P. Zhang et al., 2015) (Ye et al., 2014). Here, we report that out of the six Shelterin subunits, TRF2 is the only one whose level decrease during hippocampal aging in mice. Selective downregulation of *Terf2* in mature hippocampal neurons (caused by stereotaxic injections of AAV9-eSYN-sh*Terf2*) led to severe learning and memory impairments, which are associated with decreased neuronal activity. The causality of the relationship between this phenotype and ATM signaling was demonstrated by the fact that pharmacological inhibition of ATM in the hippocampus improved the memory impairment caused by neuronal *Terf2* downregulation. Finally, selective overexpression of *Terf2* in mature hippocampal neurons rescued the memory loss of old mice. We conclude that the expression of TRF2 declines during hippocampal aging, which contributes to memory loss by triggering unwanted activation of ATM.

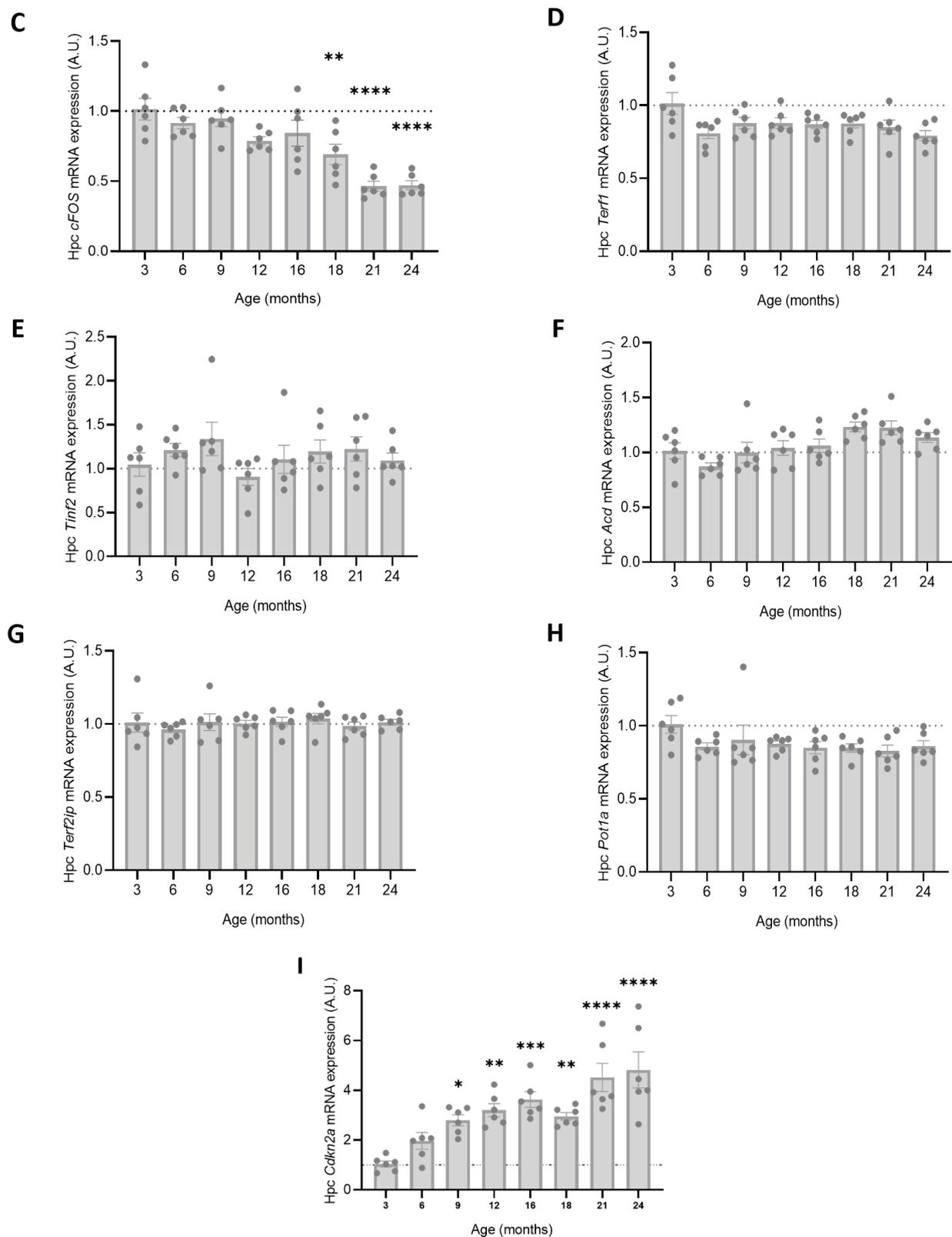
# Results

## *TRF2 levels negatively correlate with age in the murine hippocampus*

*Terf2* mRNA was detected in several regions of the brain of young mice (3 months) (Figure S1A). Via immunofluorescence analysis of hippocampi, we detected TRF2 protein in most NeuN-positive cells of the *Cornu ammonis* 1 (CA1), CA3, and Dentate gyrus (DG) regions (Figure S1B-E). Moreover, several NeuN-negative cells expressed low or undetectable levels of TRF2, indicating high expression of TRF2 in hippocampal mature neurons. Using hippocampi (Hpc) and hypothalami (Hyt) isolated from mice 3 to 24 months of age, we found an age-related decline in the *Terf2* mRNA level only in hippocampi (Figure 1A-B). This hippocampal age-related decline was confirmed at the protein level in the CA3 and DG (Figure S1F). Among the six shelterin genes, only the mRNA level of *Terf2* was significantly associated with this age-associated pattern (Figure 1 A, D-H). In parallel with the *Terf2* mRNA decline, we found a progressive decline in the mRNA level of *c-Fos*, a gene expressed when neurons fire action potentials (Dragunow et al., 1989) (Figure 1C), and an increase in the expression of the senescence marker gene *Cdkn2a* (also known as *p16*) (Figure 1I).

Overall, these results implicate TRF2 in hippocampal neuron activity, which is progressively impaired during aging.



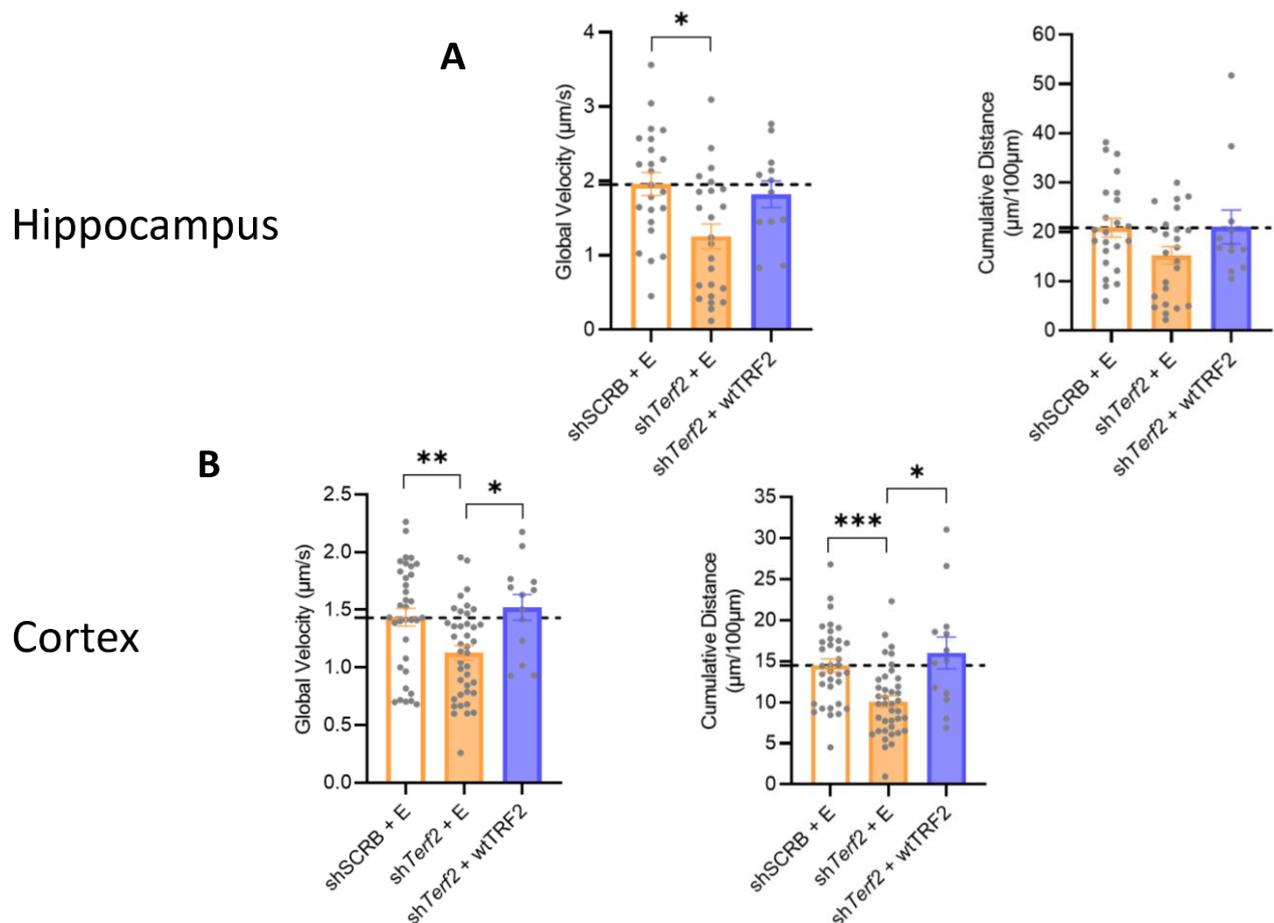


**Figure 1. Hippocampal *Terf2* downregulation during aging correlates with impaired neuronal activity**

(A-B) *Terf2* mRNA expression during aging in the (A) hippocampus [Hpc] and (B) hypothalamus [Hyt], respectively. (C) mRNA expression of the neuronal activity marker *cFOS* in the hippocampus during aging. (D-H) mRNA expression of the other Shelterin subunits in the hippocampus during aging, namely: (D) *Terf1*, (E) *Tinf2*, (F) *Acd*, (G) *Terf2ip*, and (H) *Pot1a*. (I) mRNA expression of the *bonafide* senescence marker *Cdkn2a*. Hippocampi were taken from mice of different ages (3-, 6-, 9-, 12-, 16-, 18-, 21-, 24-month-old mice) and mRNA was extracted and quantified via qPCR. For all graphs, each dot represents a mouse. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

### *Terf2* downregulation in primary neurons impairs vesicle axonal transport

To investigate the effect of *Terf2* downregulation on neuronal functions, we used primary hippocampal and cortical neuronal cell cultures established from mouse embryos (Figure S2A). The downregulation of *Terf2* mRNA was triggered by lentiviral transduction at day *in vitro* (DIV) 1 (Figure S2A-C). To assess the effect at DIV12/16 of TRF2 downregulation on axonal transport, we used microfluidic chamber devices to form networks of mature neurons expressing VAMP2-mCherry (Zala et al., 2013). In both types of neuron, TRF2 downregulation decreased the global velocity of these synaptic vesicles as well as the cumulative distance they traveled along the axons (Figure 2). Expressing wild-type TRF2 in *Terf2*-compromised neurons restored normal vesicle trafficking, thereby ruling out an off-target effect of *shTerf2*. These results hint for a role of TRF2 in neuronal vesicle trafficking and suggest that the reduction of TRF2 in neurons during aging affects intracellular dynamics and synaptic functions.



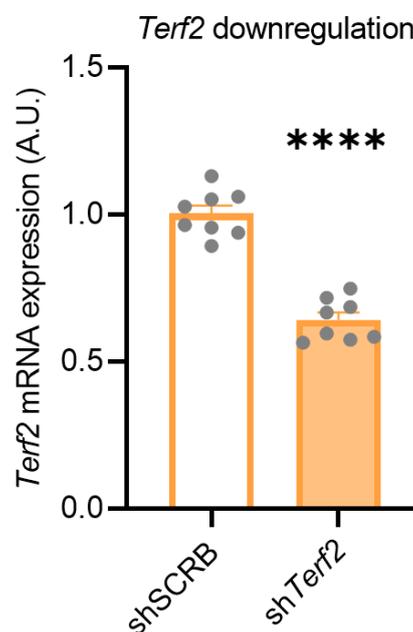
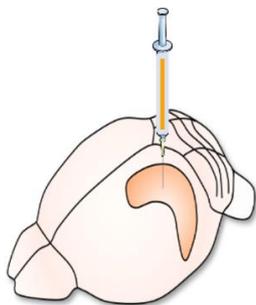
**Figure 2. *Terf2* downregulation in primary neurons impairs vesicle axonal transport**

(A-B) Quantification of velocity and cumulative distance run by the VAMP2 vesicles in (A) hippocampal and (B) cortical primary neurons. The following conditions were tested: shSCRb plus empty vector (shSCRb + E), sh*Terf2* plus empty vector (sh*Terf2* + E), and sh*Terf2* rescued by wild type TRF2 (sh*Terf2* + wtTRF2). Each dot represents the value relative to a single axon. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

### *Terf2* downregulation in hippocampal neurons impairs memory

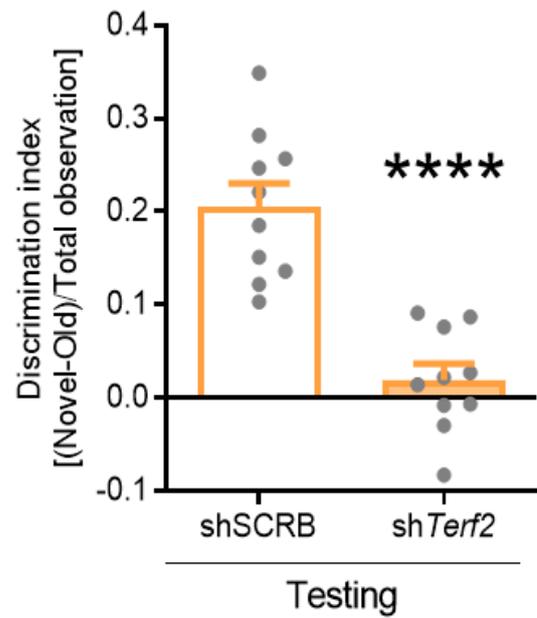
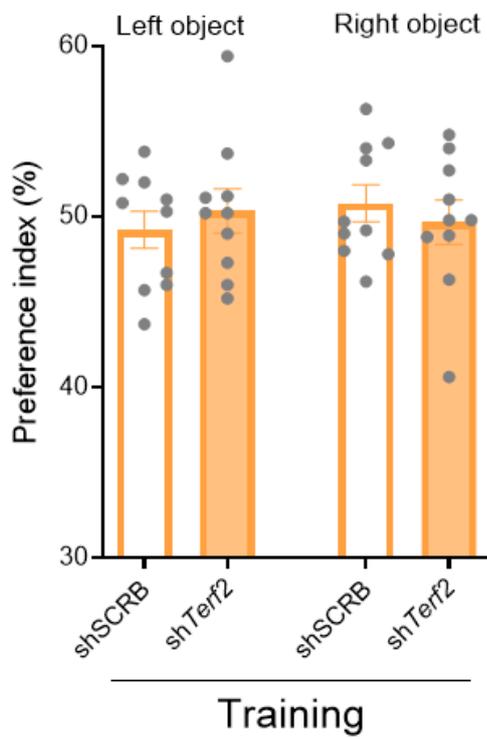
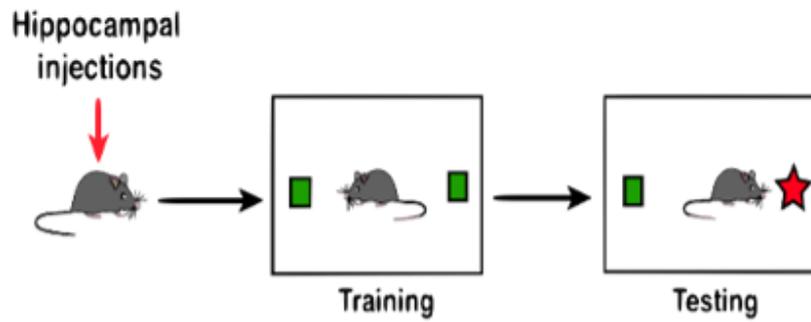
To assess *in vivo* the role of TRF2 in hippocampal neuronal functions, we stereotactically injected AAV9-*shTerf2*-eSYN into the hippocampi of young mice (3 months) to selectively downregulate *Terf2* in mature neurons. Five weeks later we performed three behavioral tests to assess the effect of *Terf2* downregulation on memory. In the novel object recognition (NOR) episodic memory test, a mouse is first placed in a box with two identical objects, and the next day is placed in the same box where one of the two objects has been replaced by a new one. Since mice are attracted by novelty, if they remember the object from the day before, they should spend more time exploring the new object. If they do not remember, they will spend equal amounts of time exploring the two objects. In the contextual fear conditioning (CFC) contextual memory test, a mouse is placed in a cage where it receives electric pulses. The next day, the mouse is taken to the same cage and if it remembers the shock of the day before, it will be scared and spend more time being still (referred to as freezing). In the Morris water maze (MWM) spatial memory test, the task is to learn and remember the localization of a platform hidden under the surface of the opaque water in a tank. A decreased *Terf2* mRNA level caused by AAV injection was confirmed in mice euthanized after behavioral testing (Figure 3A). Mice with a decreased *Terf2* mRNA level performed worse in the NOR and CFC tests (Figure 3B-C), but their performance in the MWM was unaffected (Figure 3D). Therefore, the expression of *Terf2* in hippocampal neurons is required for episodic and contextual but not spatial memory.

#### **A** Hippocampal injections of AAV9-eSYN-*shTerf2*

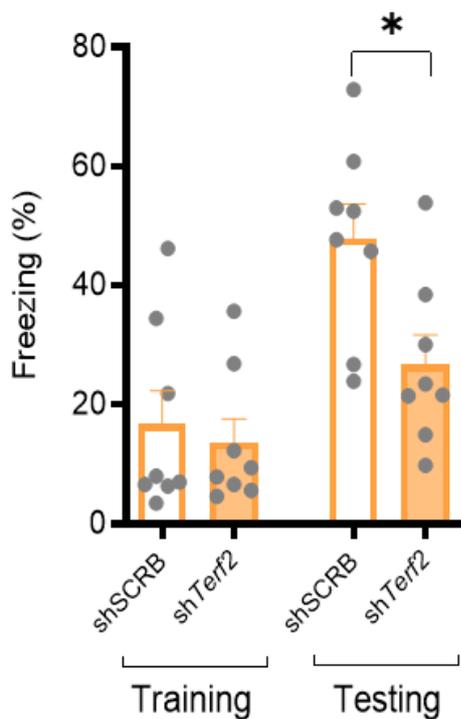
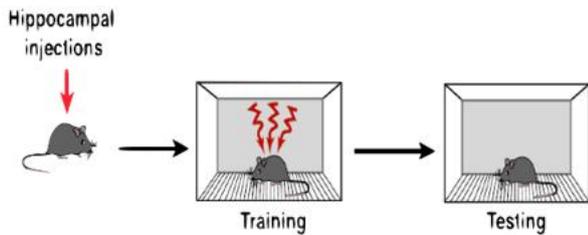


**B**

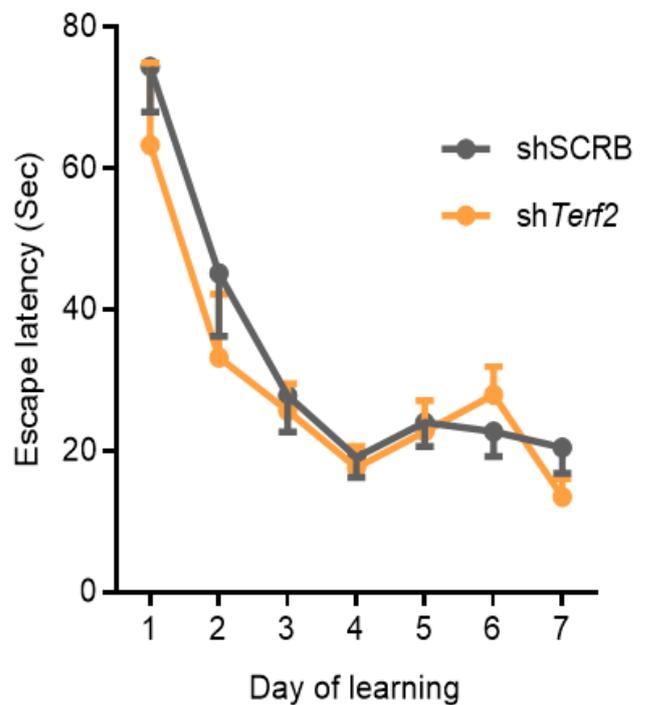
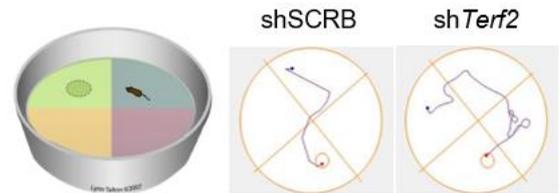
### Novel Object Recognition (Episodic memory)



**C** Contextual Fear Conditioning  
(Contextual memory)



**D** Morris Water Maze  
(Spatial memory)



**Figure 3. *Terf2* downregulation in hippocampal mature neurons impairs episodic and contextual memory**

(A) TRF2 downregulation following hippocampal stereotaxic injections in 3-month-old mice of either AAV-*Scramble*-shRNA (shSCRB) or AAV-*Terf2*-shRNA (shTerf2).

(B) Novel object recognition (NOR) (Training and testing – first and second panel, respectively), performed 5 weeks after the stereotaxic injections with either AAV-*Scramble*-shRNA or AAV-*Terf2*-shRNA. The memory performance (Discrimination index) was measured 24h after the training phase.

(C) Contextual fear conditioning (CFC) performed 6 weeks after the stereotaxic injections with either AAV-*Scramble*-shRNA or AAV-*Terf2*-shRNA. The memory performance (Freezing) was measured 24h after the training phase. For all graphs (A-C), each dot represents a mouse.

(D) Morris water maze (MWM) performed 7 weeks after the stereotaxic injections with either AAV-*Scramble*-shRNA (shSCRB) or AAV-*Terf2*-shRNA (shTerf2). The memory performance (Escape latency) was measured for 7 days, starting 24h after the training phase, with a two-day break after day 4. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

## *Terf2* downregulation in hippocampal neurons modulates the expression of proteins involved in synaptic plasticity

To evaluate the role of neuronal TRF2 in hippocampal function, we performed a proteomic analysis of hippocampi with *Terf2* downregulation, which revealed 69 downregulated proteins (including TRF2) and 127 upregulated proteins (Figure S3A) that we submitted to functional analysis. REACTOME analysis indicated enriched pathways relevant to synaptic plasticity (neurexins and neuroligins, activation of NMDA receptors and post-synaptic events, axon guidance, and voltage-gated potassium channels) (Figure 4A-B). Next, we assessed the expression and activity of Calcium-calmodulin (CaM)-dependent protein kinase II (CaMKII), which is central to synaptic plasticity, learning and memory. In agreement with the change in synaptic plasticity, the level and phosphorylation of CaMKII were decreased in *Terf2*-compromised hippocampi (Figure 4C-G).

Importantly, we could observe that knocking down *Terf2* causes the decreased expression of the immediate-early gene *cFOS*, both at the mRNA and protein level (Figure 4H-J). Noteworthy, *Terf2* downregulation also affects the mRNA expression of the neuronal gene repressor REST, and of the presynaptic factor SNAP25 (Figure 4H).

Because the autophagy-mediated induction of synaptic plasticity in hippocampal neurons is required for memory (Glatigny et al., 2019), we analyzed the levels of proteins involved in autophagy. The levels of all of these proteins in hippocampi were unaffected by the downregulation of *Terf2* (Figure S3B-E), which makes it unlikely that TRF2 controls memory by mediating the autophagy process.

**A**

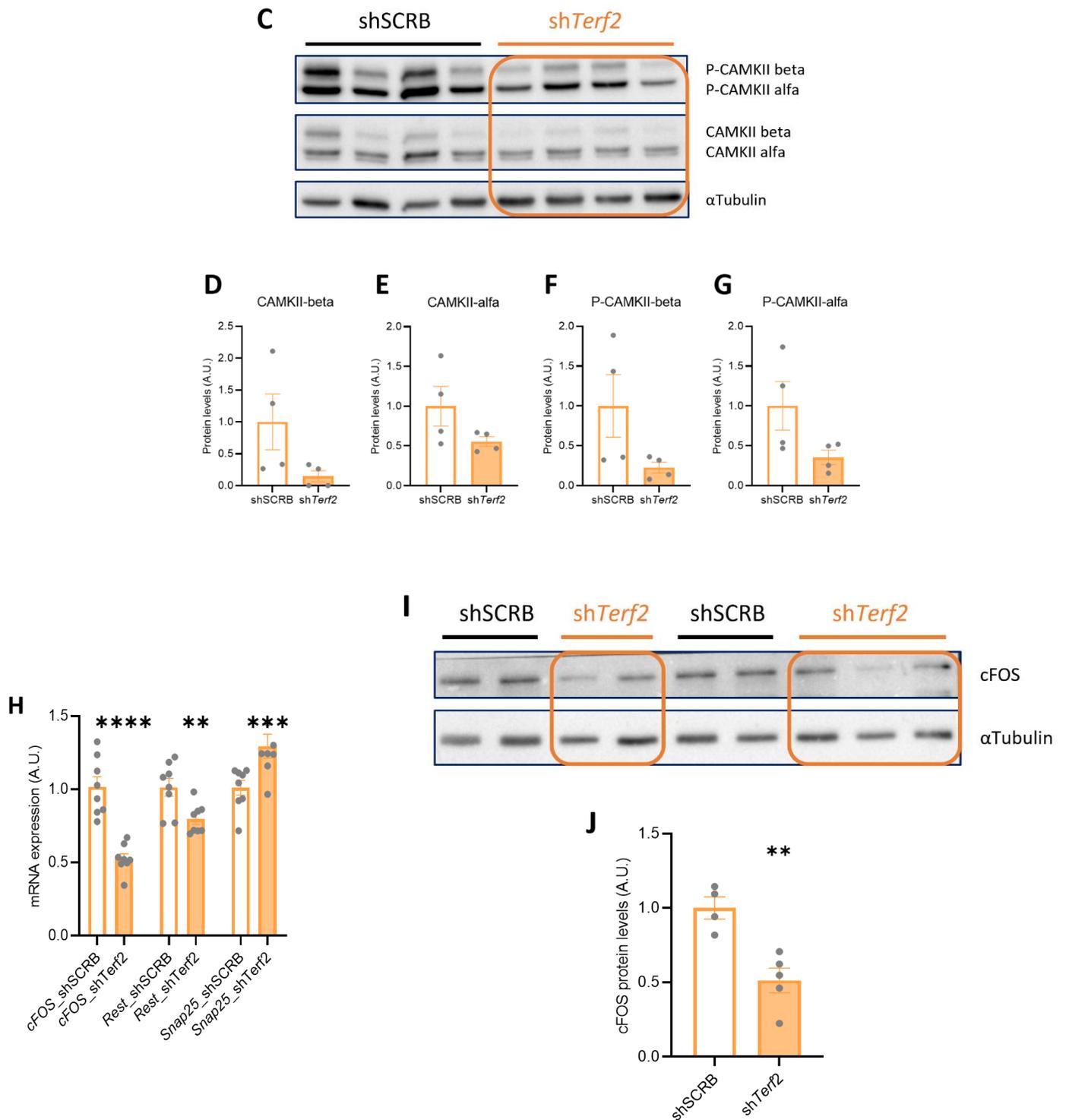
### Downregulated pathways

Molybdenum cofactor biosynthesis
RUNX2 (regulates the expression of genes involved in differentiation of myeloid cells)
RUNX1 (regulates the expression of genes involved in differentiation of myeloid cells)
Signaling by receptor tyrosine kinases
PECAM1 interactions
NGF-stimulated transcription
Platelet degranulation
Serine biosynthesis
Response to elevated platelet cytosolic Ca <sup>2+</sup>
Nuclear events (kinase and transcription factor activation)

**B**

### Upregulated pathways

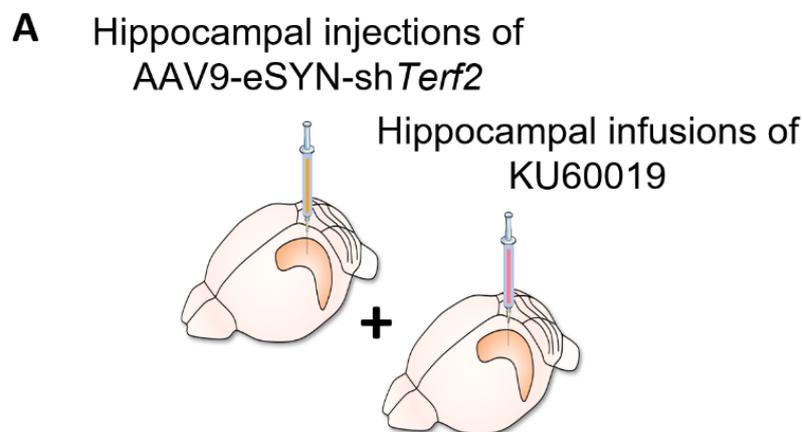
Neuronal system
Neurexins and neuroligins
Phase 1-inactivation of fast Na <sup>+</sup> channels
Activation of NMDA receptors and postsynaptic events
G-protein beta-gamma signalling
Activation of RAC1 downstream of NMDARs
Axon guidance
Regulation of TP53 degradation
Voltage-gated potassium channels
Nuclear receptor transcription pathway

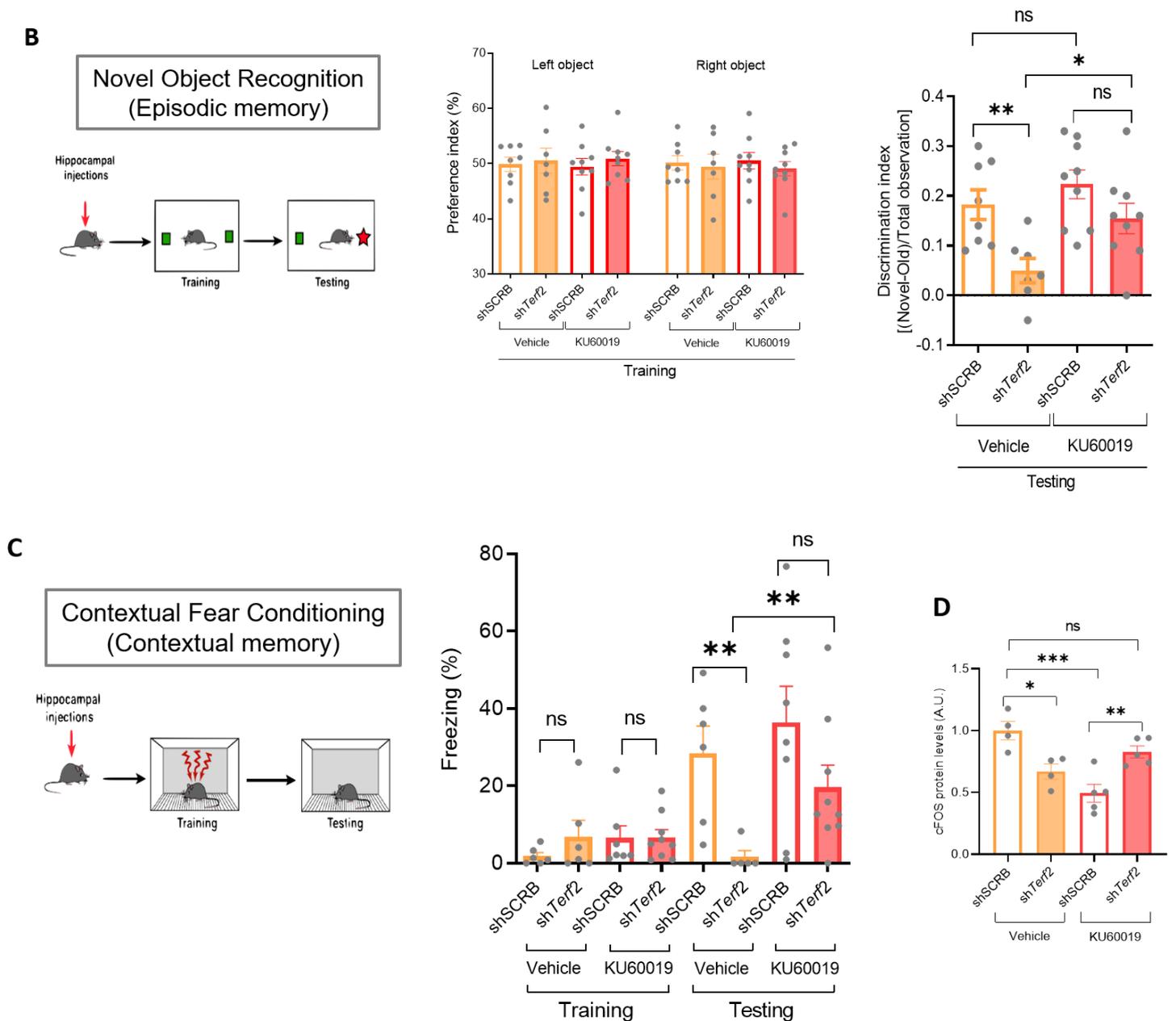


**Figure 4. *Terf2* downregulation in hippocampal mature neurons modulates the expression of genes involved in neuronal functions** (A-B) Functional analysis (Reactome) of (A) downregulated or (B) upregulated pathways as determined by proteomics analysis (the first ten pathways for each group are listed). (C) protein levels of CAMKII isoforms (alfa and beta) and their phosphorylated forms. (D-G) Quantification of the Western blot shown in figure (C): (D) CAMKII-beta, (E) CAMKII-alfa, (F) P-CAMKII-beta, and (G) P-CAMKII-alfa, respectively. (H) mRNA expression of *cFOS*, *Rest* and *Snap25* upon TRF2 downregulation. (I) Protein levels of *cFOS* upon TRF2 downregulation. (J) Quantification of the Western blot shown in (I). All mice used for (A-J) were 3-month-old when stereotaxically injected with either AAV-*Terf2*-shRNA (shTerf2) or AAV-*Scramble*-shRNA (shSCRB) and were sacrificed after the behavioral tests. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

### *ATM inhibition prevents the negative impact of Terf2 downregulation on memory*

Because TRF2 suppresses the ATM-dependent DNA-damage response (DDR) (Denchi & De Lange, 2007) (Mendez-bermudez et al., 2018), we tested whether ATM activation was upstream the cognitive impairment caused by *Terf2* downregulation. Therefore, we used KU60019, an inhibitor of ATM that is stable for a long time at the mouse body temperature, which diffuses rapidly in the brain after delivery (Vecchio et al., 2015). The efficiency of ATM inhibition was estimated by analyzing the level of CHK2 phosphorylation, which was decreased by KU60019 (Figure S4B-C). Upon infusion of KU60019 into the hippocampus, *Terf2* mRNA downregulation did not lead to episodic or contextual memory impairment, in contrast to the vehicle control (Figure 5A-C). Contrary to the vehicle control, c-Fos expression was not decreased by *Terf2* mRNA downregulation upon KU60019 infusion (Figure 5D, Figure S4D). This suggests that inhibition of ATM reverses the hippocampal changes caused by *Terf2* downregulation. Infusion of KU60019 into the shScramble control (shSCR) led to downregulation of c-Fos, indicating that inhibition of ATM interferes with hippocampal functions. However, KU60019 did not impair the memory of control mice injected with AAVs carrying shSCR (Figure 5B-C), showing that the negative impact of KU60019 on hippocampal functions is limited. We conclude that inhibition of ATM prevents the deleterious effect of *Terf2* downregulation on memory while it slightly interferes with other hippocampal functions, as expected from its roles in neuron biology (Pizzamiglio et al., 2020).





**Figure 5. Cognitive impairment due to *Terf2* downregulation is rescued by ATM inhibition**

(A) Schematic of TRF2 downregulation due to hippocampal stereotaxic injections in 3-month-old mice of either AAV-*Scramble*-shRNA (shSCRB) or AAV-*Terf2*-shRNA (shTerf2), and either infusion of the ATM inhibitor (KU60019) or not (Vehicle).

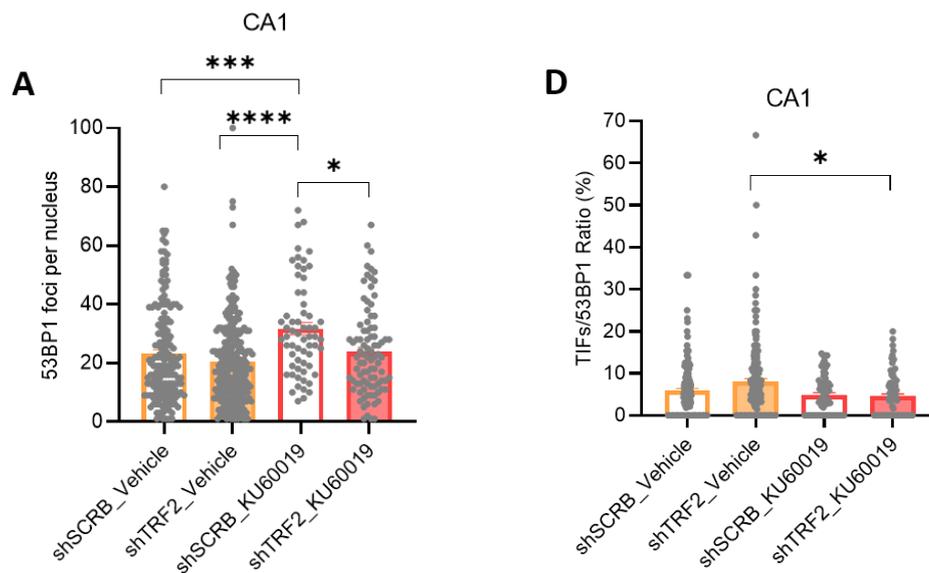
(B) Novel object recognition (NOR) (Training and testing – first and second panel, respectively), performed 5 weeks after the stereotaxic injections with either AAV-*Scramble*-shRNA (shSCRB) or AAV-*Terf2*-shRNA (shTerf2), followed by either ATM inhibitor infusion (KU60019) or not (Vehicle). The memory performance (Discrimination index) was measured 24h after the training phase.

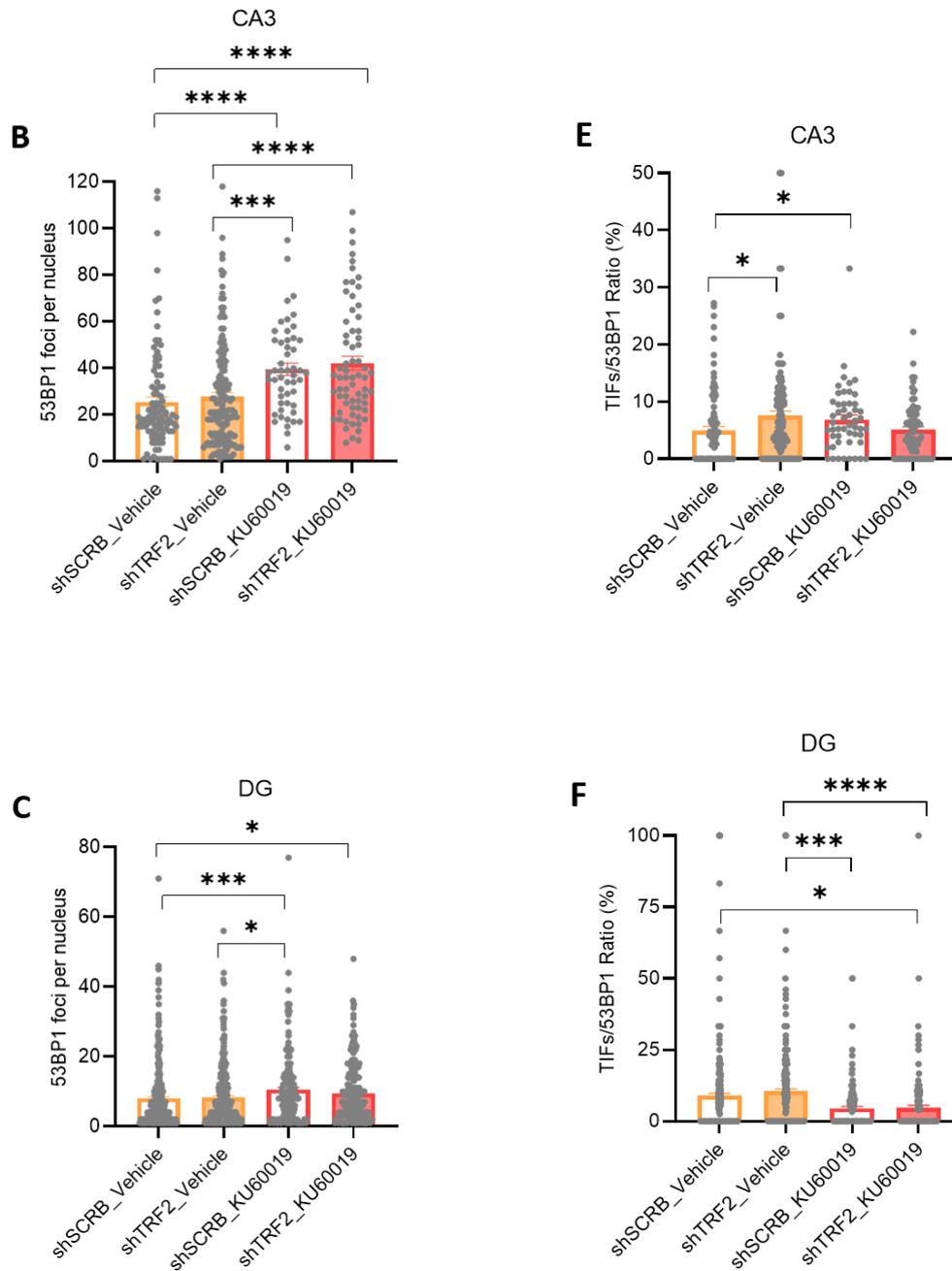
(C) Contextual fear conditioning (CFC) performed 6 weeks after the stereotaxic injections with either AAV-*Scramble*-shRNA (shSCRB) or AAV-*Terf2*-shRNA (shTerf2), followed by either ATM inhibitor infusion (KU60019) or not (Vehicle). The memory performance (Freezing time) was measured 24h after the training phase. For all graphs, each dot represents a mouse. (D) cFOS protein levels upon TRF2 downregulation (shTerf2) and ATM inhibition (KU60019). All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

### *Terf2* downregulation causes ATM-dependent telomeric DNA damage but not senescence

Next, we monitored the DDR and telomere damage in TRF2-compromised hippocampi. First, we measured the global DDR by enumerating 53BP1 foci in neuronal nuclei in the CA1, CA3, and DG. Upon *Terf2* mRNA downregulation, there was no increase in the global DDR. However, KU60019 induced a slight but detectable increase of DDR in the three areas of the hippocampus (Figure 6A-C). We measured the rate of telomere damage as the percentage of DDR foci colocalizing with a PNA telomeric probe by confocal microscopy (Figure 6D-F, Figure S5A). Telomere damage was slightly increased by *Terf2* downregulation in CA3, which was not observed upon ATM inhibition (Figure 6E, Figure S5A), in agreement with the TRF2-mediated protection against ATM activation at telomeres (Denchi & De Lange, 2007).

Since telomeric damage triggers senescence (D'Adda Di Fagagna et al., 2003), we measured the expression of factors involved in it: the cell-cycle checkpoint genes *Cdkn2a* (encoding p16) and *Cdkn1a* (p21), p53 and its phosphorylated form, *Lmnb1* (encoding Lamin B1) and *MMP2* (encoding the SASP factor MMP2). The expression of none of them was altered in hippocampi with *Terf2* downregulation (Figure S6A-F). Although it is possible that senescence of a small subset of cells is triggered by TRF2 downregulation, these results exclude a major effect of *Terf2* downregulation in the hippocampus on cellular senescence.



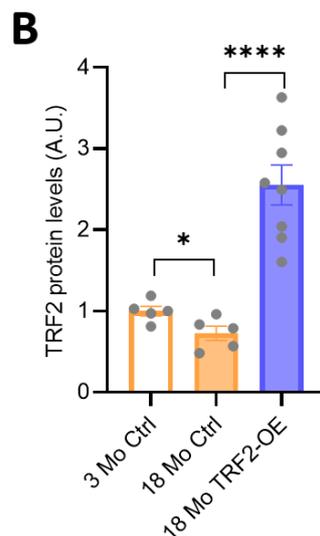
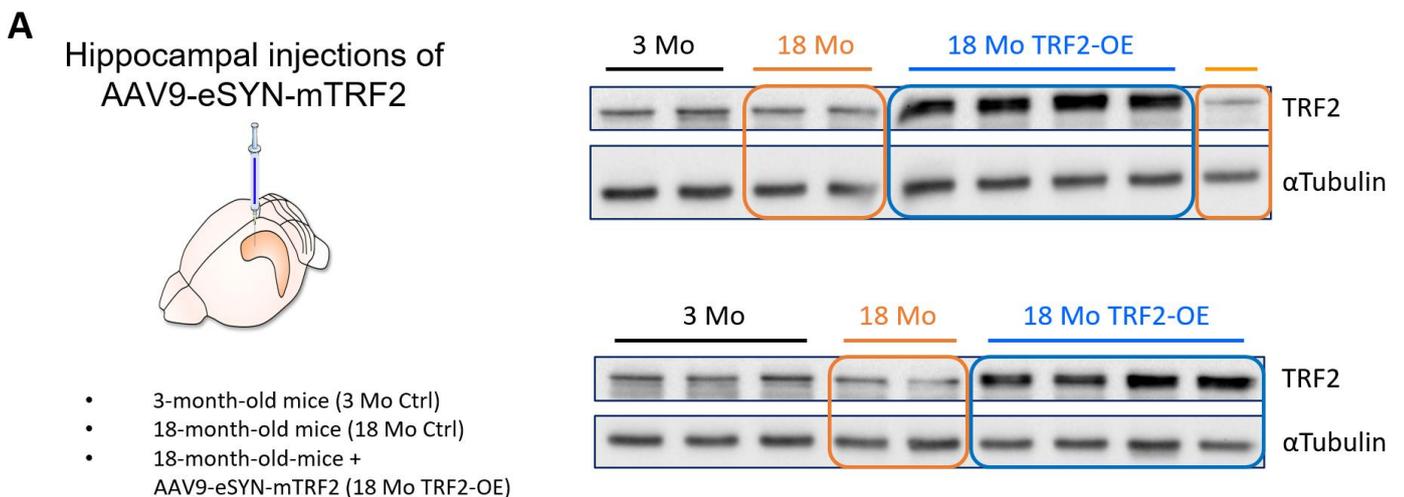


**Figure 6. *Terf2* downregulation causes ATM-dependent telomeric DNA damage**

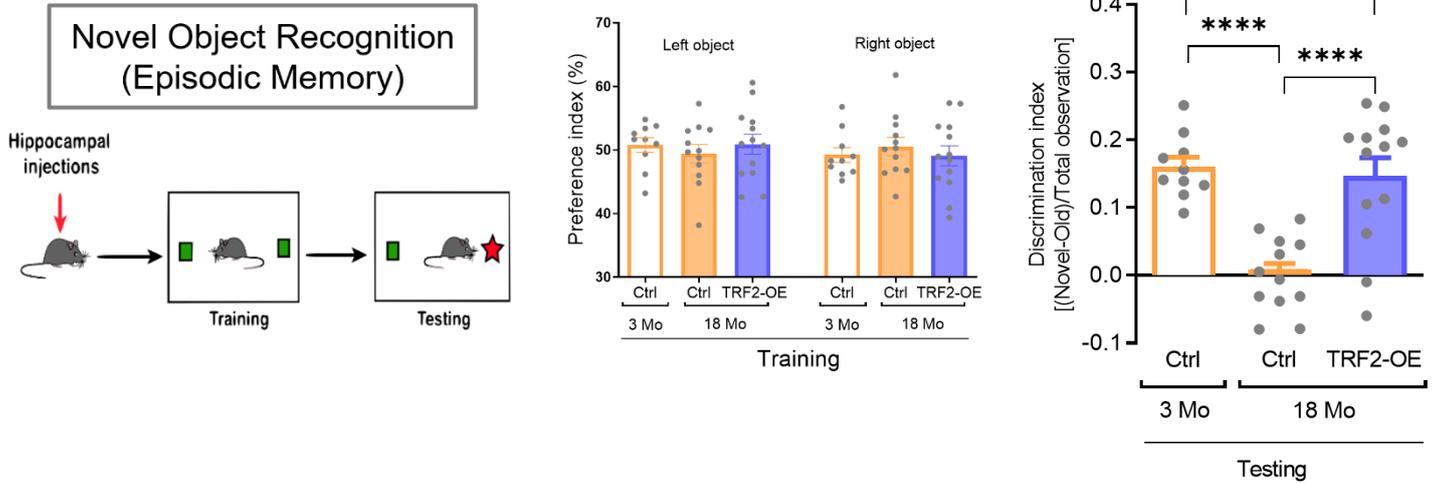
(A-C) Quantification of global DNA damage upon TRF2 downregulation (sh*Terf2*) and ATM inhibition (KU60019) in (A) CA1, (B) CA3, and (C) DG of young mice. (D-F) Quantification of telomere-specific DNA damage upon TRF2 downregulation (sh*Terf2*) and ATM inhibition (KU60019) in (D) CA1, (E) CA3, and (F) DG of young mice. Telomere-specific DNA damage (TIF = Telomere dysfunction-induced foci) is detected as the super imposition between 53BP1 foci and telomere-specific probes. Each dot in the graphs (A-F) represents a nucleus. The staining for the counting was performed on brain sections of mice stereotaxically injected with either AAV-Scramble-shRNA (shSCRB) or AAV-*Terf2*-shRNA (sh*Terf2*) at 3 months of age, followed by either ATM inhibitor infusion (KU60019) or not (Vehicle). These mice underwent the behavioral tests shown in Figure 5. For the quantification of this experiment, the following numbers of mice were used: shSCRB\_Vehicle, N=5; sh*Terf2*\_Vehicle, N=5; shSCRB\_KU60019, N=4; sh*Terf2*\_KU60019, N=4. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

### *Terf2* overexpression in old mice rescues the age-dependent cognitive impairment

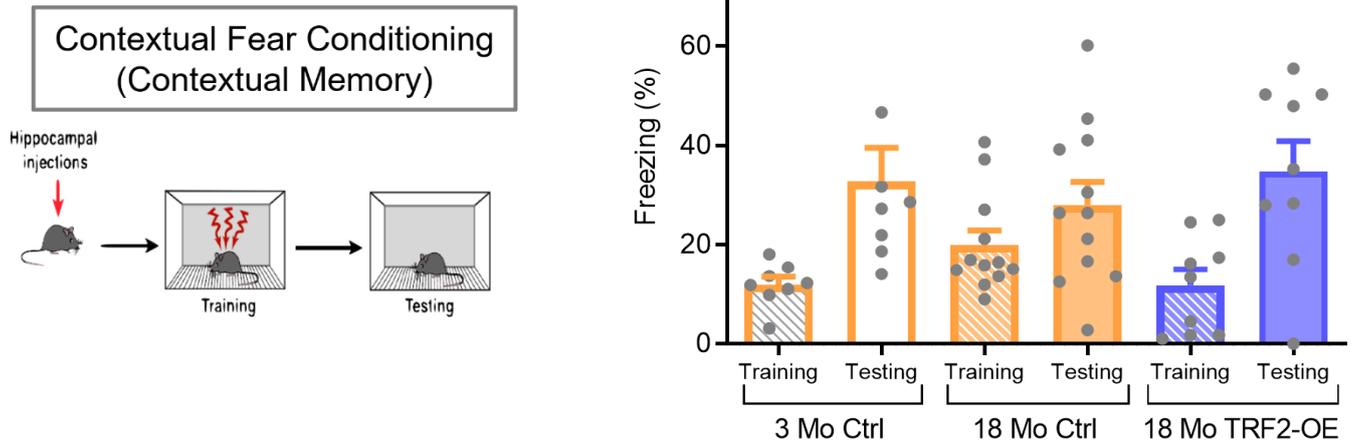
Finally, we investigated the effect of neuronal *Terf2* expression modulation on age-related neuronal and cognitive deficits. Specifically, we tested whether restoration of the TRF2 level in the aged hippocampus is necessary and sufficient to improve the age-related neuronal activity and memory decline. To this end, we stereotactically injected 18-month-old mice with AAV9 expressing the *Terf2* cDNA under the control of the eSYN promoter. The old mice exhibited a decreased level of TRF2 in their hippocampi (Figure 7A-B), confirming at the protein level the *Terf2* downregulation observed during hippocampal aging (Figure 1A). *Terf2* overexpression increased the level of TRF2 in old mice to a greater extent than that in young mice (Figure 7A-B). The old mice exhibited impaired episodic and contextual memory capacities (Figure 7C-D). These deficits were fully rescued upon *Terf2* overexpression in their hippocampi (Figure 7C-D).



C



D



**Figure 7. *Terf2* overexpression in hippocampal mature neurons rescues episodic and contextual memory of old mice**

(A) TRF2 protein levels in young (3 Mo) and old (18 Mo) mice following hippocampal stereotaxic injections of either AAV-eSYN-Empty (Ctrl) or AAV-eSYN-mTRF2 overexpressing TRF2 (TRF2-OE). (B) Quantification of the Western blots shown in (A). (C) Novel object recognition (NOR) performed with young and old mice (Training and testing – first and second panel, respectively), 5 weeks after stereotaxic injections with either AAV-eSYN-Empty (Ctrl) or AAV-eSYN-mTRF2 (TRF2-OE). The memory performance (Discrimination index) was measured 24h after the training phase. (D) Contextual fear conditioning (CFC) performed with young and old mice, 6 weeks after stereotaxic injections with either AAV-eSYN-Empty (Ctrl) or AAV-eSYN-mTRF2 (TRF2-OE). The memory performance (Freezing) was measured 24h after the training phase. For all graphs (B-D), each dot represents a mouse. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

## Discussion

Advancing age involves not only increased susceptibility to disease but also to a loss of intrinsic capacities, even in healthy individuals (defined by the WHO as mobility, cognition, vitality, psychological and sensory capacity – <https://www.who.int/publications/i/item/WHO-FWC-ALC-17.2>). This underscores the importance of understanding at the molecular level the effect of aging on cellular systems.

Here we investigated the mechanisms responsible for memory loss during physiological aging in mice. We unveiled that the expression of the telomeric factor TRF2 in hippocampal neurons acts as a limiting factor during aging, thereby sustaining the memory functions of the hippocampus. The *Terf2* mRNA and protein levels in the hippocampus were decreased in aged mice.

We evaluated the effect of this decrease by suppressing *Terf2* expression in the hippocampal neurons of young mice by injecting AAV9 expressing sh*Terf2* under the control of the eSYN promoter (specific to mature neurons). The resulting *Terf2* downregulation in young mice (~50%) was similar to that in old mice. The consequence of *Terf2* downregulation was a marked reduction in episodic and contextual memory capacities, whereas spatial memory was unaffected.

The complete restoration of episodic and contextual memory in old mice upon overexpression of *Terf2* in hippocampal neurons demonstrates that the TRF2 level in hippocampal neurons controls memory capacity during aging.

The differential effect of TRF2 on memory types can be explained by a regional effect of *Terf2* downregulation — spatial memory mainly involving the DG (Clelland et al., 2009) (Trouche et al., 2009) (Masachs et al., 2021); episodic and contextual memory mainly involving CA3 and CA1, respectively (Kesner, 2007b) (Ji & Maren, 2008). This effect may also be due to a different role of TRF2 in the distinct regions of the hippocampus or to an unequal AAV diffusion to the different hippocampal areas upon injection. Moreover, the fact that DG neurogenesis is pivotal for spatial memory and that the AAV-mediated *Terf2* downregulation was specifically targeting only postmitotic neurons, could explain why spatial memory was not affected. Nevertheless, this differential effect on memory types indicates that the effects of TRF2 downregulation in young mice are unlikely to be mediated by an effect on DG.

*Terf2* overexpression rejuvenated the hippocampi of old mice by restoring their memory capacity, suggesting that age-related *Terf2* downregulation does not lead to irreversible events, such as

neuron loss. Indeed, an effect of *Terf2* overexpression on neurogenesis is unlikely because the eSYN promoter is specific to mature neurons.

Thereafter, we analyzed the mechanism by which neuronal TRF2 affects memory. We inhibited the main target of TRF2, ATM kinase, by infusing KU60019 into hippocampi with *Terf2* downregulation. This prevented the effect of *Terf2* downregulation on memory capacity. We conclude that the effect of *Terf2* downregulation is mediated by activation of ATM kinase activity.

Classically, TRF2 inhibits ATM activity at telomeres to prevent recognition of these structures as accidental breaks. Indeed, neurons with TRF2 downregulation showed increased ATM-dependent telomeric DNA damages. However, the degree of this telomeric damage was limited and it was not associated with an increase in the DDR. Moreover, we did not detect an increase in senescence due to TRF2 downregulation in hippocampi. Therefore, although a role for telomeres in memorization cannot be ruled out, the role of the TRF2-ATM axis in memory needs to be explored in the context of the multiple roles of ATM in neuronal functions (Pizzamiglio et al., 2020).

Because synaptic plasticity is central to memorization, we examined the effect of TRF2 downregulation on this neuronal function. *In vitro*, downregulation of TRF2 slows down the transport of synaptic vesicles. *In vivo*, the expression of several components of pathways related to synaptic plasticity and neuronal activity are altered when *Terf2* is downregulated; these include products of several immediate-early genes (*c-Fos*, *Egr3*, *Nptx2*, and *Vgf*) and proteins involved in synaptic functioning and glutamate-receptor activation (e.g., CAMKII, Shank3, Homer2, Dlg3, and Slc1a1).

To the best of our knowledge, this is the first report of an ATM-dependent aging effect of the reduction of TRF2 expression in a post-mitotic cell. Investigation of the link between ATM and synaptic plasticity pathways during age-related TRF2 downregulation would provide insight about the relationship between telomeres and physiological changes during brain aging (e.g., memory decline).

This study was limited by its focus on the neuronal effect of TRF2. TRF2 may also play a role in other cell types of the hippocampus. It is therefore necessary to investigate the cellular specificity of the decrease in the expression of TRF2 in the hippocampus during aging and to modulate the expression of TRF2 in these cells.

In conclusion, our results provide insight into the roles of telomeres and TRF2 in the central nervous system and in the aging of postmitotic cells. They also reveal a novel mechanism of hippocampal aging based on *Terf2* downregulation, and the role of this telomeric protein in neuronal function by

suppressing ATM activation. Our findings will promote the development of pharmacological interventions to counteract the memory decline that accompanies the aging of the hippocampus.

# Materials & Methods

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Animals

All experiments were performed with C57BL/6J WT male mice (purchased from Janvier Laboratory, Le Genest-Saint-Isle, France). All mice were either 3- or 18-month-old at the beginning of the experiments. For all experiments, littermate controls were used. Upon arrival, mice were housed for at least two weeks before any behavioral or molecular test. Mice were housed five per cage in polycarbonate cages (35.5 x 18 x 12.5 cm), under a 12-hour light/dark cycle with *ad libitum* access to food and water prior to experimentation. All behavioral tests were performed between 10am and 5pm. In all experiments, animals were randomly assigned to treatment groups. Group sizes were determined after performing a power calculation to lead to an 80% chance of detecting a significant difference ( $p \leq 0.05$ ). All behavioral experiments were performed in accordance with the European Union directive for experimental animal use (2010/63/EU) and local ethical committee procedures and protocols (PROJET 2020120411099726 – V8 APAFiS # 30503).

## METHODS DETAILS

### Vesicle axonal transport *in vitro* assay

Microfluidic chambers were designed and built as previously described (Virlogeux *et al*, *Cell Rep.* 2018). Cortical and hippocampal primary cultures were set from E15.5 WT embryos. Dissociated cortical or hippocampal neurons were resuspended and seeded in the chambers at a final density of  $\sim 7000$  cells/mm<sup>2</sup> using growing medium (Neurobasal medium supplemented with 2% B27, 2 mM Glutamax, and 1% penicillin/streptomycin) and then placed at 37°C and 5% CO<sub>2</sub>. At DIV(day-in-vitro)<sup>1</sup>, neurons were triple-infected with lentiviruses bearing the following constructs: i) LV.CMV.VAMP2-mCherry + pLKO-shSCRB + pWPIR-Empty-eGFP; ii) LV.CMV.VAMP2-mCherry + pLKO-shTerf2 + pWPIR-Empty-eGFP; iii) LV.CMV.VAMP2-mCherry + pLKO-shTerf2 + pWPIR-mTerf2-eGFP, for 24 hours. The sequence targeted to knock down TRF2 in the *in vitro* cultures was the following: CCGGGTCCCATGACAATAAGCAGATCTCGAGATCTGCTTATTGTCATGGGACTTTTTG.

VAMP2-mCherry trafficking was observed at DIV12 and DIV16 for cortical and hippocampal neurons, respectively. An inverted microscope (Axio Observer, Zeiss) coupled to a spinning-disk confocal system (CSU-W1-T3, Yokogawa) and connected to wide-field electron-multiplying CCD camera (ProEM+1024, Princeton Instrument) was used, and neurons were kept at 37°C and 5% CO<sub>2</sub> for the whole recording. Images of VAMP2-mCherry trafficking were taken every 200 ms for a time frame of 30 s (63× oil-immersion objective, 1.46 NA). The following number of axons from at least two independent neuronal cultures were analyzed with the KymoToolBox plugin for ImageJ (Zala *et al*, *Cell* 2013):  $N = 20$  shSCRB + E,  $N = 24$  shTerf2 + E,  $N = 12$  shTerf2 + wtTRF2, for the hippocampus, and  $N = 35$  shSCRB + E,  $N = 40$  shTerf2 + E,  $N = 13$  shTerf2 + wtTRF2, for the cortex.

### Stereotaxic surgery

Mice were anesthetized via intraperitoneal injection of ketamine hydrochloride (20 mg/kg of body weight; Virbac) and xylazine (100 mg/kg of body weight; 2% Rompun, Bayer) and placed in a stereotaxic frame (900SL-KOPF). Ophthalmic eye ointment was applied to the cornea to prevent drying during surgery. The mouse head was shaved and Vetoquinol disinfectant was applied before the skin was longitudinally cut to expose the skull. The AAVs were injected bilaterally into the dorsal hippocampi using the following coordinates (from Bregma; Paxinos and Franklin, 2008):  $X = +/-1.4$  mm,  $Y = +2.0$  mm, and  $Z = -1.33$  mm. A 1  $\mu$ L volume of AAV was stereotaxically injected over 4 min (injection rate: 0.25  $\mu$ L per min). To limit the reflux along the injection track, the needle was maintained *in situ* for 4 min between each injection. After the injection, the scalp incision was sutured, and mice were kept under observation in a recovery chamber until fully motile.

### ICV cannula implantation

Three weeks after stereotaxic surgery, mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride (20 mg/kg of body weight; Virbac) and xylazine (100 mg/kg of body weight; 2% Rompun, Bayer) and placed in a stereotaxic frame (900SL; Kopf Instruments). To prevent drying during surgery, an ophthalmic eye ointment was applied to the cornea. The mouse head was shaved and Vetoquinol disinfectant was applied before the skin was longitudinally cut to expose the skull. Afterwards, a hole was made at the following coordinates:  $X = +/-1.4$  mm,  $Y = +2.0$  mm, and  $Z = -1.33$  mm. A cannula (C315GMN/SPC guide 26GA 42593 mini; cut 1.5mm below pedestal; Phymep) was inserted through the hole into the brain hippocampus and secured to the skull with dental

cement (Super-Bond C&B; Sun Medical). Lastly, the scalp incision was tightly sutured around the cannula, and mice were kept under observation in a recovery chamber until fully motile.

### KU60019 preparation and infusion

For ICV infusions, KU60019 (10 mg tube; Selleckchem) was dissolved to a concentration of 50 µg/µl in DMSO to prepare a stock solution. On the infusion day, the stock solution was further diluted (1:3000) in PBS to 16.7 ng/µl (30 µM). For thirty-three consecutive days, the experimenter gently restrained the mice and placed an ICV injector cannula in each hippocampus to infuse the drug (1.0 µl/side, at a rate of 1 µl/min) making use of a syringe pump (Harvard Apparatus) and a 1-ml plastic syringe connected to the injector cannula.

### Adeno-associated viruses expressing sh*Terf2*

Adeno-associated viruses (AAVs) expressing shRNA were purchased from Vector Biolabs (Malvern, PA). shRNAs to specifically target the TRF2 mRNA (AAV9-eSYN-eGFP-m-TRF2-shRNAmir(3) or scrambled non-targeting negative control (AAV9-eSYN-eGFP-scrmb-shRNAmir) were injected in a volume of 1 µL (bilaterally), five weeks prior to behavioral tests and brain tissue collection. The AAV titers were 3.4 and 5 x 10<sup>13</sup> genome copies(GC)/ml, respectively. AAV9-eSYN-eGFP-m-TRF2-shRNAmir(3) specifically targets mature neurons, being driven by the eSYN promoter, a hybrid promoter consisting of the 0.45-Kb human Synapsin-1 promoter fragment (hSYN1). The targeted sequence was the following:

GCTGCTGATCAGTTCTCAGCTTCTGAGTGTTTTGCCACTGACTGACACTCAGAATGAGAACTGATCAG

### Adeno-associated viruses overexpressing TRF2

AAVs overexpressing TRF2 (AAV9-eSYN-mTRF2(iso5)-2A-eGFP-WPRE) or bearing an empty vector (AAV9-eSYN-eGFP-WPRE) were purchased from Vector Biolabs at an estimated titer of 1<sup>13</sup> GC/ml. The viruses were injected in a volume of 1 µL (bilaterally), five weeks prior to behavioral tests and brain tissue collection. The sequence of the overexpressed TRF2 was the following >NM\_001371065.1 *Mus musculus* Telomeric repeat-binding factor 2 (*Terf2*), transcript variant 5, mRNA:

CCCAGAAGCCC GCGGGCGGCTCGGAACCCCGTCGGTATCATGGCTGCGGGAGCCGGGACAGCGGGCCCTG  
CTTCCGGCCCGGGCGTTGTGCGTGACCCGATGGCGTCACAGCCAAGGAAACGGCCAGTCGGGAGGGCGG  
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GGCGGGCGGCGAGCCGACGGGCATCGCGCAGCGGCGGGCGGGCTCGACGGGGGCGACACGAGCCAGGG  
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AAGTTCTATTTCCACGAGGCGCTGCGGGCCTTTCCGAGTAGCCGGTACCGGGACTTCAGGCAGATCCGGGAC  
ATCATGCAGGCGTTGCTTGTGAGGCCCTTGGGGAAGGAGCATAACGGTGTCCCGTTGCTGCGGGTTATGCAG  
TGTCTGTGCGCATTGAAGAAGGAGAAAATTTAGACTGTTCCCTTTGATATGGAGGCTGAGCTCACACCCTGG  
AATCAGCTATCAATGTGCTGGAGATGATTAACAGAGTTCACACTGACAGACTCTATGGTTGAATCCAGCAG  
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ACTTGGCCCACCCTGTTATCCAGAACTTTTCTATGAGGTCTTCCAGCAGAAGATGCTGCGTTTCTAGAGAGC  
CACCTGGATGACACGGAGCCCTACCTCCTCACGATGGCTAAAAAAGCTTTGAAATCTGAATCAGCTGCTTCAA  
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CAGAGGCCGCTTTTGCAAACTGGACCAGAAAGATCTGGTACTTGCTAATCTGGCATCCCCATCATCACCAGC  
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GACCGGCAGCCCAGGAACAGTCCCATGACAATAAGCAGATTGCTGTTGGAGGAGGACAGCCAGAGTACTGA  
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CCGGGGGAGAAGAAGCCCAAAGCATCCAAGACAAGTGGAACAGCCCTAACGGGCTTGAAGAAAAGGAA  
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ATGAAAAAATTGGCATGAACTGAGAAAGGGTTTCGTAGCCACAGGACTCACAAGAACACGGTTCTCAACA  
GTAGCCCCTGTGCTGTGACGCCTTTTCAGAAGCGGGACTGCGATGAGAATTGTGGCATCATCCTCCTGAGTC  
GGAGTCCCAGGACCCTCCCGTCACTGTTGCTGGAGATCATGGAGCTAAAAGCAAAGCCAGGACTAGCCG  
TGACCACAGCCGAGGCAACAGGCAGGCCACGGCTGGGACAGTGCCAGAAGATCATTAGTGTTCCTTTAG  
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GTCTCTCAGACACCCTTACAGACTGTCTTTTTAGCAAAAACAAAACAACAGAAAAAATTGAGTCAAGTGT  
TTTCTGTAATCTTTTTTAAATCTGAGAATGAGTTGTTCTACTTGTAACGTTCTTCATTAAGTCTTGCTTCTCT  
CAA

## Behavioral tests

### *Novel object recognition (NOR)*

The behavioral tests were recorded with a video camera. The test setting consisted of an arena with four plastic boxes (60 x 40 x 32 cm). Therefore, four animals at a time could be tested. Mice could not meet or see each other during the test. The light intensity was equal in all the parts of the arena. Two different objects were used, available in quadruplicate: (A) a blue ceramic cup (diameter; 6.5 cm; height: 7.5 cm) and (B) a clear transparent glass (diameter: 5.5 cm; height: 8.5 cm). The objects elicited equal levels of exploration as determined in pilot experiments and training phase. Mice were transported a short distance from the holding mouse facility to the testing room in their home cages and left undisturbed for at least two hours before the beginning of the test. Mice were always placed in the center of their box at the beginning of each exposure.

The NOR test consists of three phases over 3 days. On day 1 (habituation phase), mice were given 5 minutes to explore an empty arena, before being taken back to their home cage. On day 2 (training phase), mice were allowed to explore, for 10 minutes, two identical objects arranged in a symmetrical position inside the box, before being taken back to their home cage. On day 3 (testing phase), mice were given 15 minutes in the same box to explore two objects: a familiar object (the one of day 2) and a new one, placed in the same positions of day 2. The object used as a novelty (A or B), as well as the left/right localization of the objects were counterbalanced within each group. Between exposures, the boxes were cleaned with Phagospray, and the bedding was replaced. The following behaviors were considered as exploration of the objects: sniffing, touching the object with the nose or the front legs, and directing the nose to the object at a distance  $\leq 1$  cm. Investigation was not scored if the mouse was on top of the object or completely immobile. The preference index (time spent exploring the new object/total time spent exploring both objects) and the discrimination index ( $[(\text{time spent exploring the new object} - \text{time spent exploring the familiar object}) / (\text{total time spent exploring both objects})]$ ) were calculated. As control, preference index for the object location (right vs left) or for the object A versus B during the training phase were also measured. The behavior

was scored by an experimenter watching the videos and keeping track of the exploration time with a timer, unaware of the conditions being scored.

### *Contextual fear conditioning (CFC)*

Mice were left undisturbed for at least two hours before the beginning of the test. The conditioning chambers were obtained from Bioseb (France). Each chamber was located inside a larger, insulated plastic cabinet that provided protection from external light and noise (67 x 55 x 50 cm, Bioseb, France), and mice were tested individually in the conditioning chambers. The floors of the chamber consisted of twenty-seven stainless steel bars wired to a shock generator for the delivery of the foot-shock. The signals generated by the mice movements were recorded by a high sensitivity weight transducer system. The analog signal was transmitted to the Freezing software module through the load cell unit for the recording, and analysis of time active/time immobile (freezing) was performed. The CFC test was performed over two consecutive days. On day 1 (training), mice were placed in the conditioning chamber and received three foot-shocks (1 s, 0.5 mA), which were administered at 60, 120 and 180 s after the animals were placed in the chamber. On day 2 (testing), contextual fear memory was assessed by returning the mice to the conditioning chamber and measuring the freezing behavior during a 4-minute retention test, without delivering any foot-shock. Freezing behavior was considered to occur if the animals froze for at least 2 seconds. Freezing % ( $[\text{Freezing time}/\text{total time}] \times 100$ ) was automatically analyzed and scored using the Packwin 2.0 software (Bioseb, France).

### *Morris Water Maze (MWM)*

Animals were transported a short distance from the holding mouse facility to the testing room in their home cages and left undisturbed for at least two hours before the first trial. The apparatus consisted of a white circular tank (diameter: 200 cm; walls: 60 cm-high) filled with water (depth: 50 cm) maintained at  $22 \pm 1^\circ\text{C}$  and made opaque by the addition of a nontoxic white paint. A 12-cm round platform was hidden 1.0 cm below the surface of the water. The tank was set in a brightly lit room and geometric, high-contrast cues were placed on the inner wall of the tank. Each daily test consisted of four swimming trials for each mouse: the animals were placed in the tank facing the wall, in front of a specific visual cue and allowed to swim to the platform until 120 seconds had elapsed. A trial was considered over once the animals had reached the platform and remained on top of it for at least 5 seconds. After each trial, mice were placed back in their home cages for a 5-

minute inter-trial break. To prevent hypothermia, animals were gently dried with a paper towel after each trial. The starting point (visual cue) differed at each trial, and different starting points were used from day to day. The MWM was performed every day on a time frame of seven days with a two-day break after day 4. Mice movements were recorded using the ANY-maze recording software (Stoelting Co.) and the time needed to reach the platform (Escape latency) was recorded by the experimenter with a timer.

### Western blotting

Mouse dorsal hippocampi were dissected, snap-frozen and lysed in Pierce RIPA lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% Na deoxycholate, 0.1% SDS plus complete protease and phosphatase inhibitors; ThermoFisher). The lysates were loaded on a 12% SDS polyacrylamide gradient gel and transferred onto a PVDF membrane. The blots were blocked in Tris-buffered saline 0.1% Tween (TBST) 5% BSA and incubated O/N at 4°C and, according to the experiment, the following primary antibodies were used:

Rabbit anti-TRF2 (1:5000; NB110-57130, Novus Biologicals), mouse anti- $\alpha$ Tubulin (1:1000; T9026, Sigma), rabbit anti-cFOS (1:1000; ABE457, Merck), rabbit anti-P-CHK2 (1:1000; CST, 2665T), rabbit anti-GAPDH (1:1000; NB100-56875, Novus Biologicals), rabbit anti-Vinculin (1:1000; Sigma, V9131), rabbit anti-CAMKII (1:1000; 3362S, CST), rabbit anti-P-CAMKII (1:1000; 12716S, CST), mouse anti-Beclin-1 (1:1000; 612113, BD Transduction Laboratories), rabbit anti-LC3 (1:10000; L7543, Sigma), mouse anti-ATG5 (1:500; 0262-100/ATG5-7C6, Nanotools), rabbit anti-VPS34 (1:1000; 4263, CST), and guinea pig anti-p62 (1:2000; GP62-C, Progen). After the O/N incubation and three 10-minute TBST washes, the membranes were incubated, according to each specific Western blot, with the following secondary antibodies: anti-mouse IgG, HRP-linked antibody (1:10000; 7076, CST), anti-rabbit IgG, HRP-linked antibody (1:10000; 7074, CST) and anti-guinea pig IgG, HRP-linked antibody (1:10000; A5545, sigma) for 1h at RT°. The bands' signals were revealed using an ECL kit for protein detection (Clarity Western ECL Substrate, BioRad). Selected films were scanned and quantified using BioRad Image Lab software (Version 5.2).  $\alpha$ Tubulin or GAPDH bands were used for normalization. All Western blot analyses were performed on freshly prepared hippocampal lysates.

### Semiquantitative RT-PCR

Brain tissues were immediately flash-frozen after dissection and total RNA was isolated with TRIzol Reagent using a homogenizer. Single-strand cDNA was synthesized from total RNA (2 mg) by using SuperScript II Reverse Transcriptase. qRT-PCR was performed using iTAQ SYBR Green (BioRad). According to the different experiments, the following primers were used:

*Terf2* (F: AAGCCCAGAGCTCTCAACC; R: TCCACTTGTCTTTGGATGCTT)  
*Terf1* (F: GCGTCTAAGGATAGGCCAGAT; R: GTAGACTGCTGGCCATTAAC)  
*Pot1a* (F: AAGGACCGTGGACTCCTTC; R: ACATACTGCAGCGGCACA)  
*Terf2ip* (F: CCTCCCAGAAGCTCAAACG; R: CTGGCGCTCTTGTCTGT)  
*Acd* (F: TCGGATCCTAGCTCTCCTCA; R: GTCAGGGGTGATGGTTGAA)  
*Tinf2* (F: TGGTTTTGGACTCTGATGAGG; R: GCAAAGGCTTTTGGTAAGGA)  
*Rest* (F: CATGGCCTTAACCAACGACAT; R: CGACCAGGTAATCGCAGCAG)  
*Snap25* (F: CAACTGGAACGCATTGAGGAA; R: GGCCACTACTCCATCCTGATTAT)  
*cFOS* (F: CAGAGCGCAGAGCATCGCCA; R: CGATTCCGCCACTTGGCTGC)  
*FOXO3a* (F: GCTAAGCAGGCCTCATCTCA; R: TTCCGTCAGTTTGAGGGTCT)  
*Sirt3* (F: ATCCCGGACTTCAGATCCCC; R: CAACATGAAAAAGGGCTTGGG)  
*Mmp2* (F: CTGCTGGCTAGTTGCACCC; R: CCTTTGGGCTAGGTATCTCTGA)  
*p16/Cdkn2a* (F: CGCAGTTCTTGGTCACTGT; R: TGTTACGAAAGCCAGAGCG)  
*p21/Cdkn1a* (F: CGGTGTCAGAGTCTAGGGGA; R: ATTGGAGTCAGGCGCAGATC)  
*Lmnb1* (F: ATTTGGAGAATCGCTGTCAGAG; R: AAGCGGGTCTCATGCTTCC)  
*Gapdh* (F: AGGTCGGTGTGAACGGAT; R: GGGGTCGTTGATGGCAACA)

## Microscopy

Mice were sacrificed and the whole brains were immediately dissected, rinsed in 4°C PBS, and frozen in OCT embedding matrix (WVR) into plastic molds lean on a metallic support on top of dry ice plus isopropanol, to speed up the freezing process. Samples were stored at -80°C. 5-µm-thick brain cross-sections containing the hippocampi were cut with a Cryostat (CryoStar, Fisher) at -20°C, immediately placed on microscopy glass slides and stored at -80°C.

The day of the staining, the sections were let dry at RT° for 10' and fixed in PBS 4% formaldehyde for 20'. The sections were then permeabilized in PBS 0.2% Triton for 20', quickly washed in PBS three times, and blocked in PBS 0.1% Tween 4% BSA for 1h at RT°. After blocking, the slides were incubated with the primary antibodies diluted accordingly into blocking solution, O/N at 4°C. Following O/N incubation, the slides were washed quickly twice and once for 15' in PBS 0.1% Tween (60rpm on a rocking platform). After washing, the slides were incubated with the secondary antibodies diluted accordingly into blocking solution, for 1h at 37°C. Afterwards, the slides were quickly washed twice and once for 15' in PBS 0.1% Tween (60rpm on a rocking platform), let dry and set with mounting medium plus DAPI (Vectashield, Vector Laboratories).

The slides were incubated with the following primary and secondary antibodies: rabbit anti-TRF2 (1:400; NB110-57130, Novus Biologicals), mouse anti-NeuN (1:1000 ; ab104224, Abcam), anti-mouse-Cy3 (1:500; 715-165-151, Jackson ImmunoResearch), anti-rabbit-AF647 (1:500; 711-605-152, Jackson ImmunoResearch).

### TIF staining

Brain cross-sections were let dry at RT° for 10' and fixed in PBS 4% formaldehyde for 20'. Afterwards, the samples were gradually dehydrated with increasing concentrations of EtOH in dH<sub>2</sub>O: 50, 75, and 100% EtOH, 5' each. The PNA (Peptide nucleic acid) probe complementary to the telomeric DNA was prepared in 90% hybridization buffer (HB: 70% Formamide, 1 mM Tris in H<sub>2</sub>O, pH 7.2) + 10% Malic acid buffer (0.1 M Malic acid, 0.15 M NaCl, pH 7.5). The probe solution was heated up at 95°C for 5' and added on top of the samples. The samples with the probe were further heated up at 80°C for 5'. The samples were then quickly washed twice and once for 15' with HB (60rpm on a rocking platform). The same set of washes was repeated with a washing buffer (150 mM NaCl, 50 mM Tris, 0.05% Tween in dH<sub>2</sub>O, pH 7.5). The sections were then quickly washed in PBS three times and blocked in PBS 0.1% Tween, 4% BSA for 1h at RT°. After blocking, the slides were incubated with a primary anti-53BP1 antibody (1:200; NB100-305, Novus Biologicals), diluted accordingly into blocking solution, O/N at 4°C. Following O/N incubation, the slides were washed quickly twice and once for 15' (60rpm on a rocking platform) in PBS 0.1% Tween. After washing, the slides were incubated with a secondary anti-rabbit-Cy3 antibody (1:500; 711-165-152, Jackson ImmunoResearch), diluted accordingly into blocking solution, 1h at 37°C. Afterwards, the slides were washed quickly twice and once for 15' in PBS 0.1% Tween (60rpm on a rocking platform), let dry and set with mounting medium plus DAPI (Vectashield, Vector Laboratories).

### Imaging and quantification

Pictures were taken with either a Confocal microscope LSM 880 (63x, NA 1.40; Zeiss) (NeuN and TRF2 in focused pictures of CA1, CA3 and DG), a HD microscope (40x, 0.75; Zeiss) (NeuN and TRF2 in full hippocampus mosaics), or a DV Elite microscope (60x, NA 1.42; Leica) (TIF staining). The pictures were processed and analyzed with a FIJI software, and TIF quantification was performed manually counting the number of spots resulting from the superimposition of 53BP1 foci and telomeric spots (PNA probe). The counting was performed blindly by the same operator for all the pictures.

### QUANTIFICATION AND STATISTICAL ANALYSIS

All values are expressed as mean ± SEM. Alpha was set to 0.05 for all analyses. Statistical parameters including the exact sample size (n), *post hoc* tests, and statistical significance are reported in every figure and figure legend. The number of mice was estimated to be sufficient based on pilot experiments. Data were estimated to be statistically significant when  $p \leq 0.05$  by non-parametric

Mann-Whitney U test or Student's t test, and non-parametric Kruskal-Wallis or one-way/two-way ANOVAs with repeated measures when appropriate. Significant ANOVAs were followed by a two-way repeated-measures analysis of variance with a *post hoc* Tukey's HSD multiple comparisons test for pairwise differences between each mouse groups. In every figure, an asterisk denotes statistical significance (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ). Data were analyzed using GraphPad Prism v5 software.

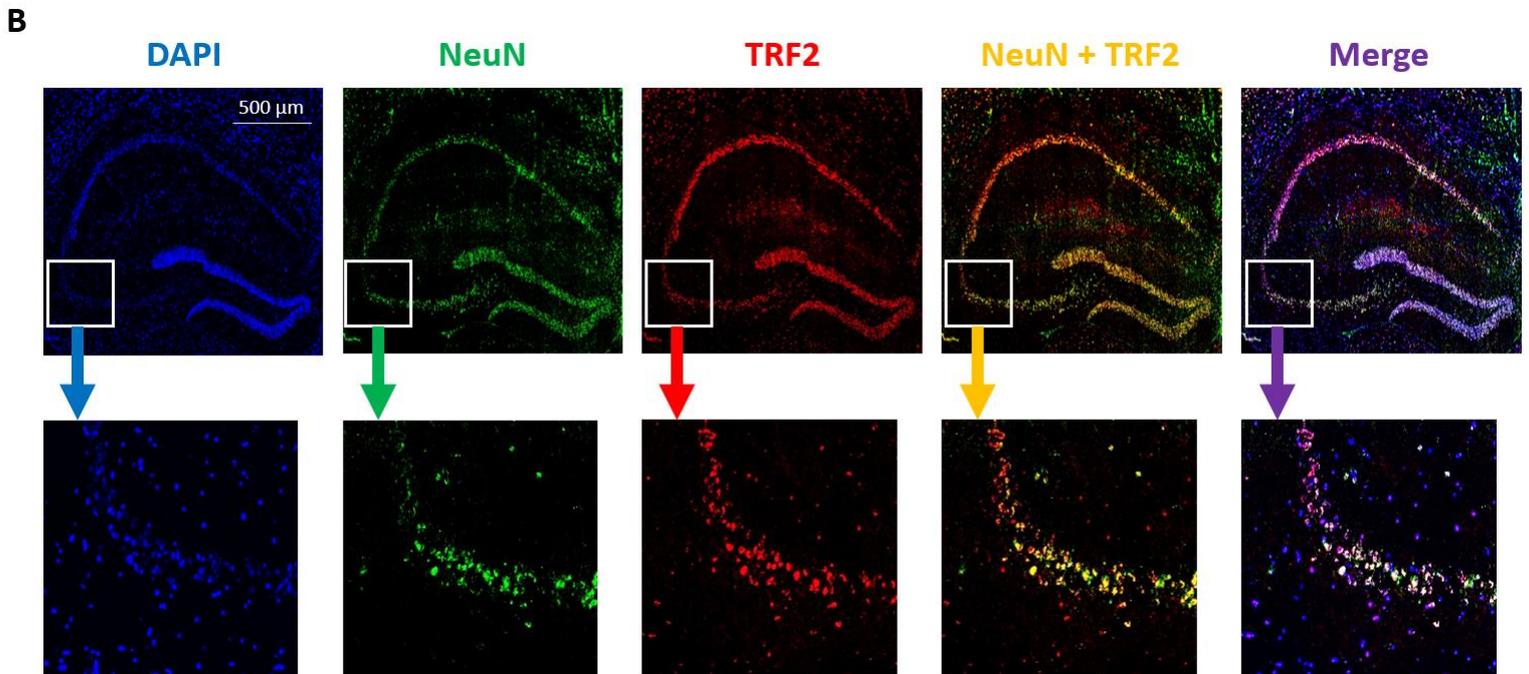
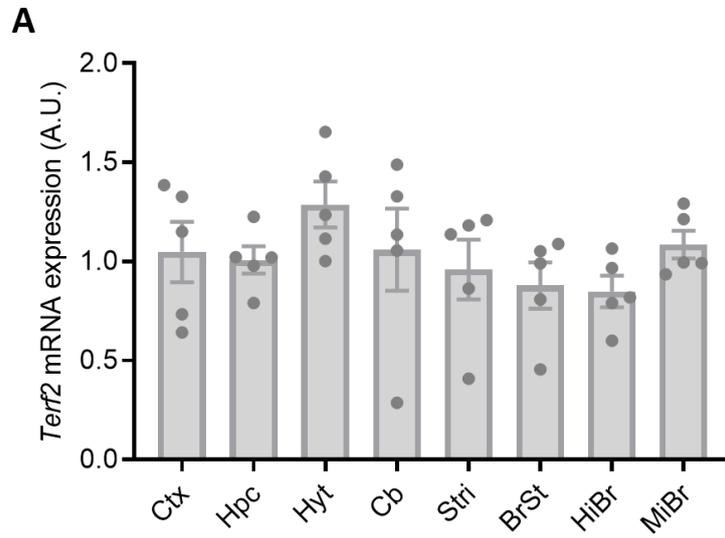
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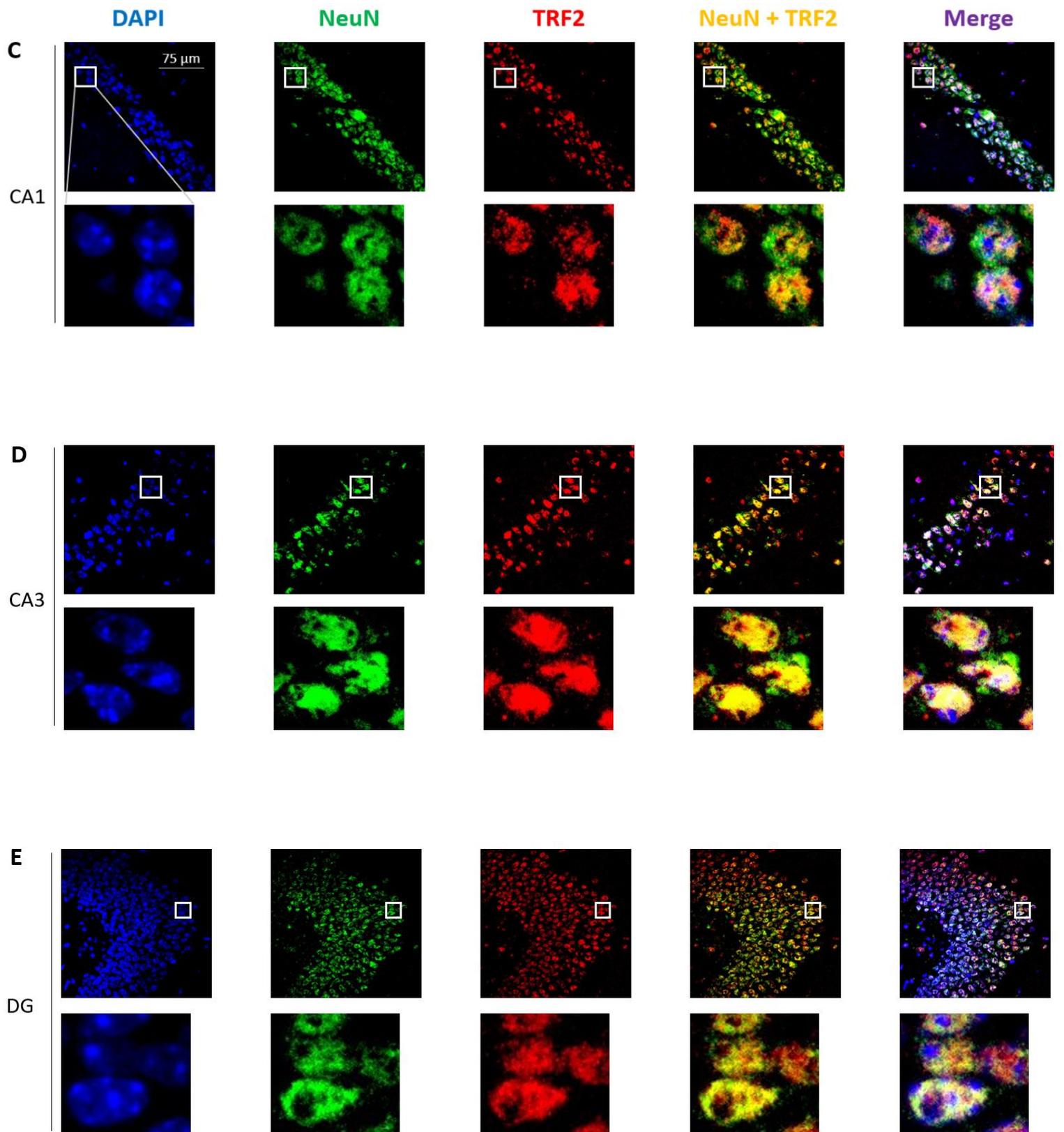
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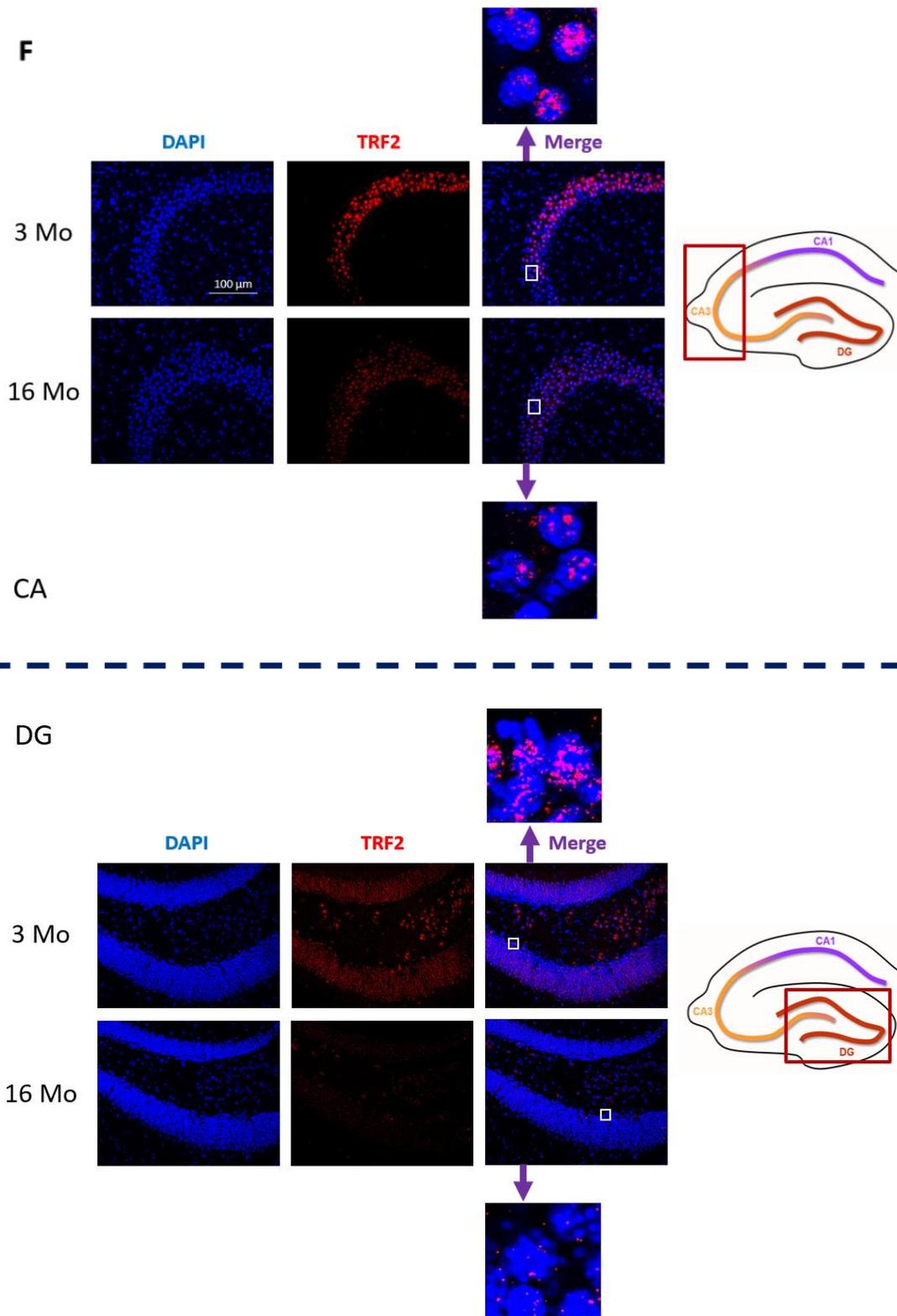
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# Supplementary Figures

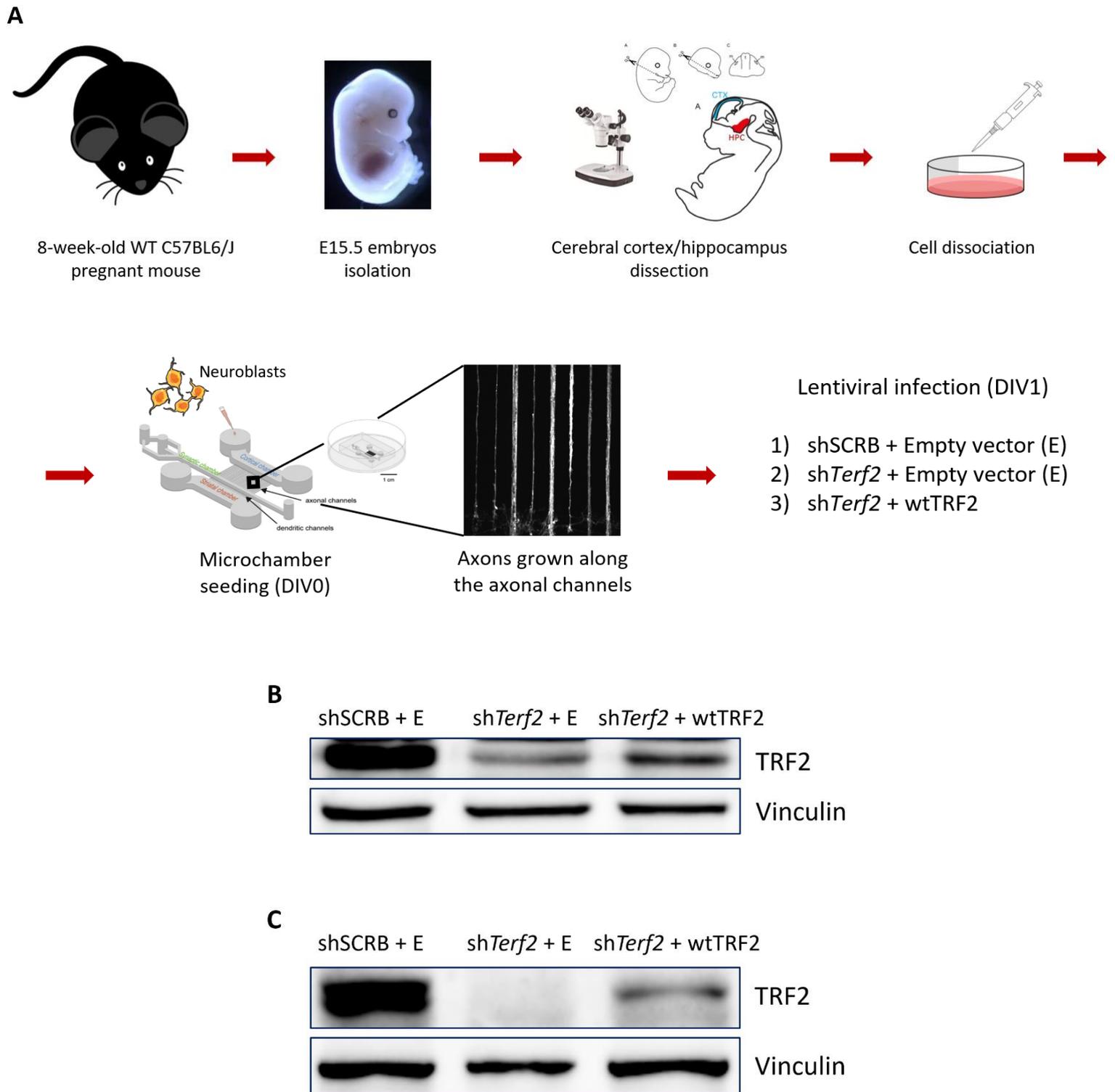






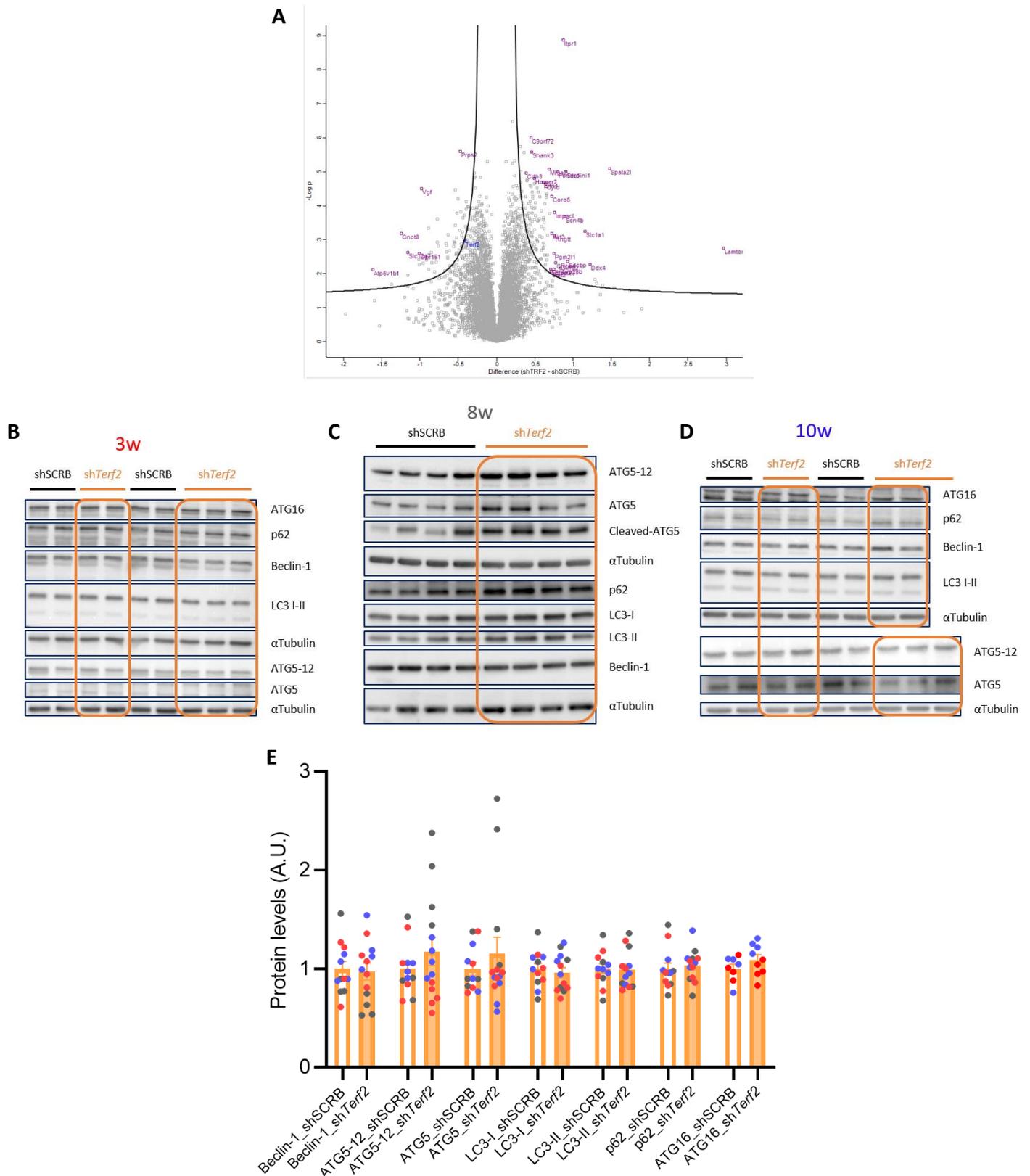
**Figure S1. TRF2 expression in the hippocampus decreases with aging**

(A) *Terf2* mRNA expression in different brain regions of 3-month-old mice: Cortex (Ctx), Hippocampus (Hpc), Hypothalamus (Hyt), Cerebellum (Cb), Striatum (Stri), Brain Stem (BrSt), Hind Brain (HiBr), and Mid Brain (MiBr). (B) NeuN (bonafide marker of mature neurons) and TRF2 expression in the whole adult hippocampus of young mice. (C-E) NeuN and TRF2 expression in the hippocampal regions (C) *Cornu ammonis 1* (CA1), (D) *Cornu ammonis 3* (CA3), and (E) *Dentate gyrus* (DG), respectively. (F) TRF2 downregulation with age: TRF2 expression in DG and CA3 in 3- (3 Mo) vs 16-month-old (16 Mo) mice. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

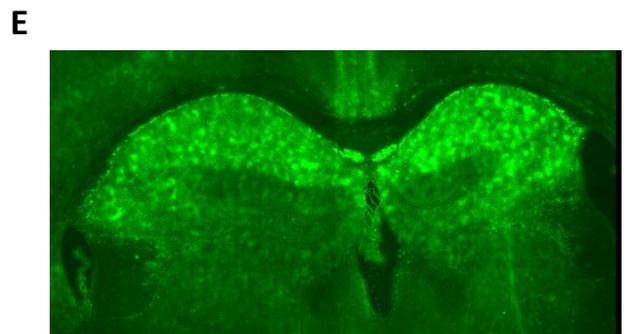
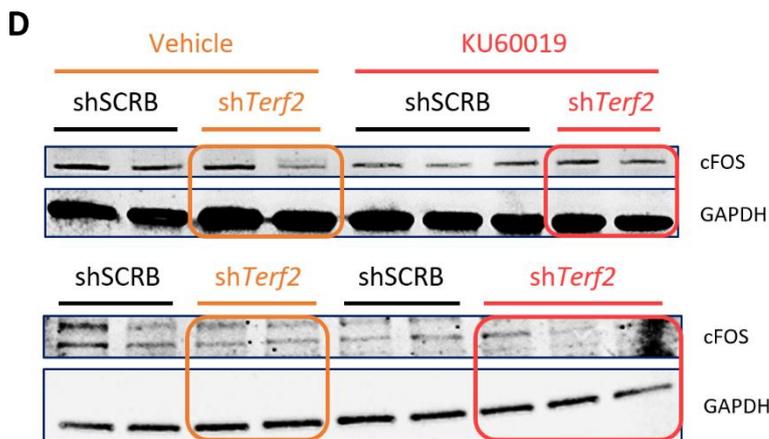
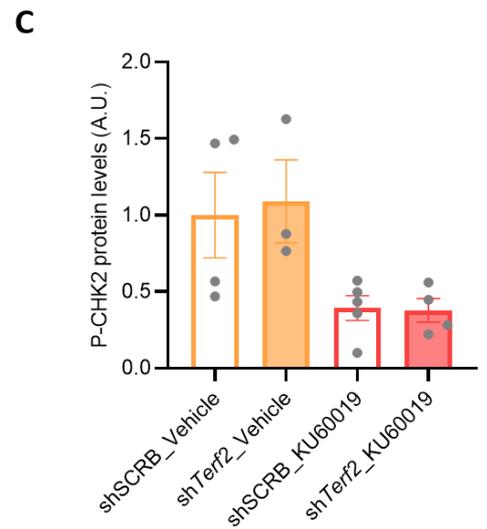
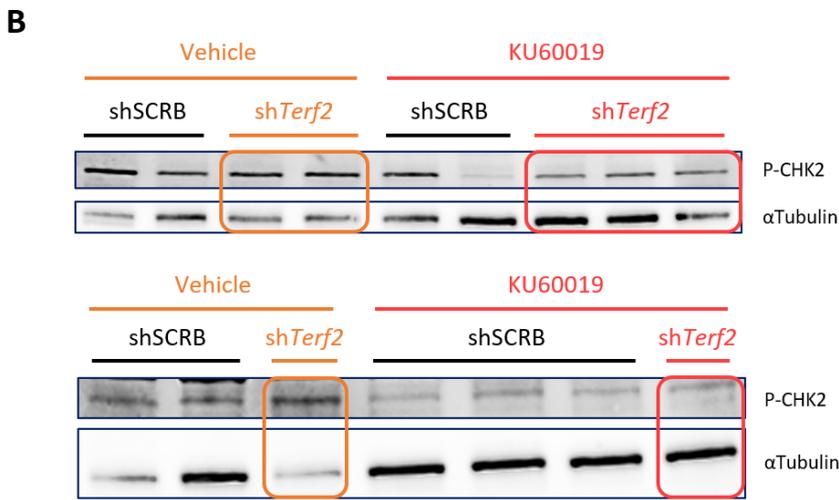
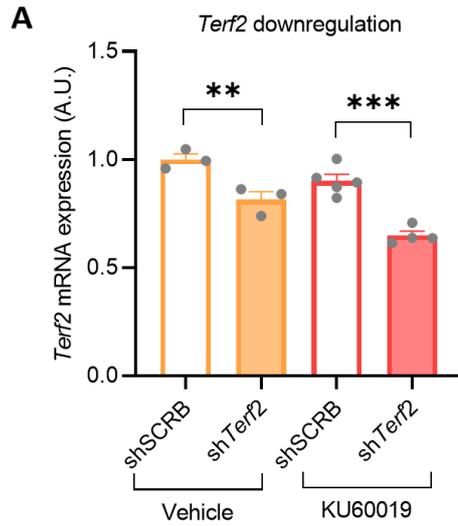


**Figure S2. Experimental setting for the vesicle axonal transport assay**

(A) Work-flow of the *in vitro* system used to knock down TRF2 in cultured primary neurons. Primary neurons were isolated from either the hippocampus or the cortex of E15.5 embryos and plated into microchambers suited for proper axonal growth along engraved channels, which allows the recording of VAMP2 vesicles movement along the axons. By means of lentiviral infection, TRF2 was either knocked down (sh*Terf2*) or not (shSCRB). Simultaneously, the neurons were either coinfecting with lentiviruses bearing an empty construct (E) or wild type TRF2 (wtTRF2). (B-C) Western blots showing TRF2 downregulation upon lentiviral infection (sh*Terf2* + E) and its rescue (sh*Terf2* + wtTRF2), in (B) cortical and (C) hippocampal neurons, respectively [DIV=Day *in vitro*].

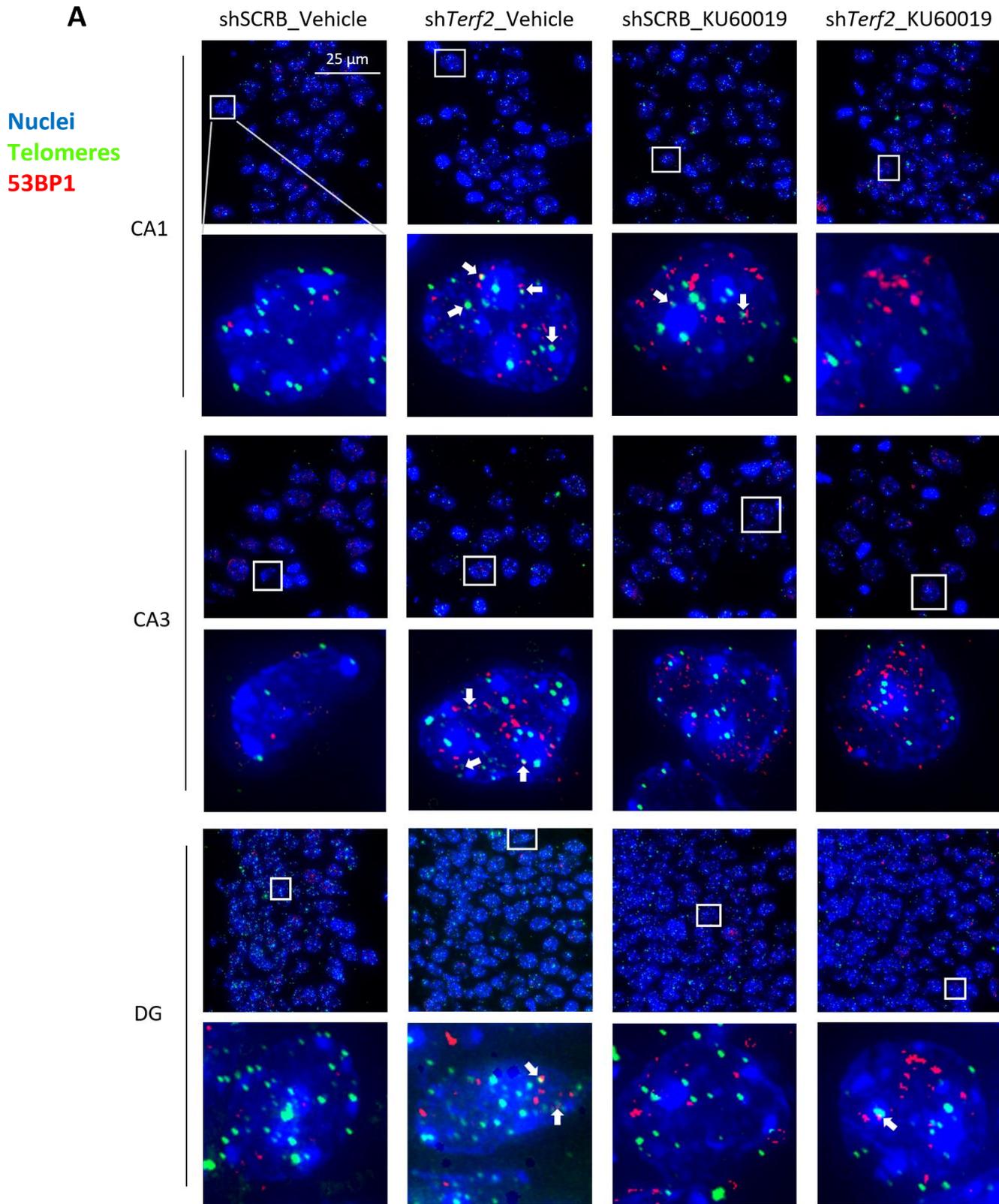


**Figure S3. *Terf2* downregulation in hippocampal mature neurons affects the levels of several proteins, but does not impair autophagy** (A) Volcano plot highlighting proteins whose expression is either up- or downregulated upon *Terf2* downregulation. (B-D) Protein levels of autophagy-related proteins in the hippocampus either (B) 3, (C) 8, or (D) 10 weeks after AAV-mediated *Terf2* downregulation. (E) Quantification of the Western blots shown in figures (B-D). The different colors represent the different Western blots, in (B) red, (C) black, and (D) blue, respectively. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

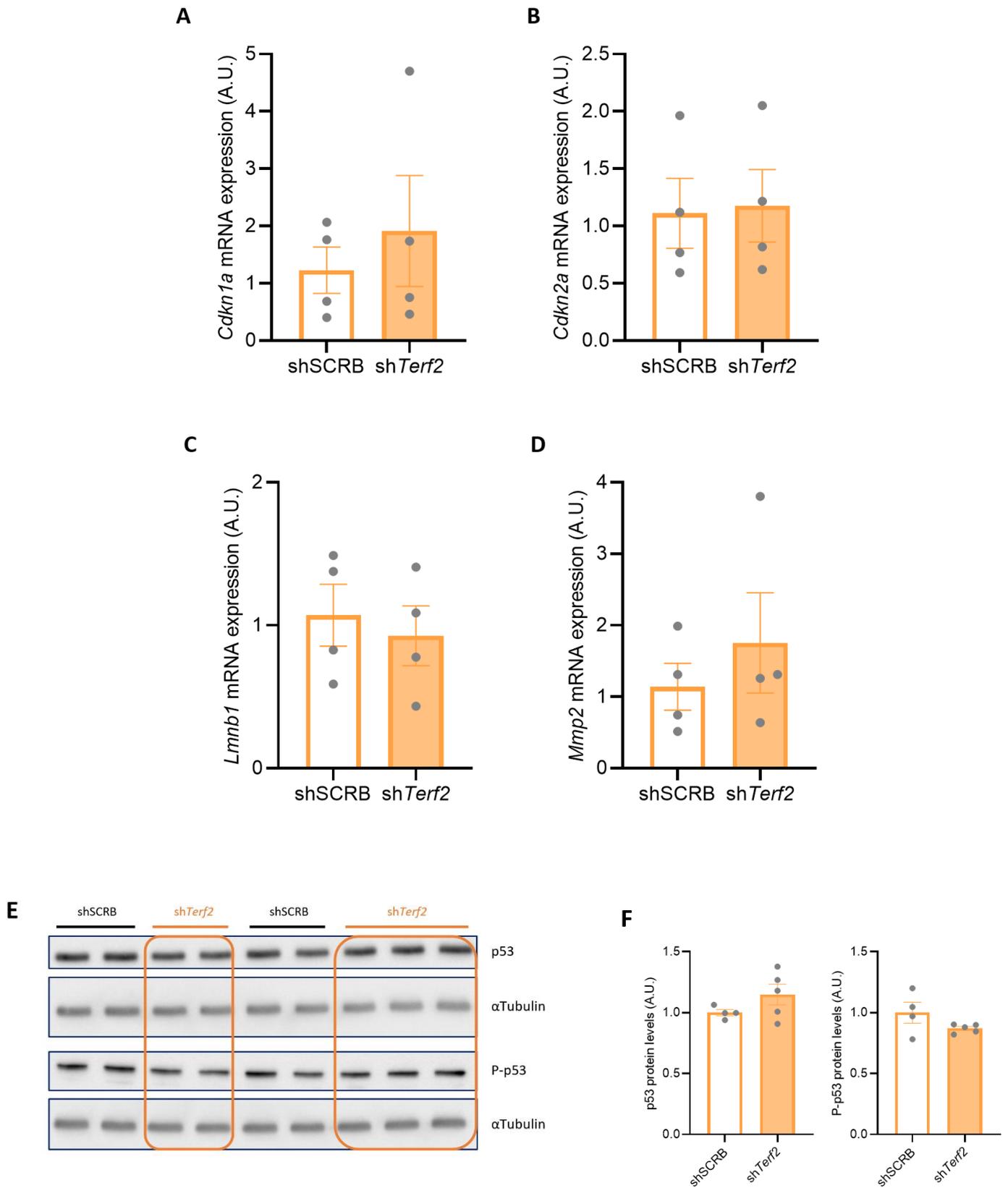


**Figure S4. Confirmation of *Terf2* downregulation and ATM inhibition**

(A) mRNA expression of TRF2 upon *Terf2* downregulation (sh*Terf2*) and ATM inhibition (KU60019). (B) Protein levels of phosphorylated CHK2 (ATM kinase target) upon TRF2 downregulation (sh*Terf2*) and ATM inhibition (KU60019). (C) Quantification of the Western blot shown in figure (B). (D) Protein levels of c-FOS upon *Terf2* downregulation (sh*Terf2*) and ATM inhibition (KU60019). (E) Visual confirmation of the AAVs targeted injections in the murine hippocampi via detection of the GFP expressed by the AAVs (representative image). All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

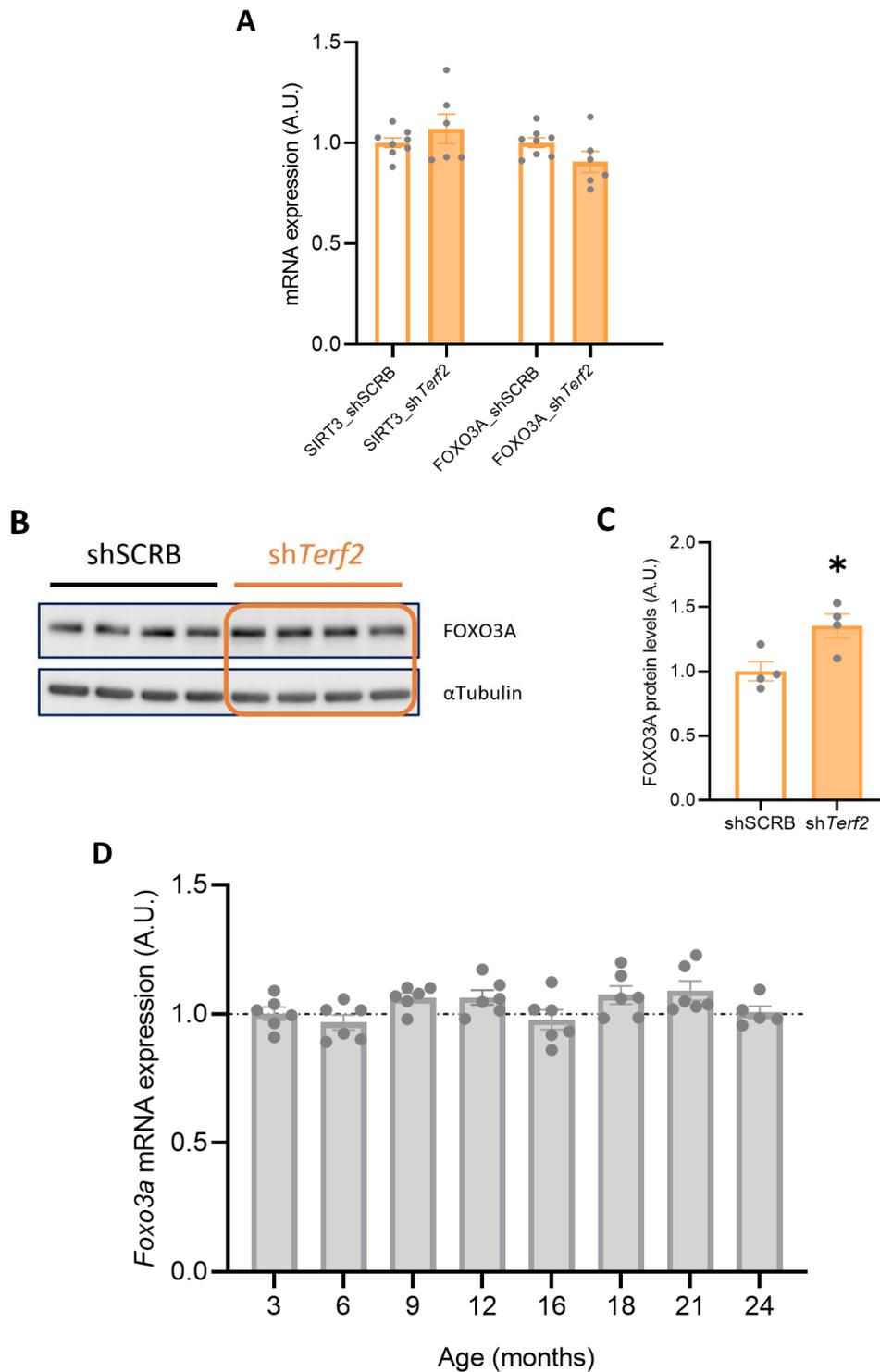


**Figure S5. *Terf2* downregulation in the hippocampus of young mice increases telomeric DNA damage, which is rescued by ATM inhibition**  
 (A) Fluorescence microscopy images of brain cross-sections of 3-month-old mice stereotaxically injected with either AAV-Scramble-shRNA (shSCRB) or AAV-*Trf2*-shRNA (sh*Terf2*), and either infused with the ATM inhibitor (KU60019) or not (Vehicle). Images of the three characteristic regions of the hippocampus were taken: *Cornu ammonis 1* (CA1), *Cornu ammonis 3* (CA3), and *Dentate gyrus* (DG). Telomere dysfunction-induced foci (TIF) are indicated by white arrows. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).



**Figure S6. *Terf2* downregulation in the hippocampus of young mice does not cause senescence**

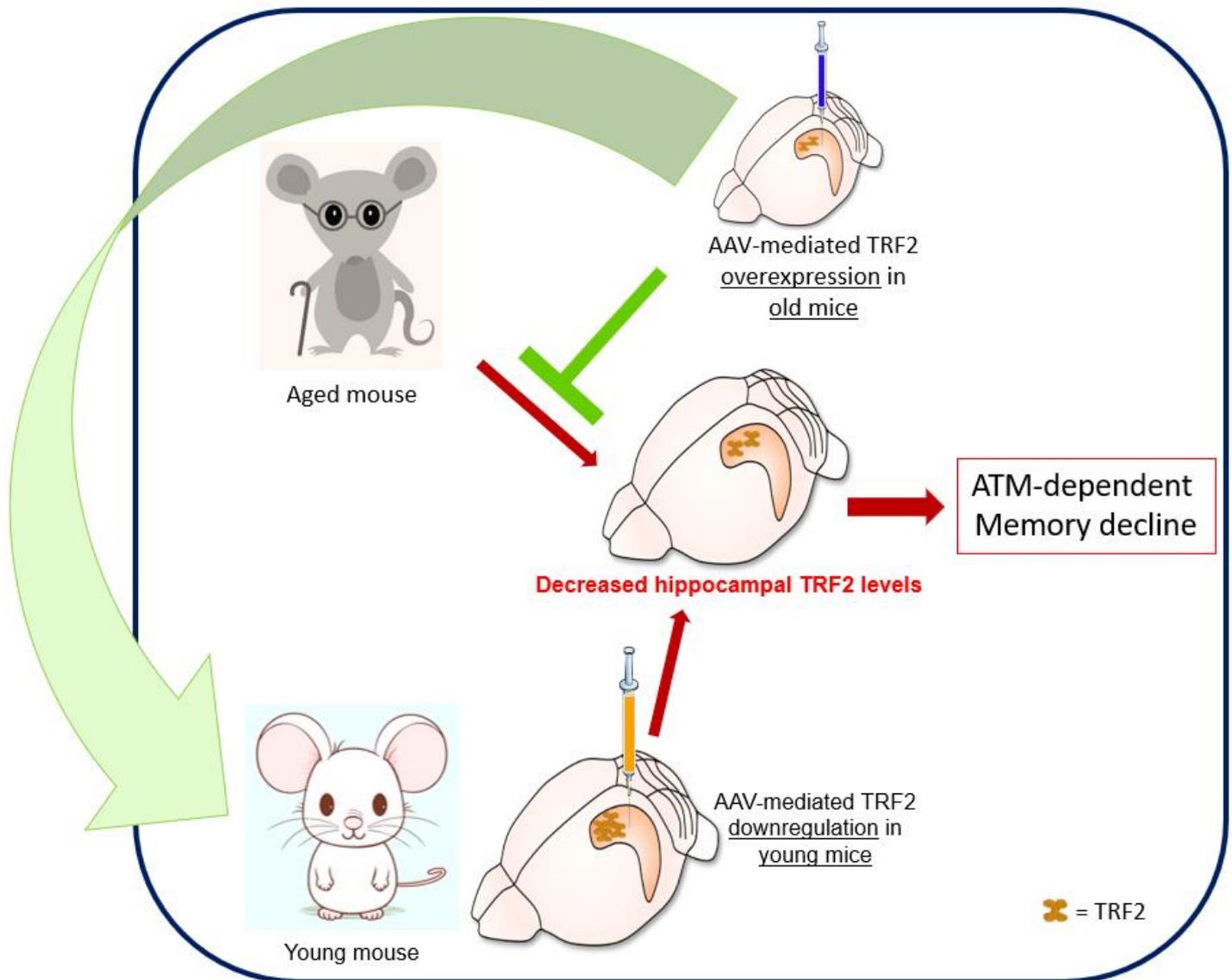
(A-D) mRNA expression of senescence-related genes upon *Terf2* downregulation (sh*Terf2*), namely: (A) *Cdkn1a*, (B) *Cdkn2a*, (C) *Lmn1*, (D) *Mmp2*. (E) Protein levels of p53 and Phospho-p53 (P-p53) upon *Terf2* downregulation (sh*Terf2*). (F) Quantification of the Western blot shown in figure (E). All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).



**Figure S7. *Terf2* downregulation in the hippocampus of young mice does not affect SIRT3 and FOXO3A mRNA expression, but does increase FOXO3A protein levels**

(A) mRNA expression of longevity factors SIRT3 and FOXO3A upon TRF2 downregulation (*shTerf2*). (B) Protein levels of the longevity factor FOXO3A upon TRF2 downregulation (*shTerf2*) in young mice. (C) Quantification of the Western blot shown in figure (B). (D) *Foxo3a* mRNA expression during aging in the hippocampus. Hippocampi were taken from mice of different ages (3-, 6-, 9-, 12-, 16-, 18-, 21-, 24-month-old mice) and mRNA was extracted and quantified via qPCR. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

## Graphical abstract



### Graphical abstract. TRF2 levels and memory decline

TRF2 hippocampal levels decline during aging. AAV-mediated *Terf2* downregulation in young mice (3-month-old) recapitulates the age-dependent TRF2 decline leading to the same ATM-dependent memory impairment. TRF2 overexpression in old mice strikingly rescues the memory phenotype with a rejuvenating effect on the old brains.

## 7. Extra Results

### *In vitro*

Autophagy is a pivotal pro-longevity phenomenon, especially in the context of long-lived postmitotic cells such as neurons, with a crucial role in memory formation (Glatigny et al., 2019) (De Risi et al., 2020). Therefore, to avoid the bulk analysis bias of molecular biology experiments performed on the entire hippocampus, hence also containing cell types other than post-mitotic neurons, we addressed the effect of *Terf2* downregulation on this important cleansing mechanism *in vitro*, making use of primary hippocampal neurons isolated from E16.5 murine embryos. After confirming the lentivirus-mediated *Terf2* downregulation in our *in vitro* setting, both at the mRNA and protein level (Figure E1A-C), we assessed the protein levels of autophagy factors involved in different steps of the autophagosome formation. Interestingly, while we observed that *Terf2* downregulation decreases the levels of early autophagy factors such as Beclin-1 and ATG5-12 (Figure E2A-D), we found that TRF2 depletion increases the levels of autophagy late factors like LC3 and p62 (Figure E2E-H). These data, simultaneously hinting for impaired autophagosome formation and autophagosome accumulation, suggest that TRF2 might be necessary not only for the initial steps of autophagy, but also for the following steps of phagosome-lysosome fusion and cargo degradation.

After assessing autophagy, we tested the effect of *Terf2* downregulation on pro-longevity factors belonging to the Sirtuin and FOXO families (Longo & Kennedy, 2006) (Willcox et al., 2008). We found that TRF2 depletion decreases both SIRT1 and FOXO3A protein levels (Figure E3A-E). Importantly, *Terf2* downregulation also increases the levels of the apoptotic marker Cleaved-Caspase-3 (Figure E3F,G), suggesting that TRF2 might have a role in neuron survival.

Mitochondria are essential organelles devoted to energy production, a particularly pivotal requisite for highly energy demanding cells such as neurons. Importantly, TRF2 depletion leads to mitochondrial dysfunction in the postmitotic context of muscle cells (Robin et al., 2020). Therefore, we set out to measure the effect of *Terf2* downregulation on the mitochondrial cell content of primary postmitotic neurons. Indeed, we could observe that knocking down TRF2 leads to decreased TIM23 levels, a bonafide readout of mitochondrial content (Figure E4A,B), hinting for a role of TRF2 in mitochondrial homeostasis.

Noteworthy, all the above-mentioned experiments were performed on primary hippocampal neurons where TRF2 was downregulated at day in vitro 4 (DIV4). Therefore, since primary neurons are canonically considered to be mature *in vitro* after DIV14, we cannot exclude that the results we found reflect an effect of TRF2 downregulation on neural progenitor cells, and not on mature neurons. Indeed, it has already been planned to repeat the same experiments making use of an IPTG-inducible lentiviral system, whereby TRF2 downregulation can be triggered via the addition of IPTG to the primary culture after DIV14, despite the cells still being infected earlier.

### *In vivo*

To pursue the investigation of a putative role of TRF2 in mitochondrial functions, we assessed the effect of *Terf2* downregulation on mitochondria *in vivo*. We found out that knocking down TRF2 in young mice (3-month-old), albeit not statistically significant, causes an increasing trend in the mRNA expression of some of the mitochondrial genes tested, including those that encode for ETC complexes and ribosomal RNAs (Figure E5A-O). On the other hand, knocking down TRF2 increases the protein levels of the ETC Complex I and III (Figure E6A-E). Noteworthy, ATM inhibition does not affect the protein levels of the ETC complexes upon *Terf2* downregulation (Figure E7A-E). Interestingly, opposite to the effect derived from knocking down TRF2 in young mice, the protein levels of the ETC Complex I decrease with aging. Moreover, the overexpression of TRF2 in old mice does not rescue the Complex I protein levels to those of young mice (Figure E8A-E).

Proteostasis, together with autophagy, is another pivotal cleansing mechanism for neurons (K. Tanaka & Matsuda, 2014). Thus, we assessed the effect of *Terf2* downregulation on the protein levels of the proteasome subunit PSMD13 and revealed that the levels of this proteasomal marker are not affected by TRF2 (Figure E9A,B).

Mitochondrial turnover via mitophagy is a crucial process for the maintenance of a functional mitochondrial pool and the recycling of these organelles when they are dysfunctional. Moreover, a physiological balance between mitochondrial fusion and fission is crucial for mitophagy to happen (Martinez-Vicente, 2017). Thus, we measured the protein levels of several factors involved in this phenomena (Figure E10A-F). Interestingly, we found that while the levels of DRP1 are increased upon *Terf2* downregulation (Figure E10B), the levels of its phosphorylated form, P-DRP1 Ser616, corresponding to an activated pro-fission version of the protein, are decreased (Figure E10C). Interestingly, ATM inhibition upon *Terf2* downregulation restores P-DRP1 Ser616 levels (Figure E11A-

C). Noteworthy, the levels of P-DRP1 Ser637, corresponding to an inactive, anti-fission form of the protein, tend to decrease upon ATM inhibition (Figure E11A-D). On the other hand, P-DRP1 Ser616 and P-DRP1 Ser637 levels tend to respectively decrease and increase during aging, which is rescued, in both cases, by TRF2 overexpression (Figure E11E-H). Interestingly, the mRNA expression of ERK2, AKT and CDK5, all potential DRP1 Ser616 residue kinases, does not vary upon *Terf2* downregulation and/or ATM inhibition (Figure E12A-C). All in all, these results suggest that TRF2 might have a role in mitochondrial turnover.

Since the canonical role of TRF2 is to protect telomeres from being misrecognized as DSBs, thereby avoiding unnecessary DDR that might lead to senescence, we assessed the effect of *Terf2* downregulation on the expression of DDR and senescence markers via immunofluorescence. In accordance with our previous results (Figure 6; Figure S6), we found that *Terf2* downregulation and/or ATM inhibition do not significantly affect total DDR, as assessed by measuring the levels of  $\gamma$ H2AX. On the same line, senescence is also unaffected, as revealed by unchanged LMNB1 levels (Figure E13A-D).

#### *How does TRF2 decline during aging?*

Intraflagellar transport protein 20 homolog (IFT20) is a crucial protein participating in cilia assembly (Follit et al., 2006). Each neuron has one cilium whose intact structure is pivotal for the process of memory formation (Rhee et al., 2016). Considering the newly discovered role of TRF2 in the memory process and the importance of IFT20 in the same phenomenon, we wondered if the two could be functionally linked to one another in this context. Therefore, we assessed the expression of TRF2 in hippocampal samples of mice that were downregulated for IFT20 (*shIft20*). Quite interestingly, we found that *Ift20* downregulation causes a decrease in the expression of *Terf2* (Figure E14A,B), suggesting that IFT20 and TRF2 might act in a same axis responsible for memory formation, with IFT20 being upstream of TRF2.

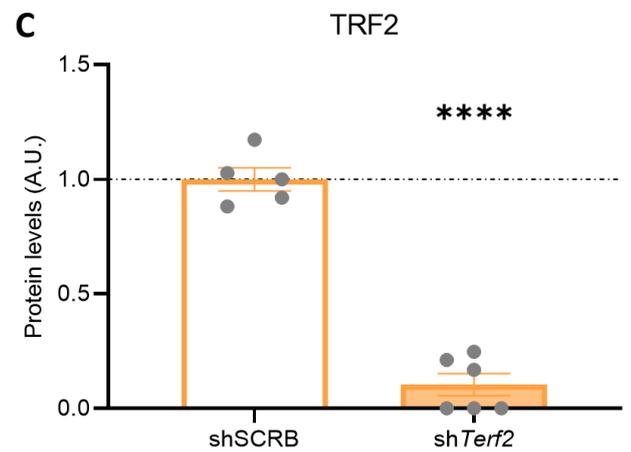
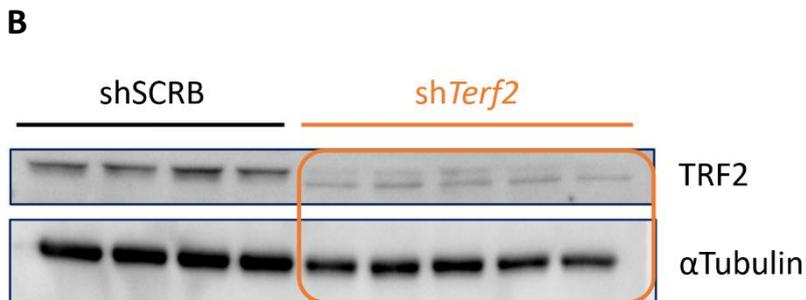
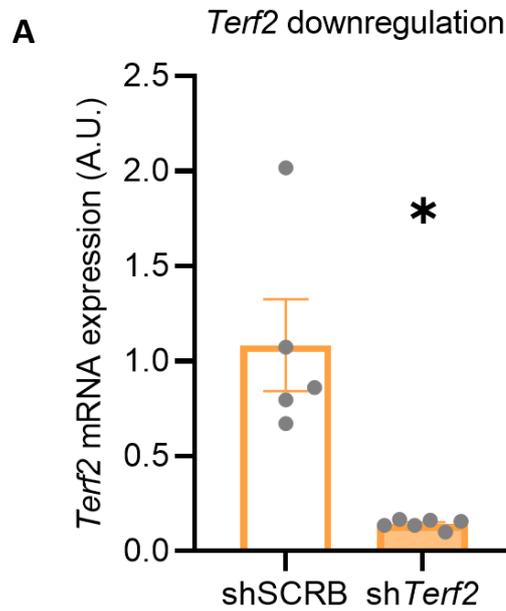
Osteocalcin (Ocn) is an osteoblast-produced neurotrophic hormone, pivotal for brain development (Oury et al., 2013) and endowed with rejuvenating functions (Khrimian et al., 2017). Therefore, since Ocn levels decrease with age, much alike TRF2, we wondered whether Ocn decline could be upstream the age-related TRF2 decline that we observed (Figure 1A). Thus, we measured the protein levels of TRF2 in young and old mice upon Ocn treatment. Interestingly, we found that while Ocn tends to increase TRF2 levels in young mice, it does not have the same effect when mice are already

old (Figure E14C,D), suggesting that an eventual pro-longevity Ocn-TRF2 axis might exist but only during the young/adult phase of murine aging.

### *The role of TRF2 in the Hypothalamus*

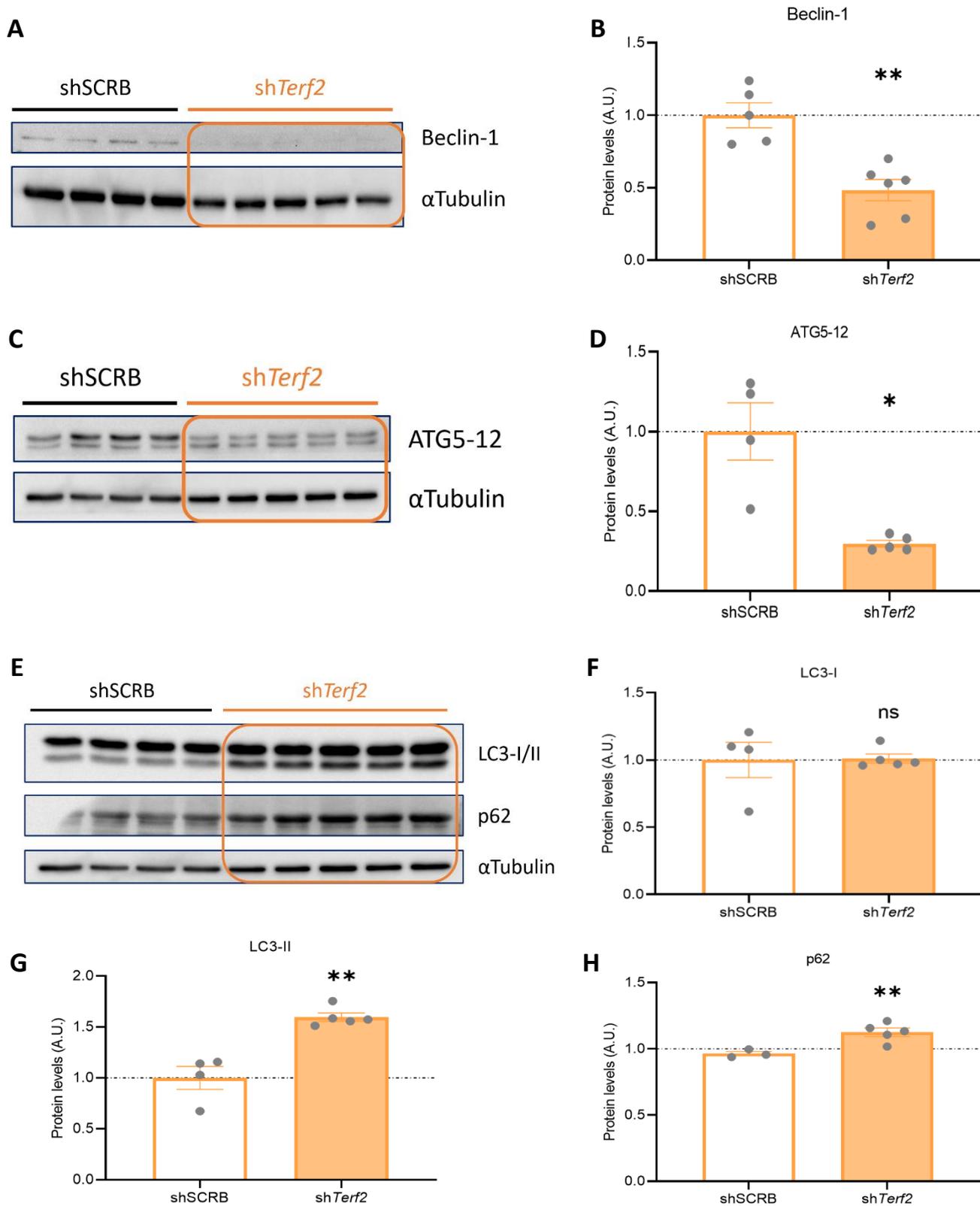
The hypothalamus is the brain region responsible (via the hypothalamic-pituitary-adrenal [HPA] axis) for the principal neuroendocrine responses of the brain, with major implications in the regulation of metabolism. HPA activation leads to glucocorticoid production by the adrenal glands (S. J. Lupien et al., 2009). Noteworthy, hippocampal alterations correlate with an impaired HPA axis function, characterized by hyperactivation and excessive or prolonged stress response. Thus, considering the effect of *Terf2* downregulation in the hippocampus, we wondered whether TRF2 could also play a role in the hypothalamus. As we did for the hippocampus, we started off by measuring the effect of *Terf2* hypothalamic downregulation *in vivo* on several phenomena including autophagy, apoptosis and senescence. We found out that knocking down TRF2 decreases the mRNA expression of the autophagy-related protein VPS34 and the longevity factor SIRT1 (Figure E15A-E). Moreover, *Terf2* downregulation tends to decrease the expression of several metabolic factors but has no effect on the expression of genes involved in ROS response and mitochondrial turnover (Figure E16A-C). Interestingly, unlike the hippocampus, the hypothalamic expression of *Terf2* does not decline with aging (Figure E16D). Moreover, the longitudinal assessment of weight gain is dramatically affected by *Terf2* downregulation, and TRF2-compromised mice exhibit lower fat and soft mass gain, but higher lean mass gain than control mice over time (Figure E17A-D). Furthermore, the hypothalamic *Terf2* downregulation leads to increased glucose tolerance and decreased insulin tolerance (Figure E17E,F). Finally, due to their leaner body mass composition, hypothalamic TRF2-compromised mice perform better in the physical test Rotarod (Figure E17G,H). Lastly, when assessed for their metabolic profile, housed inside metabolic chambers, *Terf2*-downregulated mice exhibit increased food and oxygen consumption, energy expenditure, CO<sub>2</sub> production and Respiratory exchange ratio (Figure E18A-F).

All in all, these data suggest that TRF2 might have a pivotal role also in the metabolic control mediated by the hypothalamus but in an aging-independent fashion.



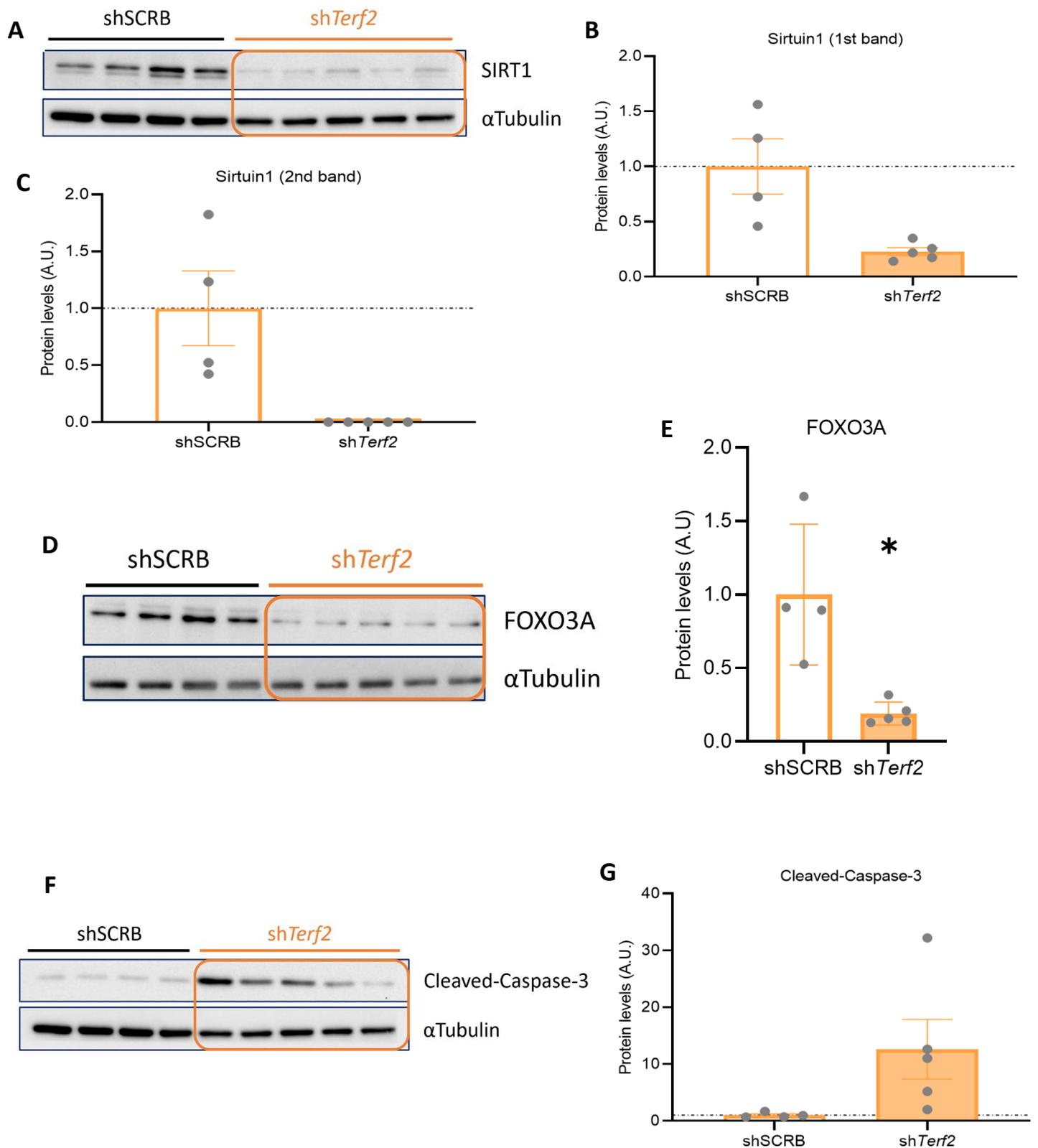
**Figure E1. Confirmation of TRF2 downregulation in hippocampal primary neurons**

(A) mRNA expression in hippocampal primary neurons upon *Terf2* downregulation (sh*Terf2*). (B) Protein levels of TRF2 in hippocampal primary neurons upon *Terf2* downregulation (sh*Terf2*). (C) Quantification of the Western blot shown in figure (B). Hippocampi were taken from E16.5 embryos. Cells were seeded and downregulated for TRF2 via lentiviral infection at DIV4. mRNA and proteins were extracted at DIV18. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

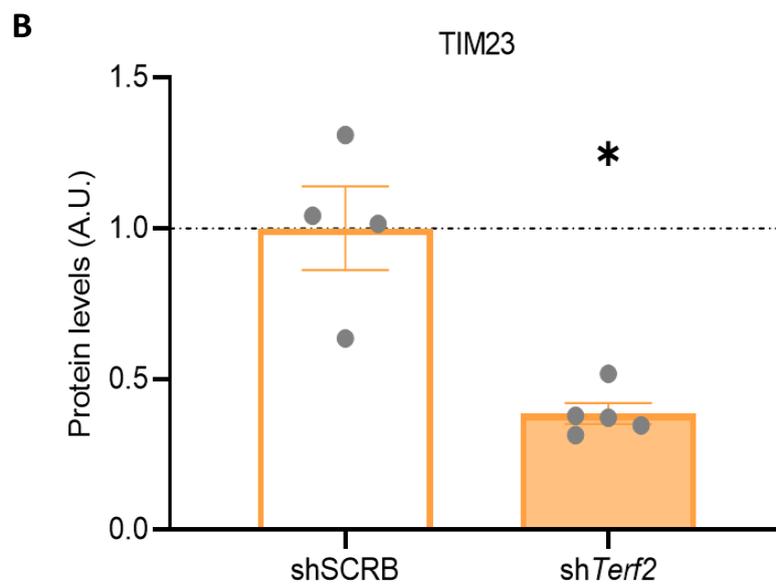
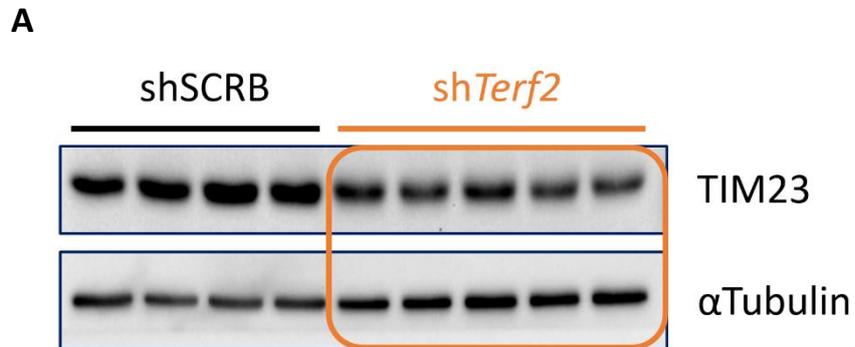


**Figure E2. *Terf2* downregulation in hippocampal primary neurons affects autophagy**

(A, C, E) Protein levels of autophagy-related proteins: (A) Beclin-1, (C) ATG5-12, (E) LC3I/II and p62. (B) Quantification of (A). (D) Quantification of (C). (F-H) Quantification of (E): (F) LC3I, (G) LC3-II, (H) p62. Hippocampi were taken from E16.5 embryos. Cells were seeded and downregulated for TRF2 via lentiviral infection at DIV4. mRNA and proteins were extracted at DIV18. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

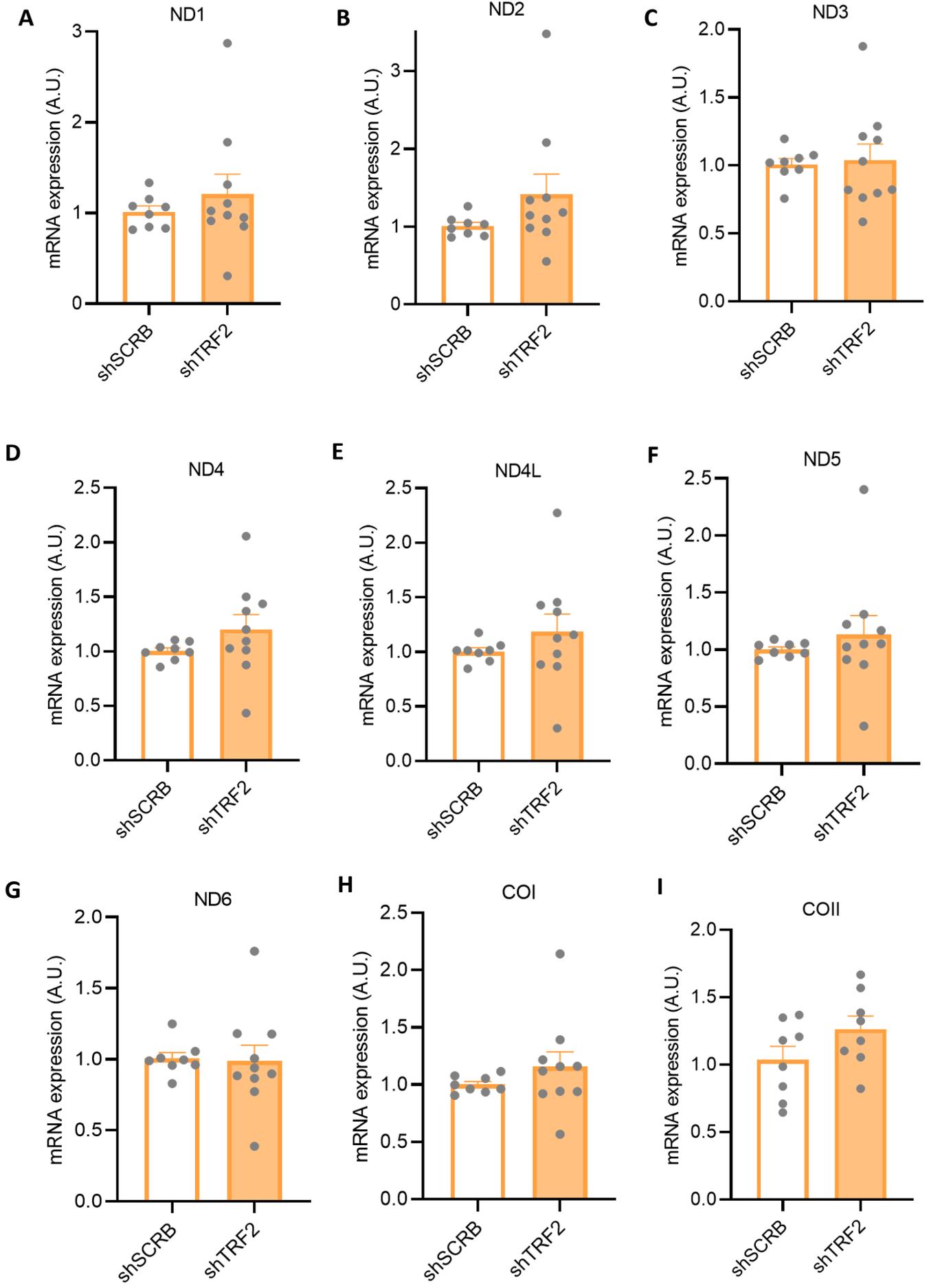


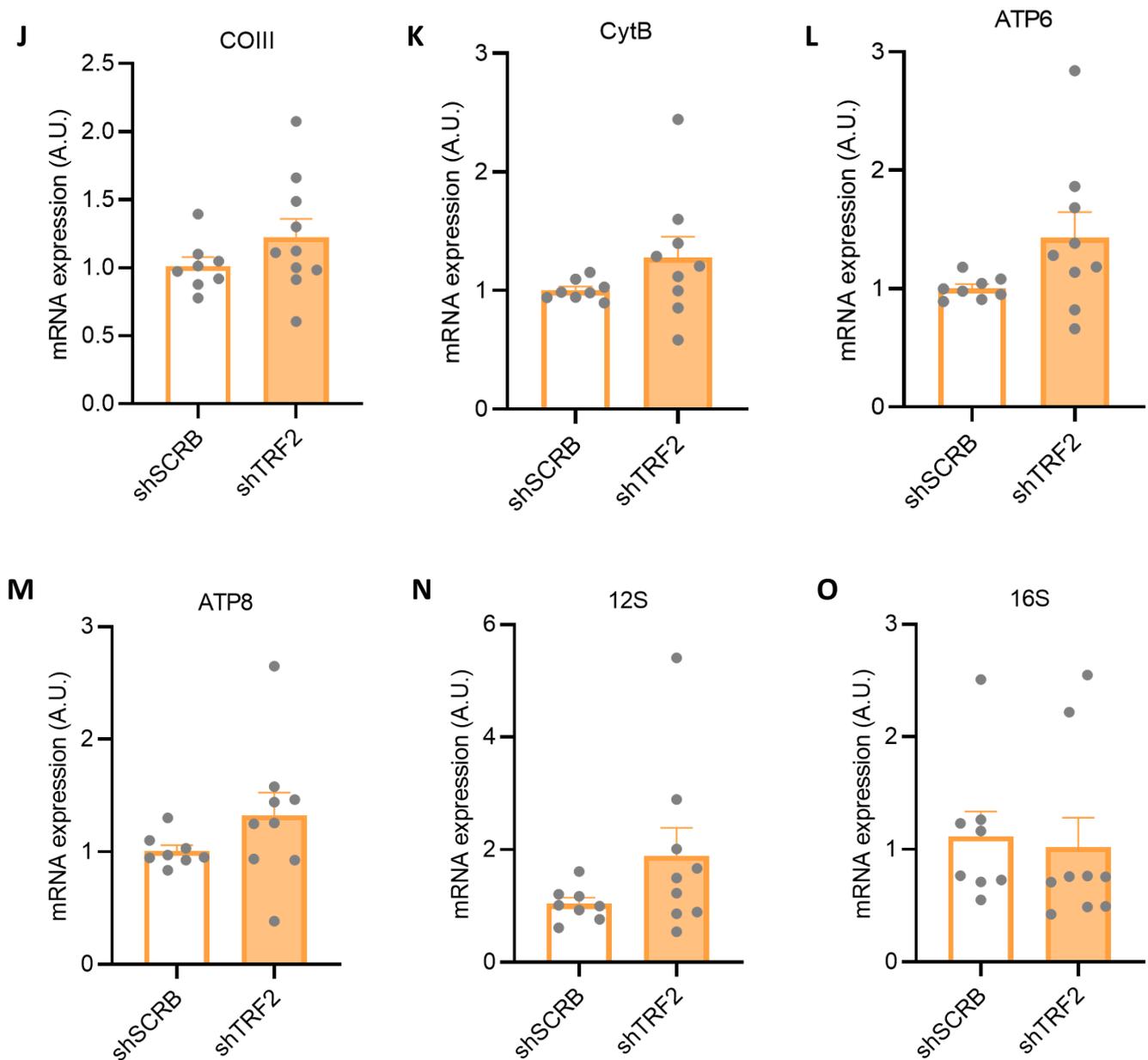
**Figure E3. *Terf2* downregulation in hippocampal primary neurons affects the expression of longevity factors and apoptosis** (A, D, F) Protein levels of (A) SIRT1, (D) FOXO3A and (F) Cleaved-Caspase-3. (B-C) Quantification of (A): (B) SIRT1, first band, (C) SIRT1, second band. (E) Quantification of (D). (G) Quantification of (F). Hippocampi were taken from E16.5 embryos. Cells were seeded and downregulated for TRF2 via lentiviral infection at DIV4. mRNA and proteins were extracted at DIV18. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).



**Figure E4. *Terf2* downregulation in hippocampal primary neurons decreases the mitochondrial content**

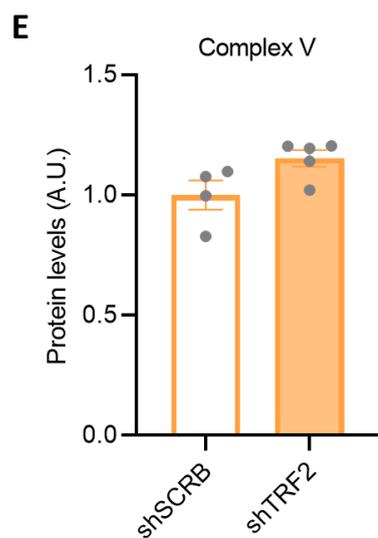
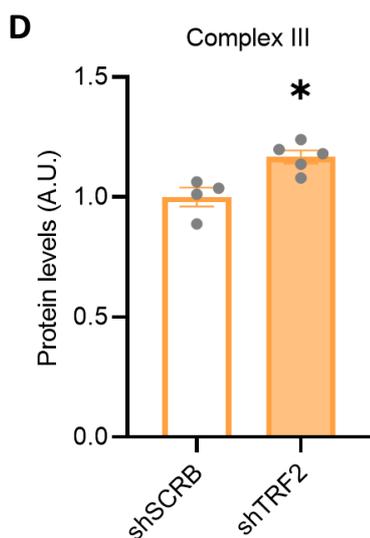
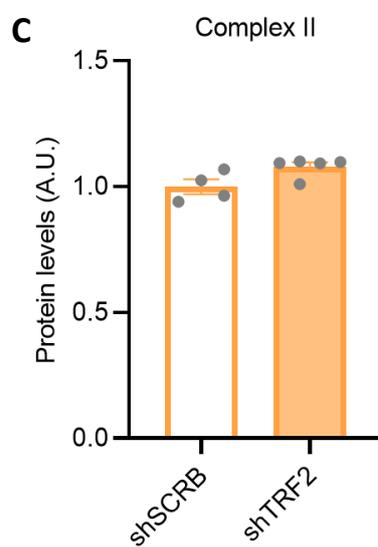
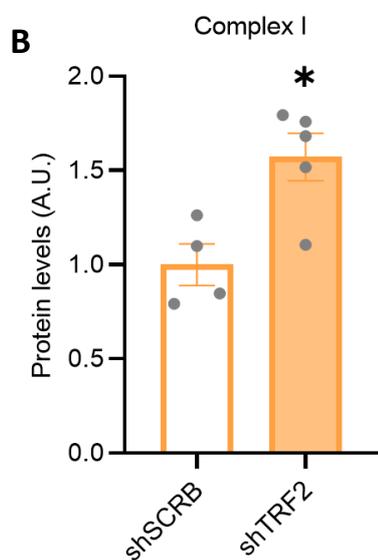
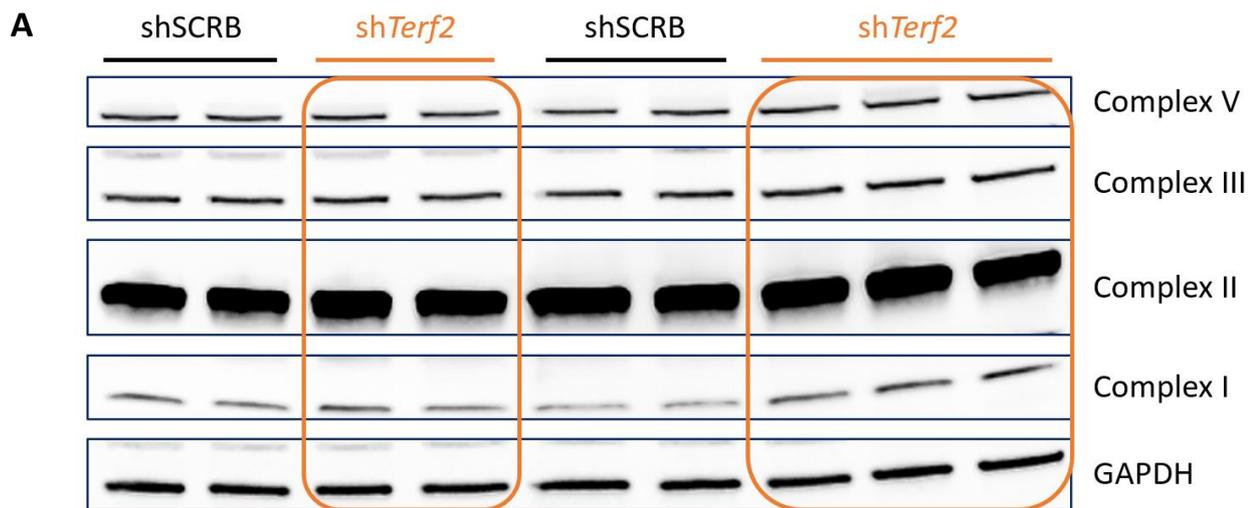
(A) Protein levels of TIM23 (Translocase of inner mitochondrial membrane 23 homolog B), bonafide marker of mitochondrial content. (B) Quantification of (A). Hippocampi were taken from E16.5 embryos. Cells were seeded and downregulated for TRF2 via lentiviral infection at DIV4. mRNA and proteins were extracted at DIV18. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).





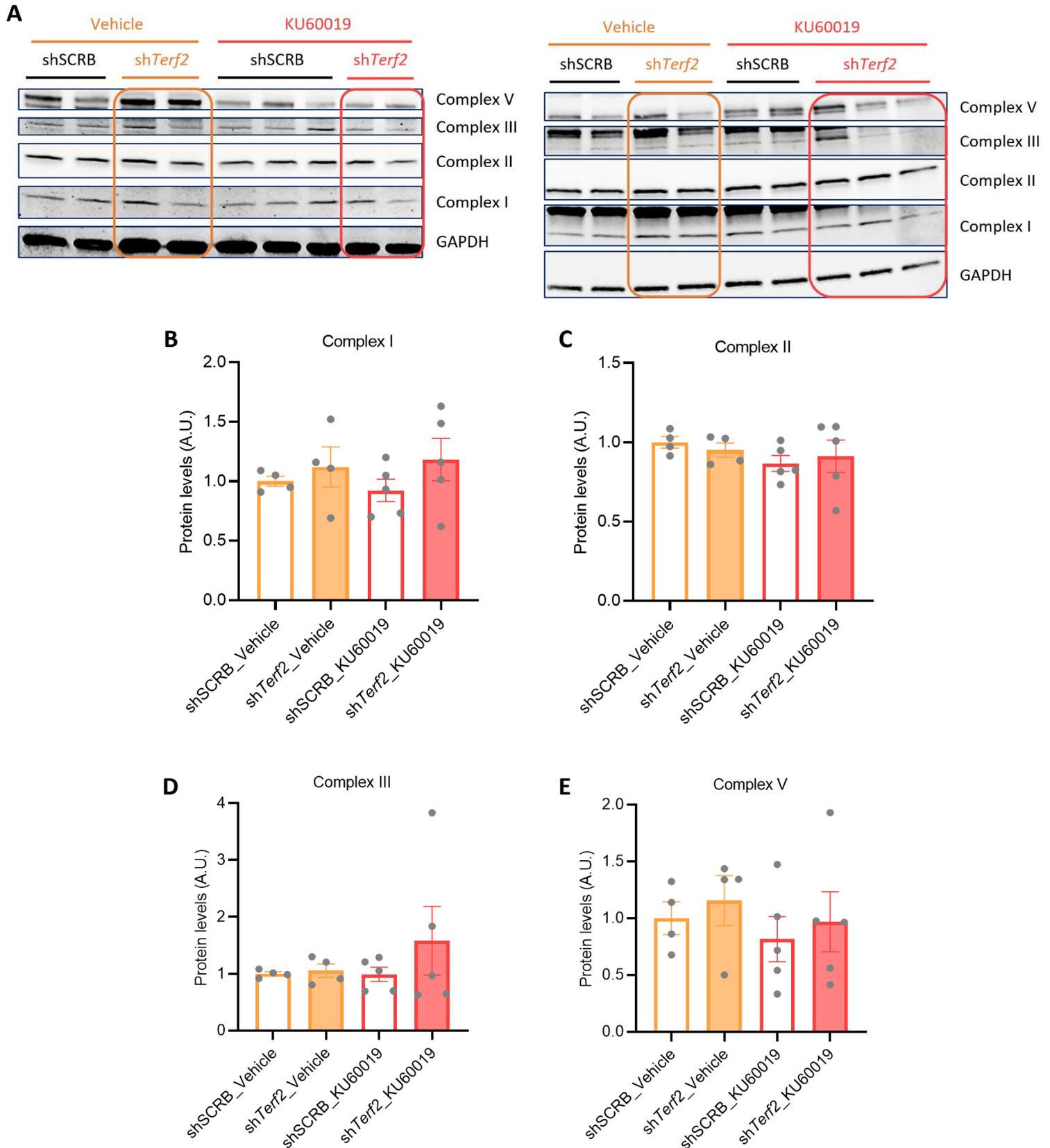
**Figure E5. *Terf2* hippocampal downregulation in live animals does not affect the expression of mitochondrial genes**

(A-G) mRNA expression of the NADH dehydrogenase subunits, which make up the Complex I of the mitochondrial electron transport chain (ETC). (H-J) mRNA expression of the Cytochrome c oxidase subunits that compose the Complex IV of the ETC. (K) mRNA expression of the Cytochrome B, pivotal component of the ETC Complex III. (L, M) mRNA expression of the ATP synthase subunits, which make up the Complex V of the ETC. (N, O) mRNA expression of mitochondrially encoded ribosomal RNAs. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ). [ND1, NADH dehydrogenase subunit 1; ND2, NADH dehydrogenase subunit 2; ND3, NADH dehydrogenase subunit 3; ND4, NADH dehydrogenase subunit 4; ND4L, NADH dehydrogenase subunit 4L; ND5, NADH dehydrogenase subunit 5; ND6, NADH dehydrogenase subunit 6; COI, Cytochrome c oxidase subunit 1; COII, Cytochrome c oxidase subunit 2; COIII, Cytochrome c oxidase subunit 3; CytB, Cytochrome B; ATP6, mitochondrially encoded ATP synthase membrane subunit 6; ATP8, mitochondrially encoded ATP synthase subunit 8; 12S, mitochondrially encoded 12S ribosomal RNA; 16S, mitochondrially encoded 16S ribosomal RNA].



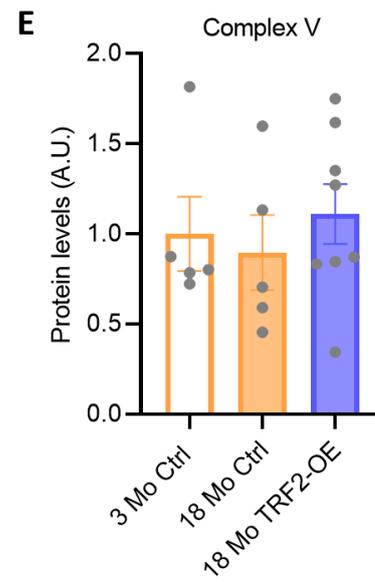
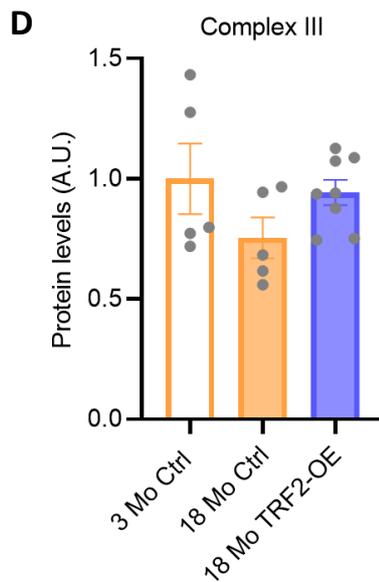
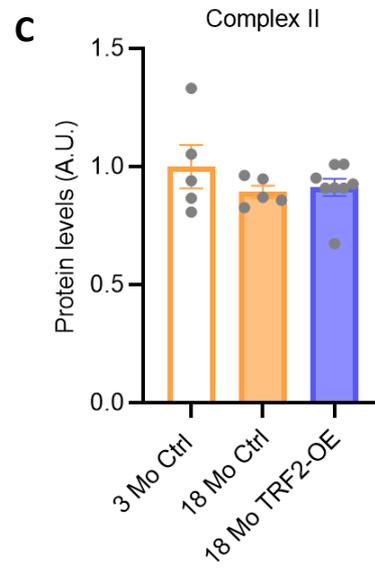
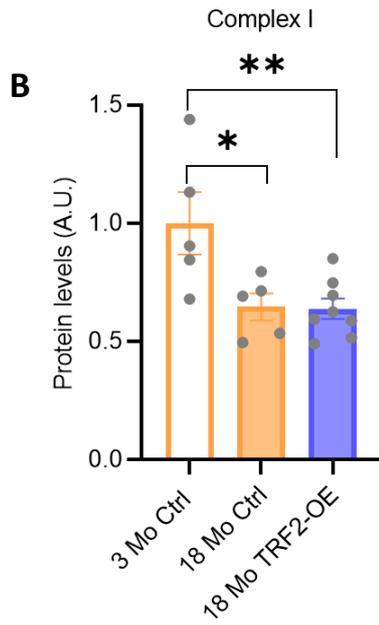
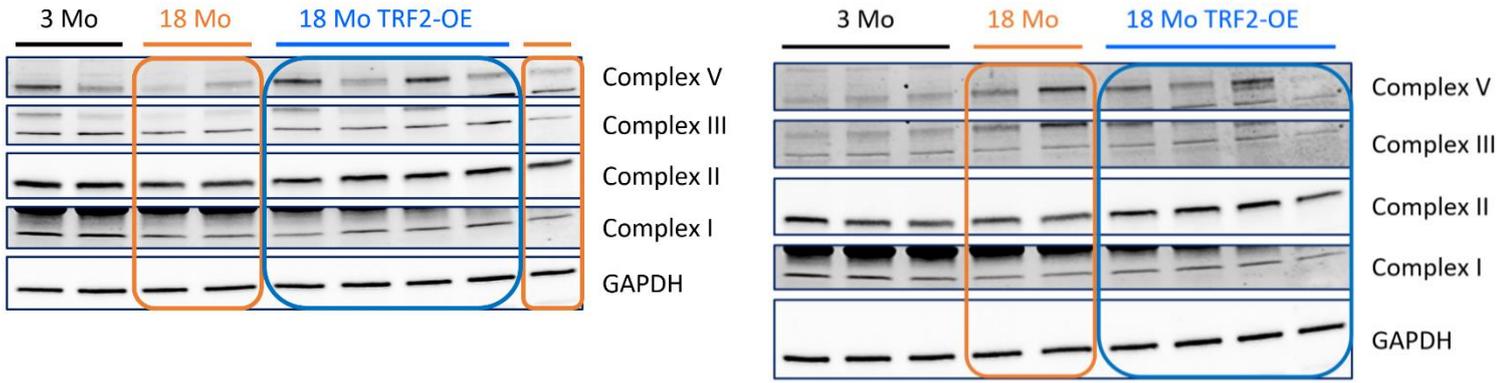
**Figure E6. *Terf2* hippocampal downregulation increases the protein levels of the electron transport chain Complex I and III**

(A) Protein levels of the Complex I, II, III and V of the electron transport chain upon TRF2 downregulation (*shTerf2*) or not (*shSCRB*). (B-E) Quantification of (B) Complex I, (C) Complex II, (D) Complex III and (E) Complex V. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).



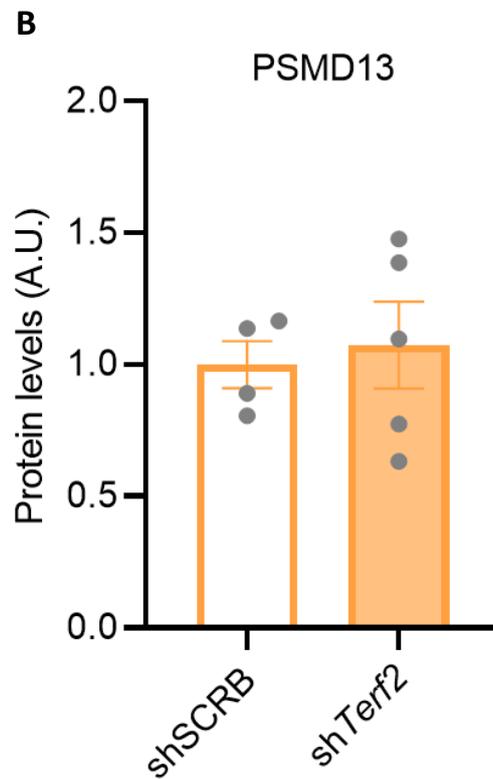
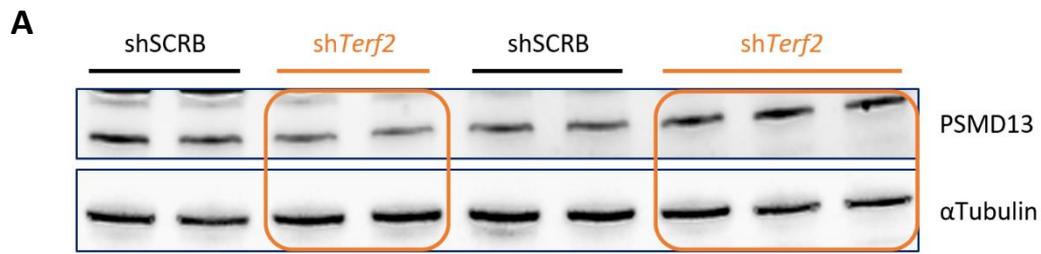
**Figure E7. ATM inhibition upon *Terf2* hippocampal downregulation does not affect the expression of the ETC complexes**

(A) Protein levels of the Complex I, II, III and V of the electron transport chain upon ATM inhibition (KU60019) or not (Vehicle) and TRF2 downregulation (sh*Terf2*) or not (shSCRB). (B-E) Quantification of (B) Complex I, (C) Complex II, (D) Complex III and (E) Complex V. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

**A**

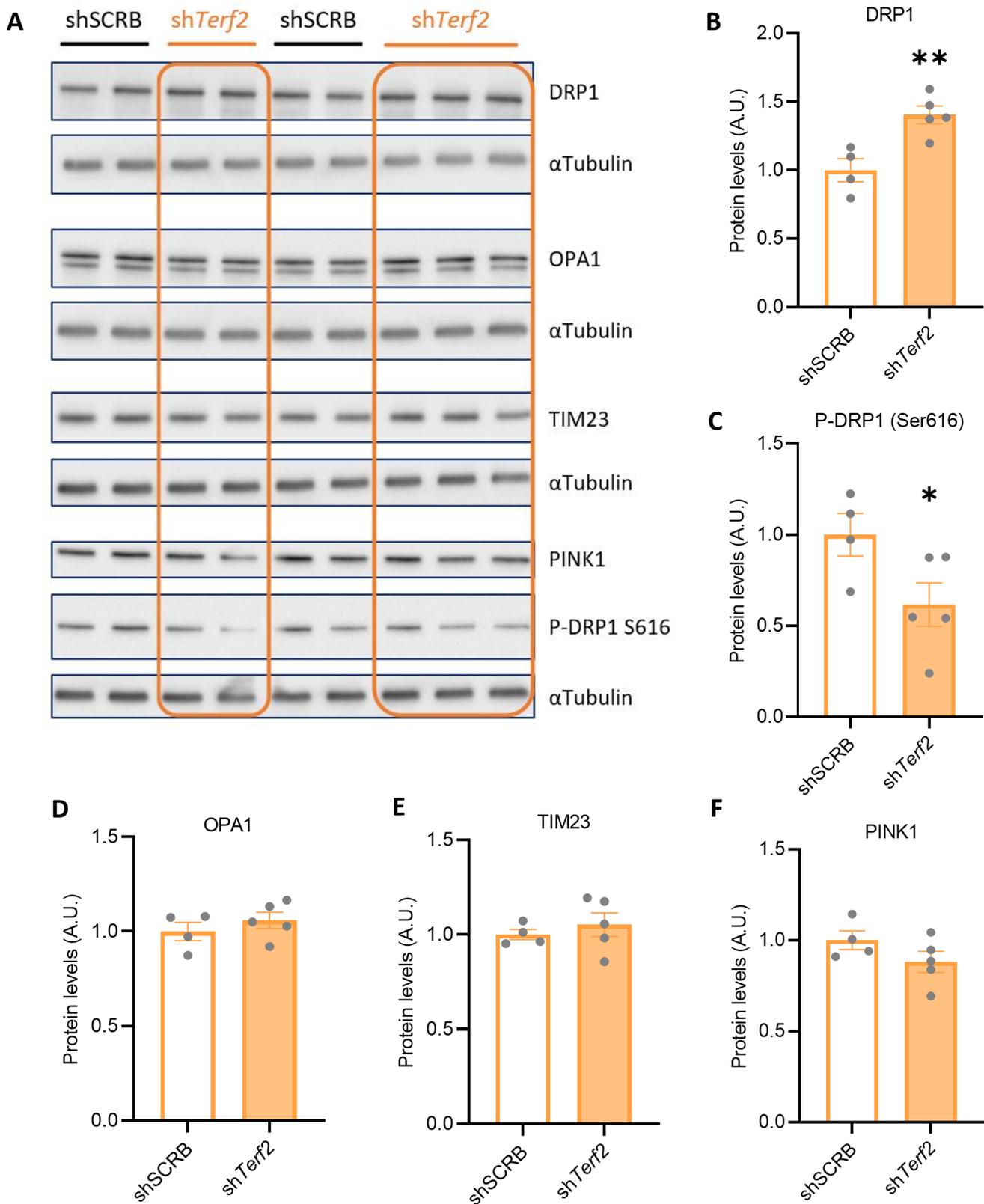
**Figure E8. Aging causes decreased expression of the ETC Complex I, which is not rescued by TRF2 overexpression**

(A) Protein levels of the Complex I, II, III and V of the electron transport chain during aging and upon TRF2 overexpression in elderly mice. (B-E) Quantification of (B) Complex I, (C) Complex II, (D) Complex III and (E) Complex V. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).



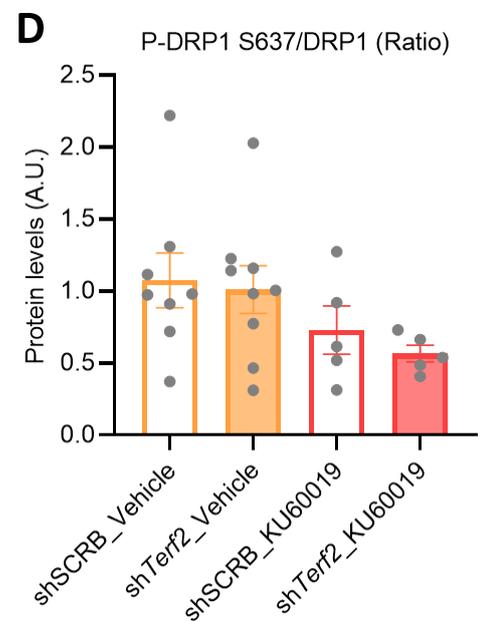
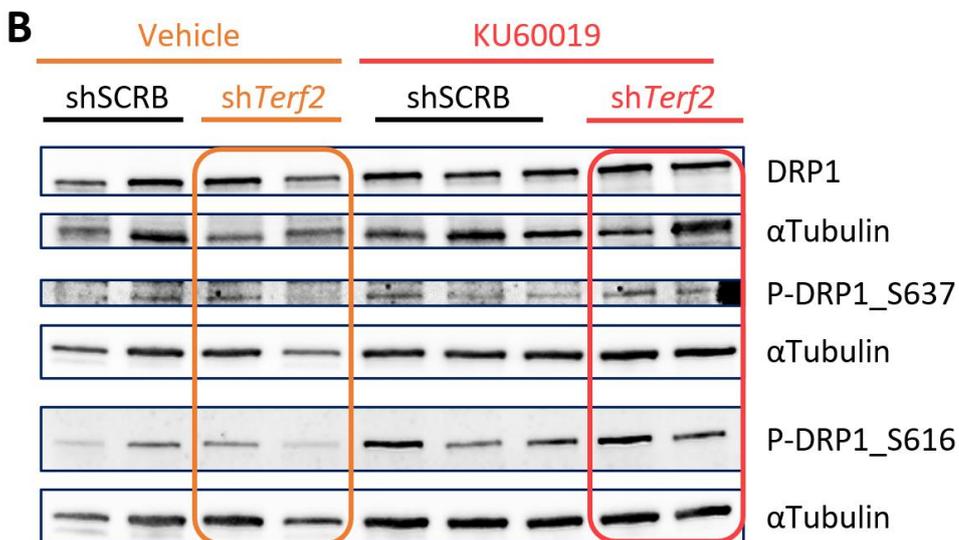
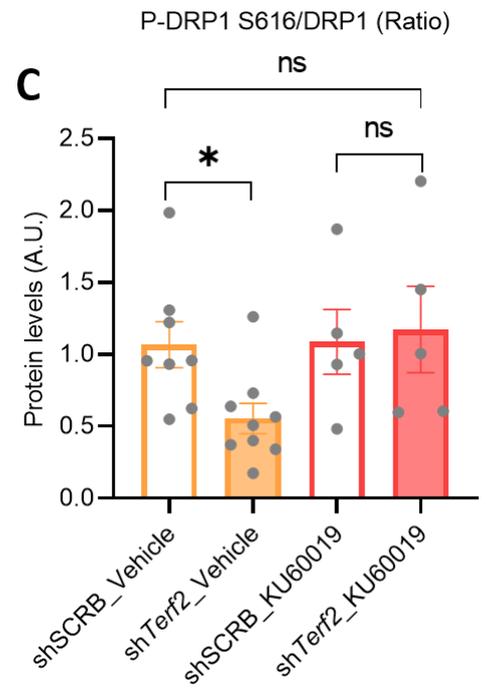
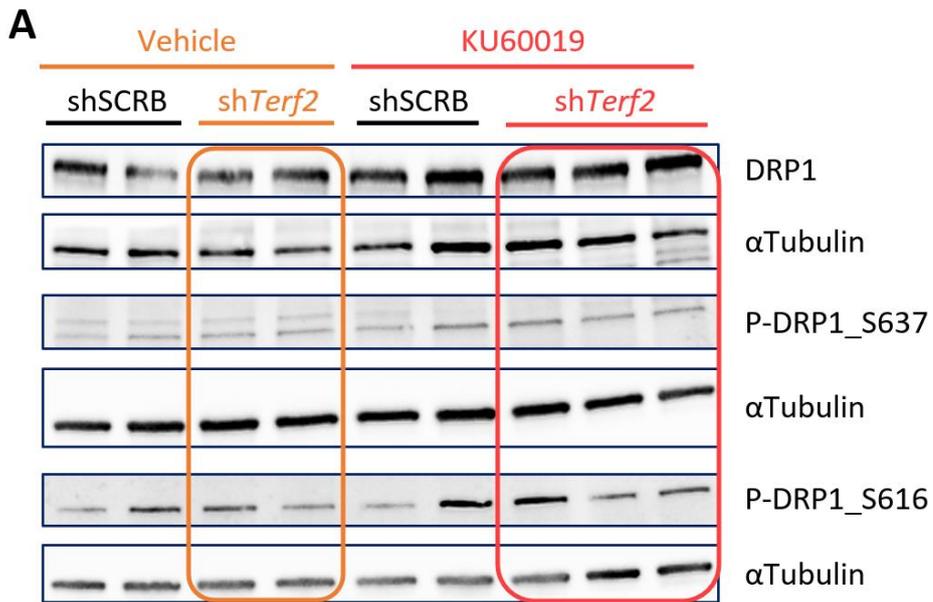
**Figure E9. *Terf2* hippocampal downregulation does not affect the protein levels of 26S proteasome non-ATPase regulatory subunit 13 (PSMD13), a subunit of the proteasome**

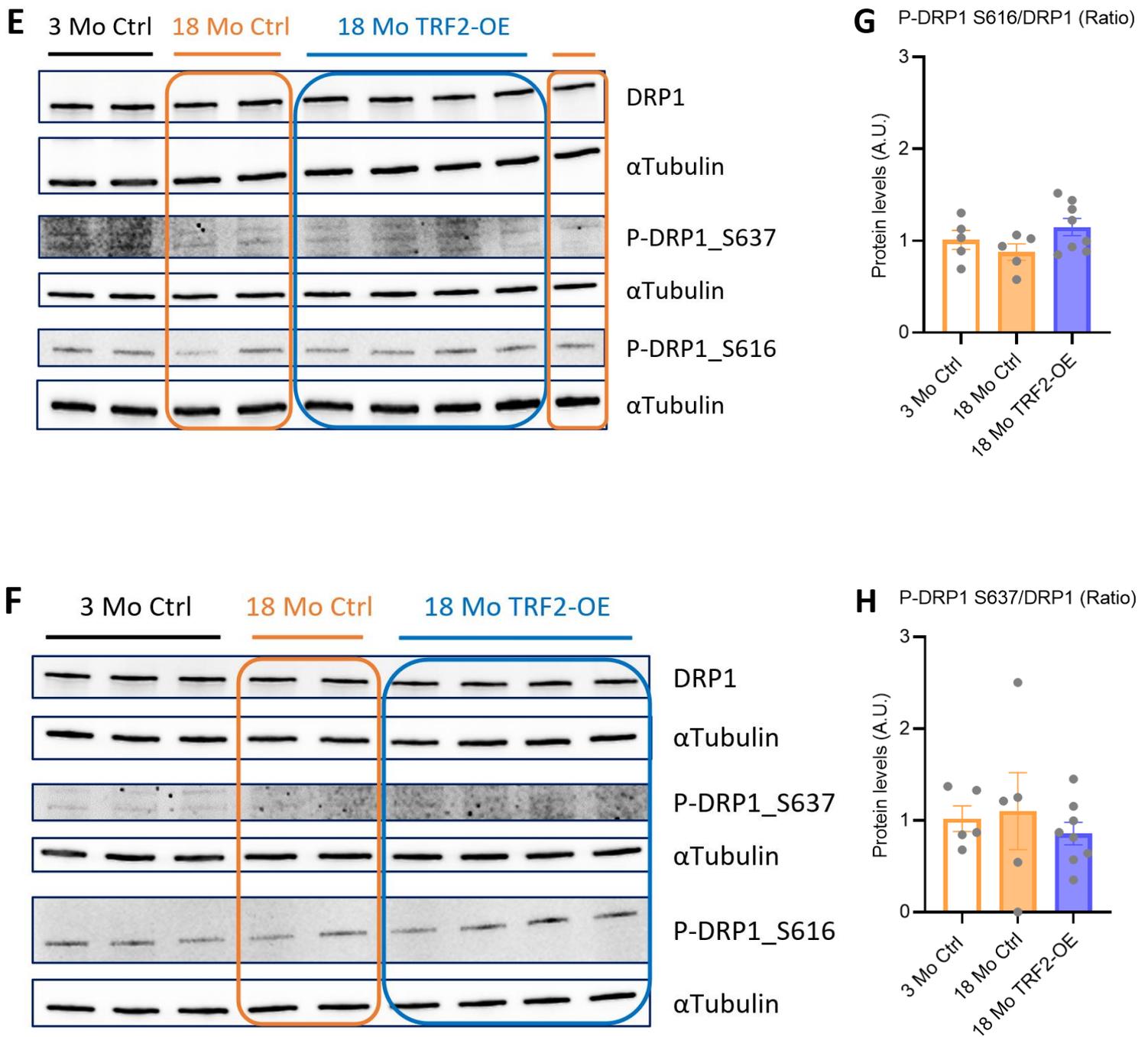
(A) Protein levels of PSMD13. (B) Quantification of (A). Hippocampi were taken from young mice that underwent TRF2 downregulation (*shTerf2*) or not (*shSCRB*) at 3 months of age and preformed NOR, MWM and CFC behavioral tests. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).



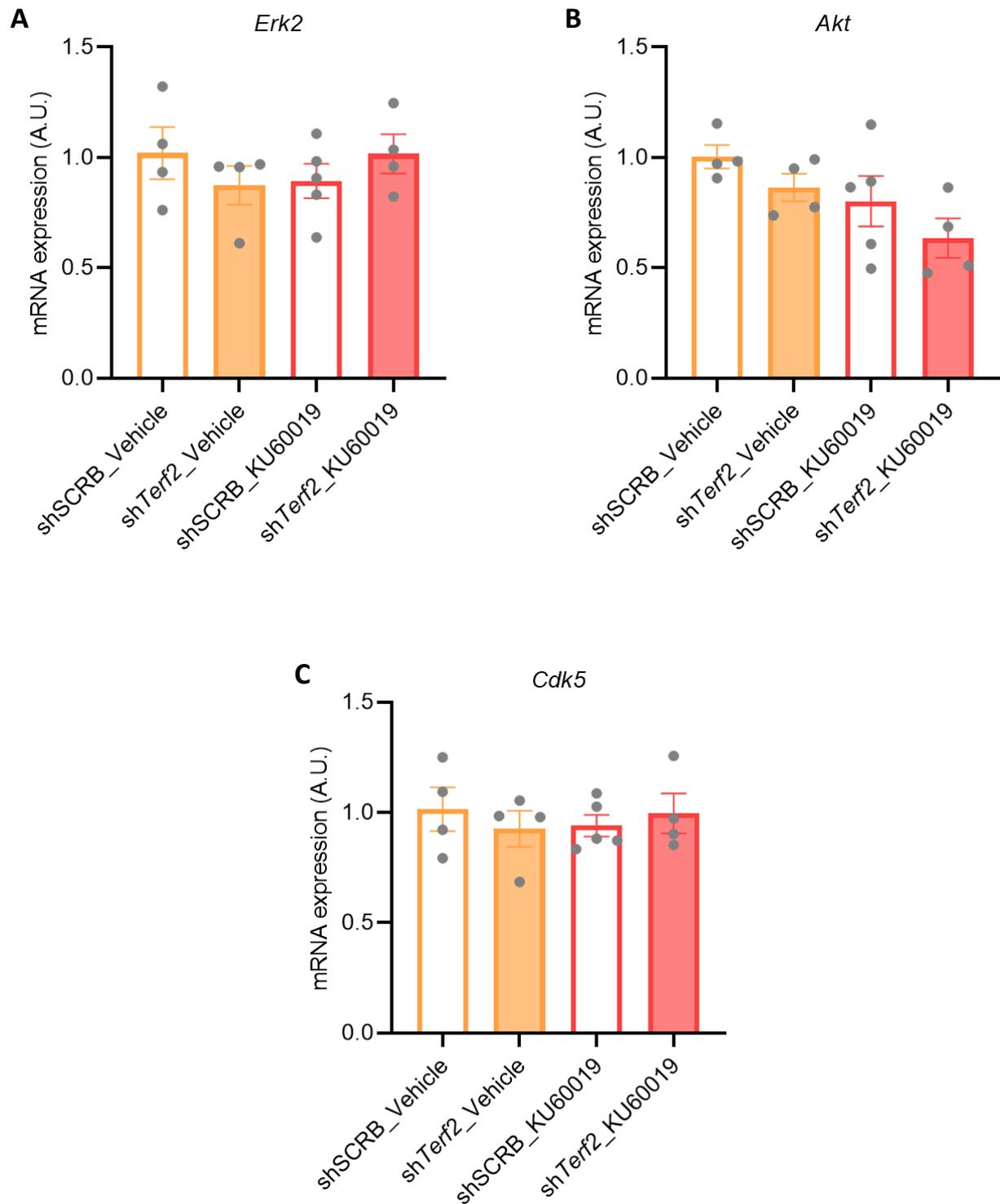
**Figure E10. *Terf2* hippocampal downregulation affects Dynamin-related protein 1 (DRP1) levels and its pro-mitochondrial-fission phosphorylation**

(A) Protein levels of DRP1, P-DRP1, OPA1 (Optic atrophy 1), TIM23 and PINK1 (PTEN-induced protein 1). (B-F) Quantification of (B) DRP1, (C) P-DRP1, (D) OPA1, (E) TIM23 and (F) PINK1. Hippocampi were taken from young mice that underwent TRF2 downregulation (*shTerf2*) or not (*shSCRB*) at 3 months of age and preformed NOR, MWM and CFC behavioral tests. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).



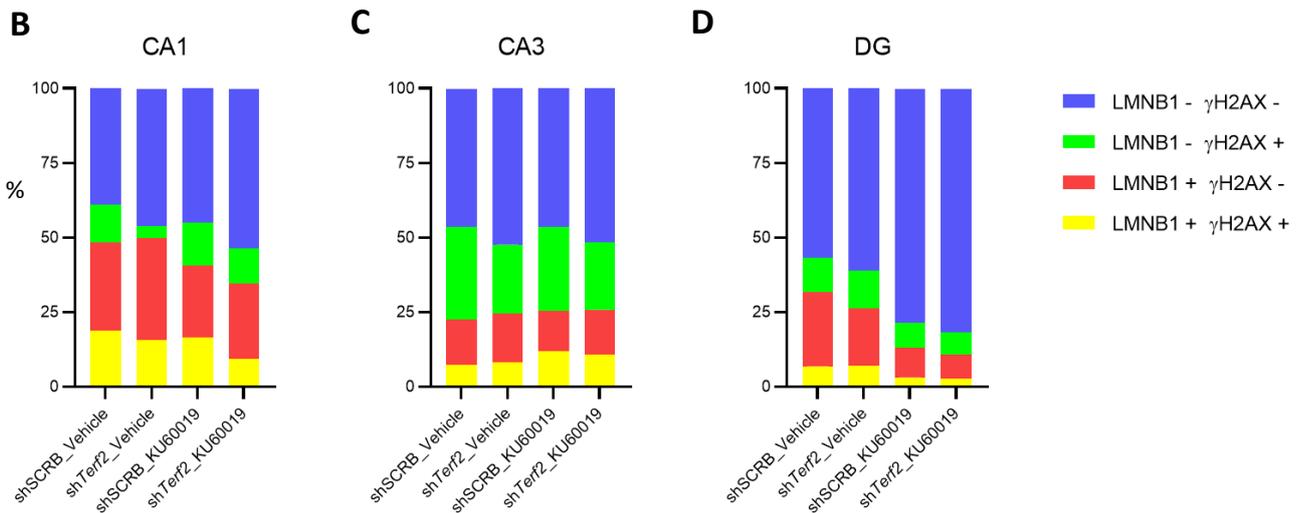
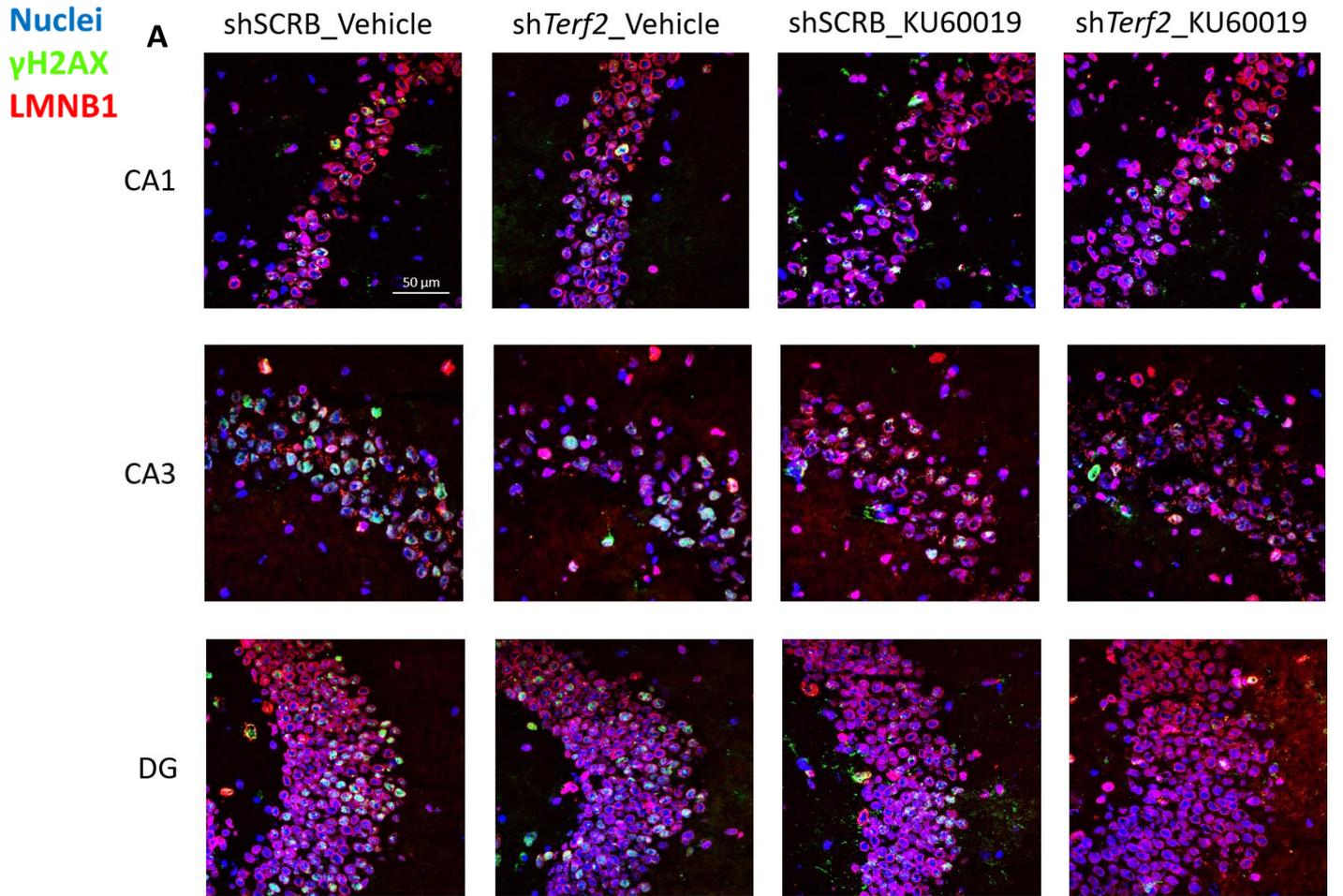


**Figure E11. Impaired DRP1 S616 phosphorylation is rescued by ATM inhibition, and increased upon TRF2 overexpression in old mice**  
 (A-B) Hippocampal protein levels of DRP1, P-DRP1 Ser616 (Activating phosphorylation) and P-DRP1 Ser637 (Inhibitory phosphorylation), upon TRF2 downregulation (shSCRB vs shTRF2) and ATM inhibition (KU60019 vs Vehicle). (C-D) Quantification of the Western blots shown in (A-B). (E-F) Hippocampal protein levels of DRP1, P-DRP1 Ser616 (Activating phosphorylation) and P-DRP1 Ser637 (Inhibitory phosphorylation) in young (3 Mo Ctrl), old mice (18 Mo Ctrl), and old mice overexpressing TRF2 (18 Mo TRF2-OE). (G-H) Quantification of the Western blots shown in (E-F). Hippocampi were taken from young mice that underwent *Terf2* downregulation (sh*Terf2*) or not (shSCRB) at 3 months of age and preformed NOR, MWM and CFC behavioral tests. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).



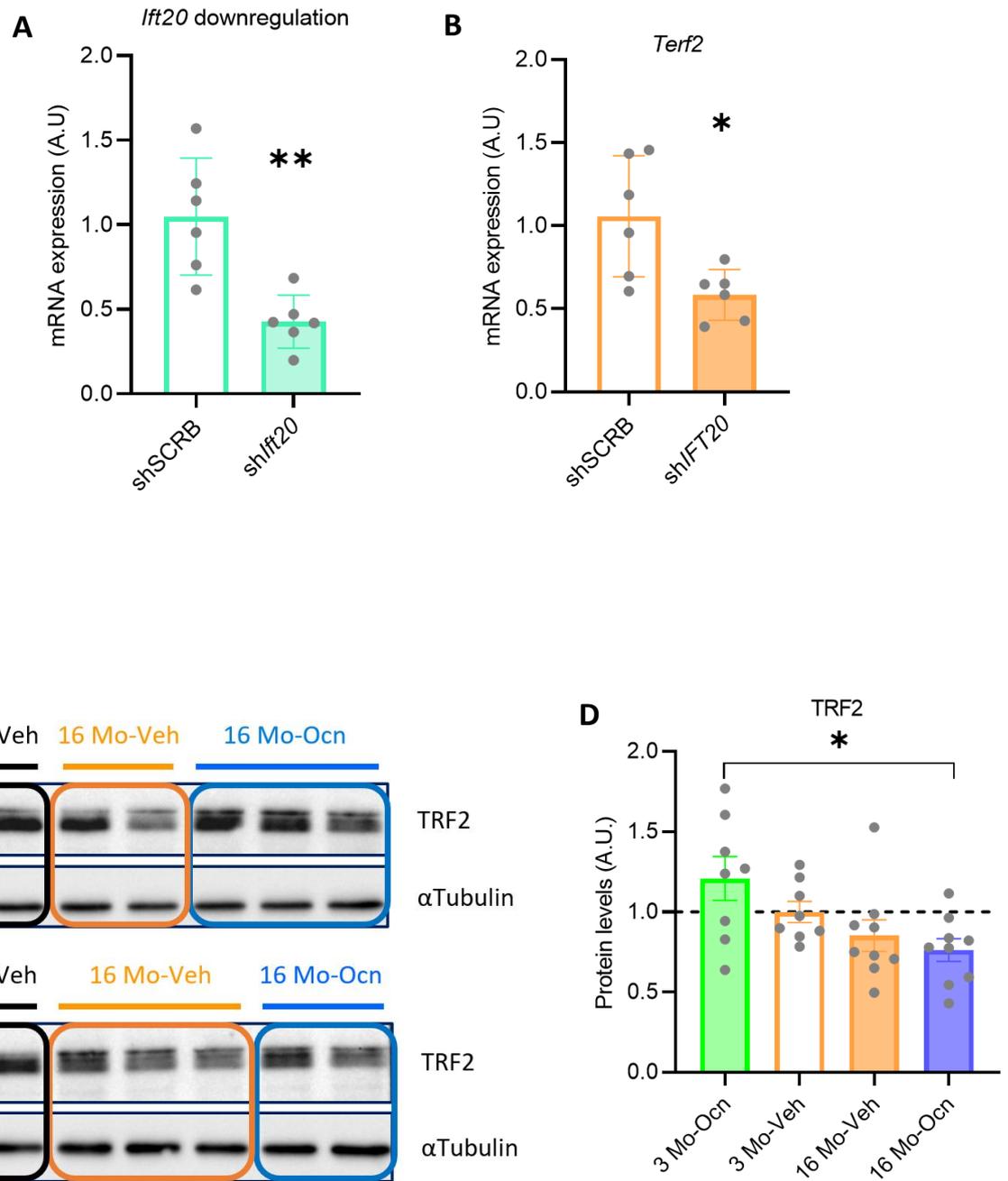
**Figure E12. *Terf2* hippocampal downregulation and ATM inhibition do not affect the expression of DRP1 kinases**

(A-C) mRNA expression of reported DRP1 kinases. (A) mRNA expression of Mitogen-activated protein kinase 1 (Erk2). (B) mRNA expression of Serine/threonine kinase 1 (Akt). (C) mRNA expression of Cyclin-dependent kinase 5 (Cdk5). Hippocampi were taken from young mice that underwent *Terf2* downregulation (sh*Terf2*) or not (shSCRB) and ATM inhibition (KU60019) or not (Vehicle) at 3 months of age. The animals performed NOR, MWM and CFC behavioral tests. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).



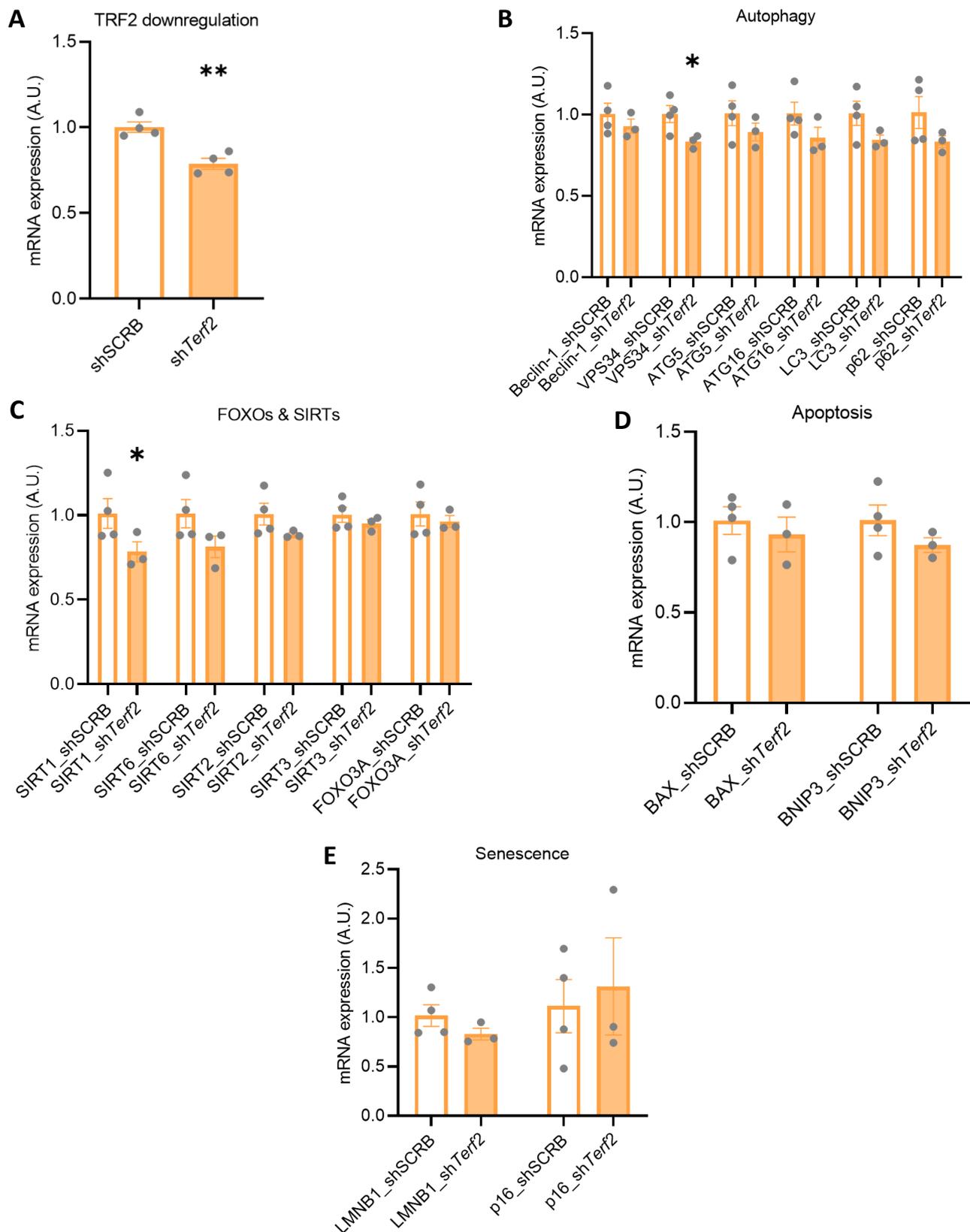
**Figure E13. *Terf2* hippocampal downregulation and ATM inhibition do not significantly affect LMNB1 and  $\gamma$ H2AX levels**

(A) Staining of  $\gamma$ H2AX and LMNB1 in CA1, CA3 and DG. (B-D) Quantification (%) of LMNB1 and  $\gamma$ H2AX positive/negative nuclei in (B) CA1, (C) CA3 and (D) DG. Brains were taken from young mice that underwent *Terf2* downregulation (sh*Terf2*) or not (shSCRB) and ATM inhibition (KU60019) or not (Vehicle) at 3 months of age. The animals preformed NOR, MWM and CFC behavioral tests. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).



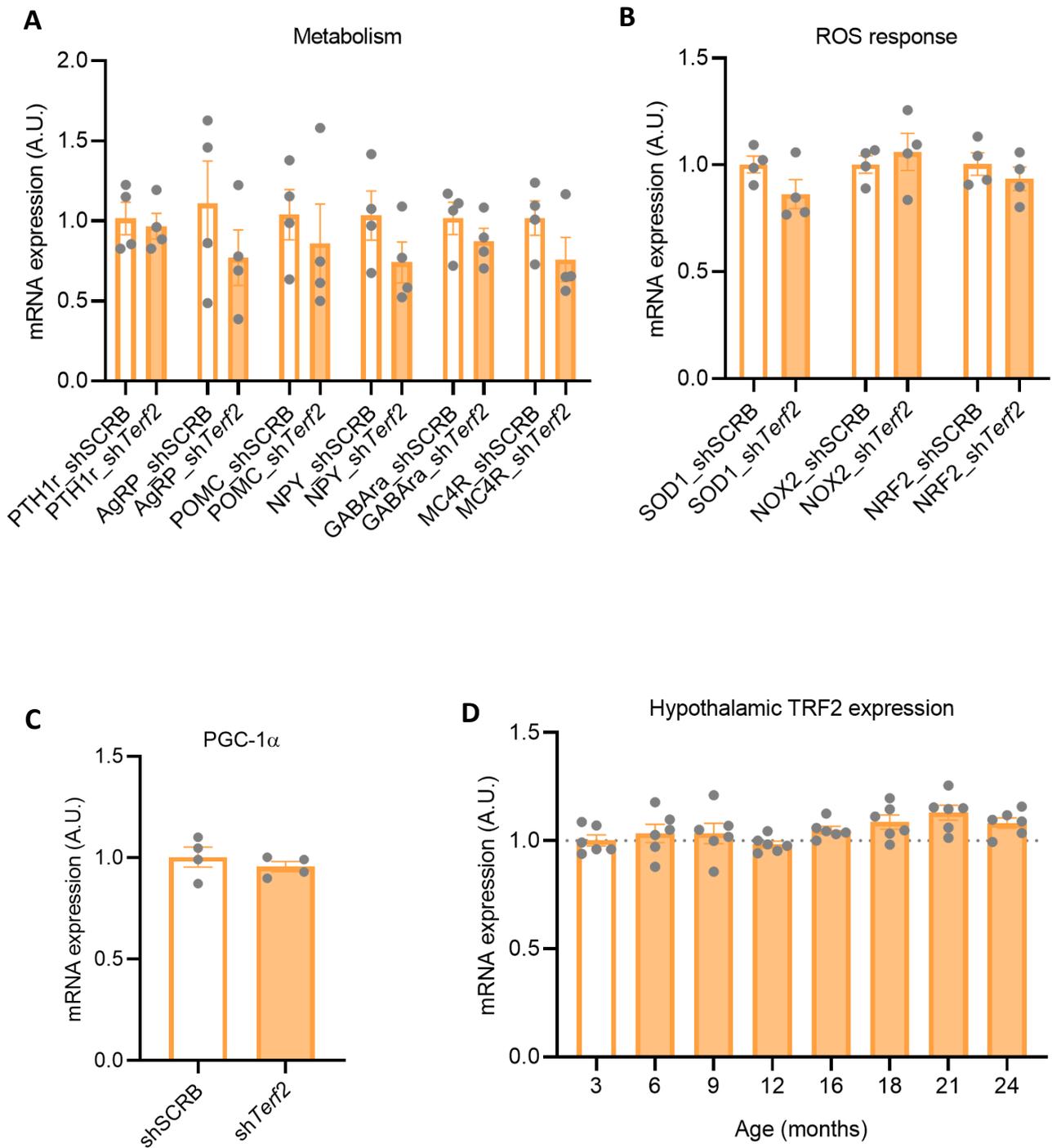
**Figure E14. IFT20 hippocampal downregulation causes a decrease in *Terf2* expression. Osteocalcin treatment increases TRF2 levels in young mice, but not in the elderly**

(A) AAV-mediated hippocampal downregulation of *Ift20* in 3-month-old mice. (B) *Terf2* expression upon *Ift20* downregulation. (C) Protein levels of TRF2 in young and old mice upon Osteocalcin (Ocn) treatment or not (Veh – Vehicle) (Representative Western blot) (D) Quantification of (C). All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ). [*Ift20*, Intraflagellar transport protein 20 homolog].



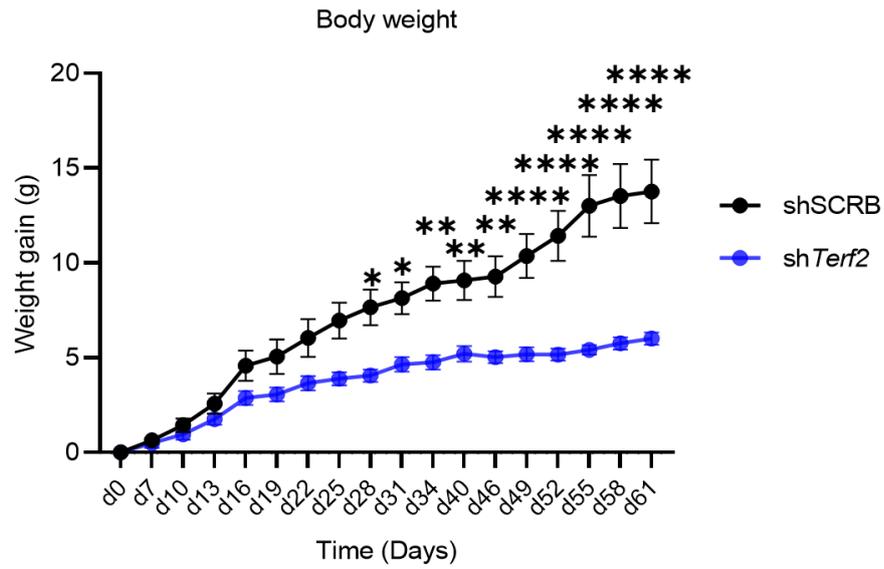
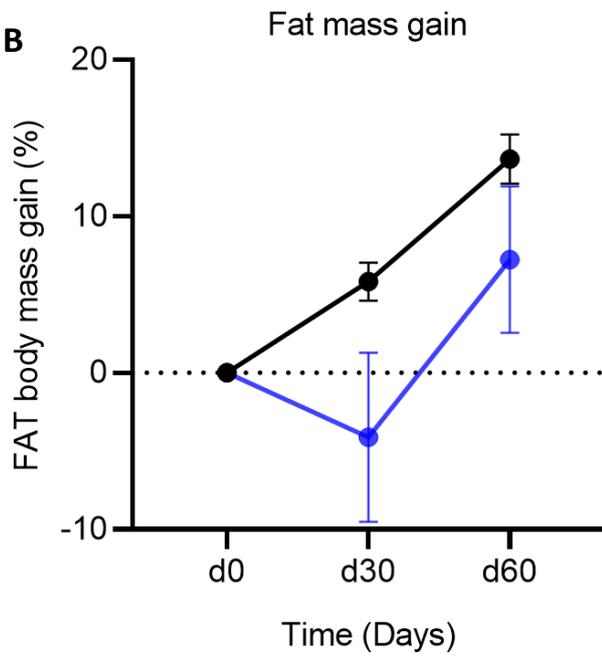
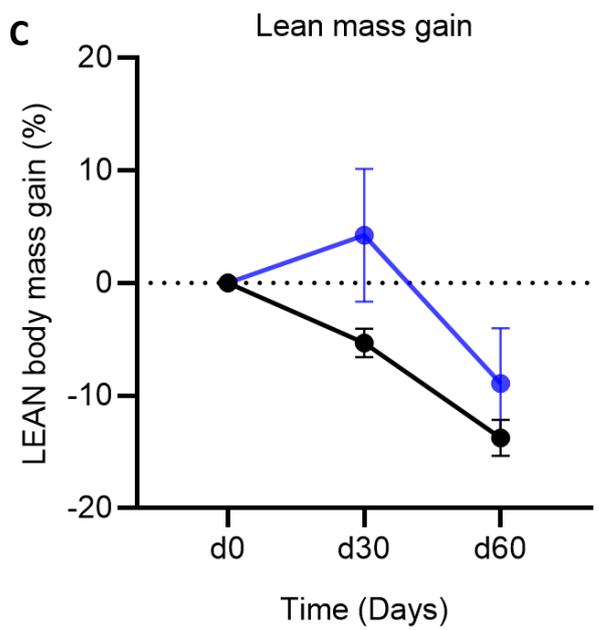
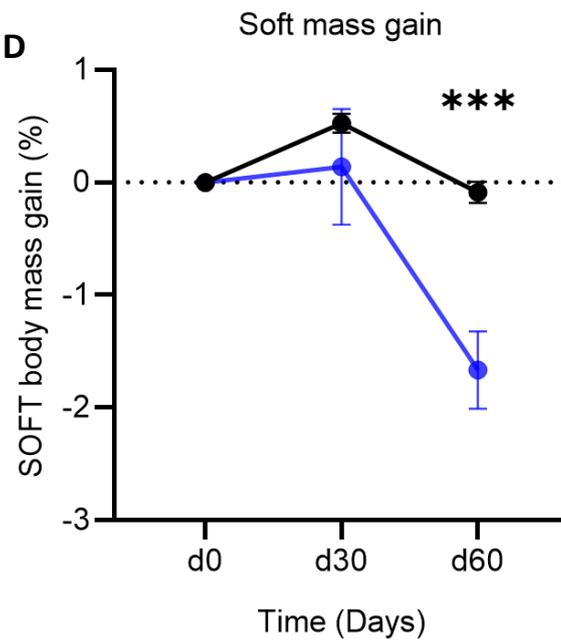
**Figure E15. *Terf2* hypothalamic downregulation impairs VPS34 and SIRT1 mRNA expression**

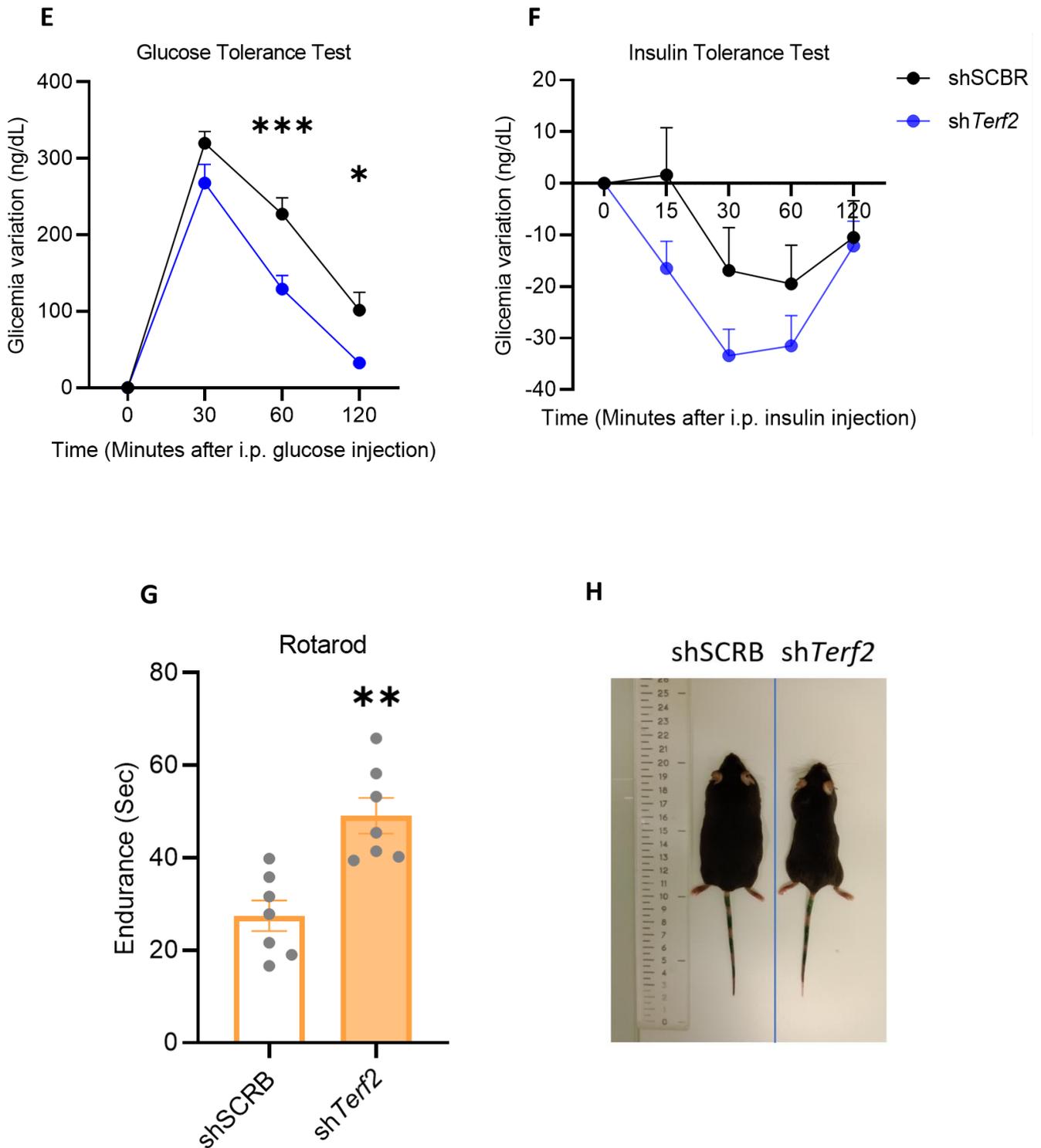
(A-E) Transcriptomic analysis of the effect of *Terf2* hypothalamic downregulation on several phenomena. (A) Confirmation of the AAV-mediated *Terf2* downregulation in the hypothalamus. (B-E) Effect of *Terf2* downregulation on (B) Autophagy, (C) longevity factor expression, (D) Apoptosis and (E) Senescence. Hypothalami were taken three weeks after the AAV injections were performed on 3-month-old mice. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ) [VPS34, Vacuolar protein sorting 34 homolog; BAX, BCL2-associated X protein; BNIP3, BCL2-interacting protein 3].



**Figure E16. *Terf2* hypothalamic downregulation does not affect the expression of genes involved in metabolism, ROS response and mitochondrial turnover**

(A-C) Effect of the AAV-mediated hypothalamic *Terf2* downregulation on (A) metabolism, (B) ROS response and (C) mitochondrial turnover. (D) *Terf2* hypothalamic expression during aging. Hypothalami were taken five weeks after the AAV injections were performed on 3-month-old mice. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ). [PTH1r, Parathyroid hormone 1 receptor; AgRP, Agouti-related neuropeptide; POMC, Proopiomelanocortin; NPY, Neuropeptide Y; GABAra, GABA receptor alpha; MC4R, Melanocortin 4 receptor; SOD1, Superoxide dismutase 1; NOX2, NADPH oxidase 2; NRF2, Nuclear factor erythroid 2-related factor 2; PGC-1 $\alpha$ , Peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$ ].

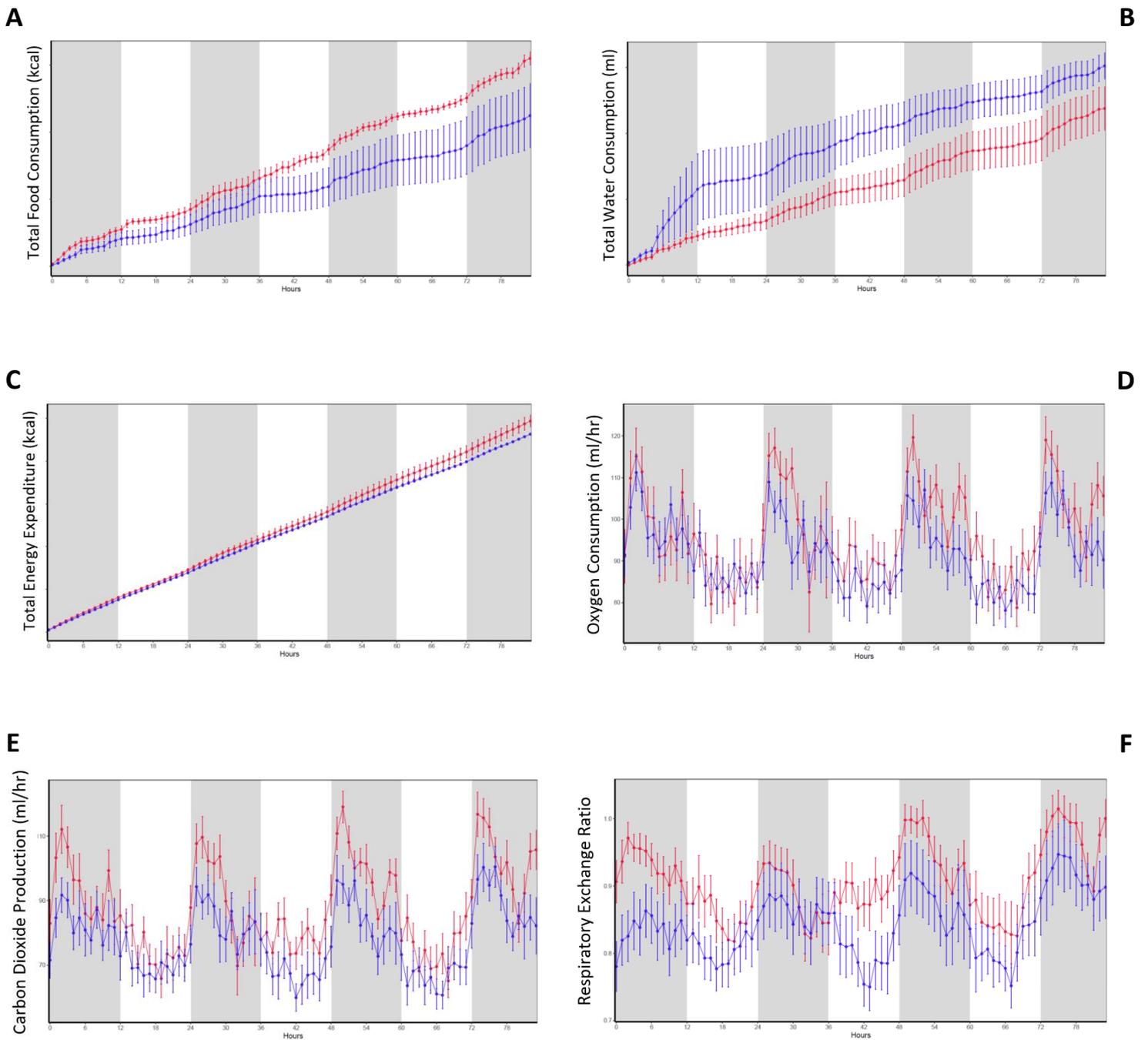
**A****B****C****D**



**Figure E17. Effect of *Terf2* hypothalamic downregulation on the metabolic profile**

(A) Longitudinal analysis concerning the effect of the AAV-mediated hypothalamic *Terf2* downregulation on body weight. (B-D) Body mass composition analysis upon *Terf2* downregulation 0, 30 and 60 days after AAV-mediated *Terf2* downregulation: (B) Fat mass gain, (C) Lean mass gain and (D) Soft mass gain. (E) Glucose tolerance test and (F) Insulin tolerance test at 0, 30, 60 and 120 minutes after i.p. injections of either glucose or insulin, respectively. (G) Rotarod test. (H) Representative picture of mice downregulated for TRF2 (*shTerf2*) or not (*shSCRB*) sixty days after AAV-mediated *Terf2* downregulation. All values are expressed as mean  $\pm$  SEM (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ).

• shSCRB  
• shTerf2



**Figure E18. Metabolic chambers: *Terf2* downregulation increases food and oxygen consumption, energy expenditure, carbon dioxide production and the respiratory exchange ratio**

(A-F) Mice were either downregulated for TRF2 (sh*Terf2*) or not (shSCRB) via stereotaxic hypothalamic injections of AAVs. Five weeks after the AAV injection, mice were housed into metabolic chambers to assess the effect of *Terf2* downregulation on several parameters. (A) Total food consumption (Kcal). (B) Total water consumption (ml). (C) Total energy expenditure (Kcal). (D) Oxygen consumption (ml/hr). (E) Carbon dioxide production (ml/hr). (F) Respiratory exchange ratio (CO<sub>2</sub> ml/O<sub>2</sub> ml).

# Materials & Methods

## Primary neuronal cell culture

The day prior to dissection, 12- and 24-well plates were coated with Poly L-Lysine [Sigma – L2020-25mg] in PBS [Gibco – 14190-094] (400 and 100 µl per well, respectively). On dissection day, plates were washed three times with PBS and let dry. After drying, plates were filled with complete (10%FBS, 1%P/S) DMEM media [Life Technologies – 61965059] (1 and 0.5 ml per well, respectively). Two pregnant mice (E16.5) were sacrificed to get their embryos. Embryo brains dissection of hippocampi was performed in ice-cold Leibowitz's L-15 medium [Gibco – 11415064]. After dissection, the hippocampi of each mouse were collected in a 15-ml falcon tube and centrifuged for 3' at 60g, at RT°. After centrifugation, Leibowitz's L-15 media was removed and 5 ml of pre-warmed Trypsin were added to each tube. Tubes with Trypsin were incubated for 15' at 37°C. After incubation, tubes were centrifuged again (3', 60g, RT°), Trypsin was removed and 7 ml of pre-warmed complete DMEM were added to each tube. Tubes were swung three times and three washes with complete pre-warmed DMEM were performed with 5'-centrifuges (60g, RT°). Pellets were resuspended with 1 ml complete pre-warmed DMEM and tissues were dissociated by pipetting twenty times up and down with a p1000, carefully avoiding the formation of bubbles. Once dissociation was complete, other 2 ml of DMEM per tube were added and resuspended six more times. Cells from both tubes were pooled and counted. 250 000 and 120 000 cells per well were plated for 12- and 24-well plates, respectively. Seeded plates were put at 37°C in 5% CO<sub>2</sub>. The next day, the media was removed from each well using a p1000 and pre-warmed complete (B27-50x [Gibco – 17504-044], Mycozap-0.3% [Lonza – VZA-20-21], Glutamax-100x [Gibco – 35050-038]) Neurobasal media [Life Technologies – 21103-049] was added carefully. Half of the media was changed twice a week until cells were used for molecular biology experiments at DIV18.

## Stereotaxic surgery

Mice were anesthetized via intraperitoneal injection of ketamine hydrochloride (20 mg/kg of body weight; Virbac) and xylazine (100 mg/kg of body weight; 2% Rompun, Bayer) and placed in a stereotaxic frame (900SL-KOPF). Ophthalmic eye ointment was applied to the cornea to prevent drying during surgery. The mouse head was shaved and Vetoquinol disinfectant was applied before the skin was longitudinally cut to expose the skull. The AAVs were injected bilaterally into the

hypothalami using the following coordinates (from Bregma; Paxinos and Franklin, 2008): X = +/-0.4 mm, Y = -1.4 mm, and Z = -5.67 mm. A 1  $\mu$ L volume of AAV was injected stereotaxically over 4 min (injection rate: 0.25  $\mu$ L per min). To limit the reflux along the injection track, the needle was maintained *in situ* for 4 min between each injection. After the injection, the scalp incision was sutured, and mice were kept under observation in a recovery chamber until fully motile.

### Adeno-associated viruses expressing shTerf2

Adeno-associated viruses (AAVs) expressing shRNA were purchased from Vector Biolabs (Malvern, PA). shRNAs to specifically target the TRF2 mRNA (AAV9-eSYN-eGFP-m-TRF2-shRNAmir(3) or scrambled non-targeting negative control (AAV9-eSYN-eGFP-scrmb-shRNAmir) were injected in a volume of 1  $\mu$ L (bilaterally), five weeks prior to behavioral tests and brain tissue collection. The AAV titers were 3.4 and 5 x 10<sup>13</sup> genome copies(GC)/ml, respectively. AAV9-eSYN-eGFP-m-TRF2-shRNAmir(3) specifically targets mature neurons, being driven by the eSYN promoter, a hybrid promoter consisting of the 0.45-Kb human Synapsin-1 promoter fragment (hSYN1). The used shRNA sequence was the following:

GCTGCTGATCAGTTCTCAGCTTCTGAGTGTTCCTGGCCACTGACTGACACTCAGAATGAGAACTGATCAG

### Western blotting

Mouse dorsal hippocampi were dissected, snap-frozen and lysed in Pierce RIPA lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% Na deoxycholate, 0.1% SDS plus complete protease and phosphatase inhibitors; ThermoFisher). The lysates were loaded on a 12% SDS polyacrylamide gradient gel and transferred onto a PVDF membrane. The blots were blocked in Tris-buffered saline 0.1% Tween (TBST) 5% BSA and incubated O/N at 4°C and, according to the experiment, the following primary antibodies were used:

Rabbit anti-TRF2 (1:5000; NB110-57130, Novus Biologicals), mouse anti- $\alpha$ tubulin (1:1000; T9026, Sigma), rabbit anti-cFOS (1:1000; ABE457, Merck), rabbit anti-P-CHEK2 (1:1000; CST, 2665T), rabbit anti-GAPDH (1:1000; NB100-56875, Novus Biologicals), rabbit anti-Vinculin (1:1000; Sigma, V9131), rabbit anti-CAMKII (1:1000; 3362S, CST), rabbit anti-P-CAMKII (1:1000; 12716S, CST), mouse anti-Beclin-1 (1:1000; 612113, BD Transduction Laboratories), rabbit anti-LC3 (1:10000; L7543, Sigma), mouse anti-ATG5 (1:500; 0262-100/ATG5-7C6, Nanotools), rabbit anti-VPS34 (1:1000; 4263, CST), and guinea pig anti-p62 (1:2000; GP62-C, Progen), mouse anti-ATG5-12 (1:1000, 0262-100, Nanotools), rabbit anti-SIRT1 (1:10000, 07-131, Millipore/Upstate), rabbit anti-P-SIRT1 (1:1000,

2314S, CST), rabbit anti-FOXO3A (1:1000, 75D8, CST), rabbit anti-Cleaved-Caspase-3 (1:1000, 96645, CST), mouse anti-TIM23 (1:1000, 311222, BD Biosciences), rabbit anti-PSMD13 (1:1000, NBP-80961, Biotechne), rabbit anti-DRP1 (1:1000, NB110-55288, Novus Biologicals), rabbit anti-P-DRP1 [S616] (1:1000, 3455, CST), rabbit anti-P-DRP1 [S637] (1:1000, 4867S, CST), rabbit anti-OPA1 (1:1000, NB110-55290, Biotechne), rabbit anti-PINK1 (1:1000, Ab23707, Abcam), OXPHOS antibody cocktail (1:1000, Ab110411, Abcam). After the O/N incubation and three 10-minute TBST washes, the membranes were incubated, according to each specific Western blot, with the following secondary antibodies: anti-mouse IgG, HRP-linked antibody (1:10000; 7076, CST), anti-rabbit IgG, HRP-linked antibody (1:10000; 7074, CST) and anti-guinea pig IgG, HRP-linked antibody (1:10000; A5545, sigma) for 1h at RT°. The bands' signals were revealed using an ECL kit for protein detection (Clarity Western ECL Substrate, BioRad). Selected films were scanned and quantified using BioRad Image Lab software (Version 5.2).  $\alpha$ Tubulin or GAPDH bands were used for normalization. All Western blot analyses were performed on freshly prepared hippocampal lysates.

### Semiquantitative RT-PCR

Brain tissues were immediately flash-frozen after dissection and total RNA was isolated with TRIzol Reagent using a homogenizer. Single-strand cDNA was synthesized from total RNA (2 mg) by using SuperScript II Reverse Transcriptase. qRT-PCR was performed using iTAQ SYBR Green (BioRad). According to the different experiments, the following primers were used:

*Terf2* (F: AAGCCCAGAGCTCTCAACC; R: TCCACTTGTCTTTGGATGCTT)  
*Rest* (F: CATGGCCTTAACCAACGACAT; R: CGACCAGGTAATCGCAGCAG)  
*Snap25* (F: CAACTGGAACGCATTGAGGAA; R: GGCCACTACTCCATCCTGATTAT)  
*cFOS* (F: CAGAGCGCAGAGCATCGCCA; R: CGATTCCGCCACTTGGCTGC)  
*FOXO3a* (F: GCTAAGCAGGCCTCATCTCA; R: TTCCGTCAGTTTGAGGGTCT)  
*Sirt3* (F: ATCCCGGACTTCAGATCCCC; R: CAACATGAAAAAGGGCTTGGG)  
*Mmp2* (F: CTGCTGGCTAGTTGCACCC; R: CCTTTGGGCTAGGTATCTCTGA)  
*p16/Cdkn2a* (F: CGCAGTTCTTGTCCTACTGT; R: TGTTACGAAAGCCAGAGCG)  
*p21/Cdkn1a* (F: CGGTGTCAGAGTCTAGGGGA; R: ATTGGAGTCAGGCGCAGATC)  
*Lmnb1* (F: ATTTGGAGAATCGCTGTCAGAG; R: AAGCGGGTCTCATGCTTCC)  
*Gapdh* (F: AGGTCGGTGTGAACGGAT; R: GGGGTCGTTGATGGCAACA)  
*16S* (F: CCAGCTATCACCAAGCTCGT; R: CTAGAAACCCCGAAACCAAA)  
*12S* (F: AATGAAGTACGCACACACCG; R: GGGTGTAGGCCAGATGCTTT)  
*Nd1* (F: CCATTTGCAGACGCCATAA; R: TGAAATTGTTTGGGCTACGG)  
*Nd2* (F: CTCCGTGCTACCTAACACCTTATC; R: GACCTAAGAAGATTGTGAAGTAGATGATG)  
*Nd3* (F: AGCTCTGCACGTCTACCATT; R: TGTGTTTCATTCATATGCTAGGCC)  
*Nd4* (F: CACTTCTATGACAAACCGACGA; R: TTAGTGGCAGTAATCAGGCTGT)  
*Nd4l* (F: CCTCACCATAGCCTTCTCACT; R: TGGGGATTGGTATGGAGCTT)  
*Nd5* (F: TCCTACTGGTCCGATTCCAC; R: TGATGTCGTTTTGGGTGAGA)  
*Nd6* (F: CCCAGCTACTACCATCATTCAAGT; R: GATGGTTTGGGAGATTGGTTGATGT)  
*Col* (F: ACTCCTACCACCATCATTCTCC; R: GGCTAGATTTCCGGCTAGAGG)

*Coll* (F: CTTGGTCTACAAGACGCCAC; R: CTATTGGCAGAACGACTCGG)  
*Coll3* (F: AGTATCATGCTGCGGCTTCA; R: TACCAAGGCCACCACTCC)  
*Atp8* (F: TGCCACAAC TAGATACATCAACA; R: GTTGGGGTAATGAATGAGGCA)  
*Atp6* (F: AAGCTCACTTGCCCACTTCC; R: GTAAGCCGGACTGCTAATGC)  
*CytB* (F: TTTTATCTGCATCTGAGTTTAATCCTGT; R: CCACCTCATCTTACCATTTATTATCGC)  
*Erk* (F: CCTACGGCATGGTTTGCTCT; R: AGGTCTGGTGCTCAAAAGGA)  
*Akt* (F: CCGCCTGATCAAGTTCTCCT; R: GGCTTCTGGACTCGGCAAT)  
*Cdk5* (F: CAGAGCTGGCTAATGCAGGA; R: TGTCCTAGCAGTCGGAAGA)  
*Bax* (F: AGACAGGGGCCTTTTTGCTAC; R: AATTCGCCGGAGACTCG)  
*Bnip3* (F: ACACAAGCGTTATGAAGAAAGGG; R: CTTCCAATGTAGATCCCCAAGC)  
*Foxo6* (F: TCATGGACAGTGACGAAATGG; R: ACCCAGCTCTGGTTAGGGG)  
*Sirt6* (F: CCTGGTCAGCCAGAACGTAG; R: TACTGCGTCTTACTACTTGGGA)  
*Foxo4a* (F: GTGCTCGCATCTCCTACTGAAG; R: CATGTCGCACTCCAGGTTCT)  
*Gadd45a* (F: AGACCGAAAGGATGGACACG; R: GTACACGCCGACCGTAATG)  
*Cdkn1b* (F: TCTCTTCGGCCCGGTCAAT; R: AAATTCCACTTGCGCTGACTC)  
*Pgc1a* (F: AAAGTTGCTAGCGGTCTCTCA; R: TGGCTGGTGCCAGTAAGAG)  
*Sod1* (F: AACCAGTTGTGTTGTCAGGAC; R: CCACCATGTTTCTTAGAGTGAGG)  
*Nox2/Ncf1* (F: ACACCTTCATTCGCCATATTGC; R: TCGGTGAATTTTCTGTAGACCAC)  
*Nox2/Cybb* (F: TGTGGTTGGGGCTGAATGTC; R: CTGAGAAAGGAGAGCAGATTTTCG)  
*Maoa* (F: GCCCAGTATCACAGGCCAC; R: CGGGCTTCCAGAACCAAGA)  
*Maob* (F: ATGAGCAACAAAAGCGATGTGA; R: TCCTAATTGTGTAAGTCCTGCCT)  
*Nrf2* (F: TAGATGACCATGAGTCGCTTGC; R: GCCAACTTGCTCCATGTCC)  
*Ppp2r2c* (F: ACGGACACGCGGAAAATTAAC; R: CAGTTCCCACTGTGGTTGA)  
*Atg5* (F: TGTGCTTCGAGATGTGTGGTT; R: GTCAAATAGCTGACTCTTGGCAA)  
*Beclin-1* (F: ATGGAGGGGTCTAAGGCGTC; R: TCCTCTCCTGAGTTAGCCTCT)  
*Ift20* (F: AGGCAGGGCTGCATTTTGAT; R: CAAGCTCAATTAGACCACCAACA)  
*Pink1* (F: TTCTTCCGCCAGTCGGTAG; R: CTGCTTCTCCTCGATCAGCC)  
*Opa1* (F: TGGAAAATGGTTCGAGAGTCAG; R: CATTCCGTCTCTAGGTTAAAGCG)  
*Drp1* (F: CAGGAATTGTTACGTTCCCTAA; R: CCTGAATTAAGTTGTTCCCGTGA)  
*Tim23* (F: GAAGGTGGCGGAAGAAGTAGC; R: GGGGGTTCATACCAGTCAGC)  
*Vps34* (F: CCTGGACATCAACGTGCAG; R: TGTCTTGGTATAGCCCAGAAA)  
*Atg16* (F: CAGAGCAGCTACTAAGCGACT; R: AAAAGGGGAGATTCGGACAGA)  
*Lc3* (F: GACCGCTGTAAGGAGGTGC; R: CTTGACCAACTCGCTCATGTTA)  
*p62* (F: CCGGCTGATTGAGTCCCTC; R: CCCCAGATGTCGTAATTCTTGG)  
*Sirt1* (F: TGATTGGCACCGATCCTCG; R: CCACAGCGTCATATCATCCAG)  
*Sirt2* (F: CCACGGCACCTTCTACACATC; R: CACCTGGGAGTTGCTTCTGAG)  
*Agrp* (F: ATGCTGACTGCAATGTTGCTG; R: CAGACTTAGACCTGGGAAGTCT)  
*Npy* (F: ATGCTAGGTAACAAGCGAATGG; R: TGTCGCAGAGCGGAGTAGTAT)  
*Pomc* (F: ATGCCGAGATTCTGCTACAGT; R: TCCAGCGAGAGGTCGAGTTT)  
*Mc4r* (F: CCCGGACGGAGGATGCTAT; R: TCGCCACGATCACTAGAATGT)  
*GabaRa* (F: GGAAGCTACGCTTACACAACC; R: CCATCGGGAGCAACCTGAA)  
*Pth1r* (F: CCTGAGTCAGCTCCTCAACC; R: GCCGGCCCAATTGTGTGTC)

## Microscopy

Mice were sacrificed and the whole brains were immediately dissected, rinsed in 4°C PBS, and frozen in OCT embedding matrix (WVR) into plastic molds lean on a metallic support on top of dry ice plus isopropanol, to speed up the freezing process. Samples were stored at -80°C. 5- $\mu$ m-thick brain cross-sections containing the hippocampi were cut with a Cryostat (CryoStar, Fisher) at -20°C, immediately placed on microscopy glass slides and stored at -80°C.

The day of the staining, the sections were let dry at RT° for 10' and fixed in PBS 4% formaldehyde for 20'. The sections were then permeabilized in PBS 0.2% Triton for 20', quickly washed in PBS three times, and blocked in PBS 0.1% Tween 4% BSA for 1h at RT°. After blocking, the slides were incubated with the primary antibodies diluted accordingly into blocking solution, O/N at 4°C. Following O/N incubation, the slides were washed quickly twice and once for 15' in PBS 0.1% Tween (60rpm on a rocking platform). After washing, the slides were incubated with the secondary antibodies diluted accordingly into blocking solution, for 1h at 37°C. Afterwards, the slides were quickly washed twice and once for 15' in PBS 0.1% Tween (60rpm on a rocking platform), let dry and set with mounting medium plus DAPI (Vectashield, Vector Laboratories).

The slides were incubated with the following primary and secondary antibodies: rabbit anti- LMNB1 (1:200; Ab16058, Abcam), mouse anti- $\gamma$ H2AX (1:200; 05-636, Millipore), anti-mouse-Cy3 (1:500; 715-165-151, Jackson ImmunoResearch), anti-rabbit-AF647 (1:500; 711-605-152, Jackson ImmunoResearch).

## Insulin and glucose tolerance tests

Mice were maintained in a normal light/dark cycle according to the standard protocols and fasted for at least 16 hours prior to the test. One by one, mice were weighed and their tails cut at the very tip for blood draw. Baseline glucose was measured using a glucometer. Mice were then intraperitoneally (i.p.) injected with either 30% Glucose or Insulin with a volume ( $\mu$ l) equal to their weight (g) multiplied by a factor of 6.6. The glycaemia was then measured again at 15, 30, 60 and 120 minutes from the injection. After the test, mice were allowed time to recover and food was regularly supplied again.

### Body mass composition

One by one, mice were anesthetized with Isoflurane, put inside a Faxitron machine (Hologic) and X-ray-scanned. Images were taken and the selected ROI were analyzed via the Faxitron software to obtain the percentages of fat, lean and soft mass of the animals.

### Rotarod

Mice were trained to walk on a horizontally-placed rotating cylinder in groups of five. Once the animals could sustain the walk for at least 20 seconds without falling off the cylinder, they were considered properly trained. After training, mice underwent five rounds each of Rotarod walks and their endurance (Seconds) was scored as the average of the five walks.

### QUANTIFICATION AND STATISTICAL ANALYSIS

All values are expressed as mean  $\pm$  SEM. Alpha was set to 0.05 for all analyses. Statistical parameters including the exact sample size (n), *post hoc* tests, and statistical significance are reported in every figure and figure legend. The number of mice was estimated to be sufficient based on pilot experiments. Data were estimated to be statistically significant when  $p \leq 0.05$  by non-parametric Mann-Whitney U test or Student's t test, and non-parametric Kruskal-Wallis or one-way/two-way ANOVAs with repeated measures when appropriate. Significant ANOVAs were followed by a two-way repeated-measures analysis of variance with a *post hoc* Tukey's HSD multiple comparisons test for pairwise differences between each mouse groups. In every figure, an asterisk denotes statistical significance (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$ ). Data were analyzed using GraphPad Prism v5 software.

## 8. Discussion

Aging is characterized by increased disease susceptibility and loss of intrinsic capacity, even in healthy individuals (being intrinsic capacity defined by the WHO as mobility, cognition, vitality, sensorial perception and psychological ability – <https://www.who.int/publications/i/item/WHO-FWC-ALC-17.2>). The world aged population (older than 65-year-old) is right now (as of 2024) in a phase of steep increase and will reach and overcome the youngest fractions of the world population (0→14-year-old and 15→24-year-old) by 2060. Therefore, it results evident that advances in the understanding of the molecular mechanisms underlying intrinsic capacity are of the utmost importance for the future of our societies. From a brain perspective loss of intrinsic capacity means decreased sensorial perception, compromised motor coordination and impaired memory and learning. In this work, we focused on the latter by studying in a murine *in vivo* model the mechanisms responsible for memory loss during physiological aging.

The main result of this study is that the expression of the telomere-binding protein TRF2 in hippocampal neurons is a limiting factor during aging for the maintenance of memory function.

First, we showed that the expression of TRF2 decreases in the murine hippocampus during aging, both at the transcriptional and protein level. The impact of this decrease was studied by reducing the expression of TRF2 in hippocampal neurons of young mice by means of stereotaxic injections of adeno-associated viruses (AAVs) expressing sh*Terf2* under the control of the mature neuron-specific eSYN promoter. Noteworthy, the reduction of *Terf2* expression in young mice by means of this viral strategy (approximately 50%) is alike the reduction of TRF2 expression observed in old mice, validating this approach for the sake of understanding the effect of TRF2 decreased levels in aging mice. As a consequence of such a decreased *Terf2* expression we revealed a drastic impairment of episodic and contextual memory, whereas spatial memory was not affected. The complete and stunning recovery of episodic and contextual memory in old mice upon *Terf2* overexpression in hippocampal neurons corroborates the fact that TRF2 levels affect memory during aging.

The differential effect of TRF2 on memory types can be explained by a regional effect of *Terf2* downregulation — spatial memory mainly involving the DG (Clelland et al., 2009) (Trouche et al., 2009) (Masachs et al., 2021); episodic and contextual memory mainly involving CA3 and CA1, respectively (Kesner, 2007b) (Ji & Maren, 2008). This effect may also be due to a different role of TRF2 in the distinct regions of the hippocampus or to an unequal AAV diffusion to the different

hippocampal areas upon injection. Moreover, the fact that DG neurogenesis is pivotal for spatial memory and that the AAV-mediated *Terf2* downregulation was specifically targeting only postmitotic neurons (mature neuron-specific eSYN promoter), could explain why spatial memory was not affected. Nevertheless, this differential effect on memory types indicates that the effects of TRF2 downregulation in young mice are unlikely to be mediated by an impact on DG.

A striking result is that TRF2 overexpression can rejuvenate the hippocampi of old mice by restoring their mnemonic capacity, suggesting that the age-related TRF2 downregulation does not lead to irreversible events, such as neuron loss. Indeed, an effect of TRF2 overexpression on neurogenesis seems unlikely since, as previously mentioned, the eSYN promoter used in this study is specific for mature neurons.

Subsequently, we aimed at deciphering the molecular mechanism by which hippocampal TRF2 affects memory. As the main known target of TRF2 is the Ataxia telangiectasia mutated (ATM) kinase, we decided to inhibit it by infusing the drug KU60019 (a specific ATM inhibitor) while simultaneously downregulating *Terf2*. This treatment was sufficient to prevent the downregulation of *Terf2* from affecting memory. Thus, we conclude that the effect of TRF2 downregulation leading to a memory phenotype is mediated by the ATM kinase activity. Classically, TRF2 inhibits ATM activity at telomeres to prevent these structures from being mistakenly recognized as double strand breaks by the DNA damage response machinery. Indeed, neurons with decreased TRF2 expression show a mild increase in the telomere-specific DNA damage response (DDR) in an ATM activity-dependent fashion. However, it should be noted that the increment of telomeric damage is limited and not associated with an increase in total DDR. Moreover, telomere damage usually triggers senescence, but we were unable to detect an increase in senescence upon hippocampal TRF2 downregulation. Therefore, although a telomeric effect in the mnemonic process cannot be ruled out, the role of the TRF2-ATM axis in memory needs to be explored also in the light of the multiple functions that ATM has in neurons (Pizzamiglio et al., 2020).

As synaptic plasticity is essential for the mnemonic process, we assessed the effect of TRF2 downregulation on this pivotal neuronal phenomenon. We have shown that TRF2 downregulation *in vitro* leads to a slowing down of synaptic vesicle transport, while *in vivo* it modulates the levels of proteins involved in pathways related to synaptic plasticity and neuronal activation. In particular, TRF2 downregulation affects the protein levels of several immediate early genes (*e.g.*, c-Fos, Egr3, Nptx2 and Vgf) and also those of proteins involved in synapsis formation and glutamate receptor trafficking and activation (*e.g.*, CamKII, Shank3, Homer2, Dlg3 and Slc1a1).

To our knowledge, this is the first time that an ATM-dependent effect is described upon TRF2 downregulation in a post-mitotic cell context. Therefore, understanding how ATM is linked to synaptic plasticity when the expression of TRF2 is decreased during aging has the potential to shed new light on the links between telomere dynamics and the physiological changes occurring during brain aging (*e.g.*, memory decline).

A limitation of our study is to focus on the neuronal effects of TRF2. Indeed, it is possible that TRF2 also plays a role in the other cell types of the hippocampus (*e.g.*, Astrocytes, Oligodendrocytes, and microglia) and might decrease during aging also in these hippocampal cells. It will therefore be necessary to study the cellular specificity of the TRF2 decrease that we observed in the hippocampus during aging and to eventually modulate the expression of TRF2 in these cells to understand whether the selective TRF2 downregulation in these cell types can recapitulate the results obtained with the downregulation of TRF2 in the hippocampal neurons.

Moreover, our AAV-mediated system allowed us to specifically downregulate TRF2 in all the mature neurons of the hippocampus, but making no distinction among the neuronal subtypes (*e.g.*, Glutamatergic, GABAergic, dopaminergic, et cetera). This further level of distinction might be pivotal for the sake of a deeper understanding of the mechanisms underlying the loss of memory upon TRF2 decrease in the murine hippocampus during aging.

In conclusion, this study opens up a new field of research on telomeres and TRF2 in the central nervous system, in the context of post-mitotic cell aging. This work also reveals a radically novel mechanism contributing to hippocampal aging based on the downregulation of the telomere-capping factor TRF2. Moreover, it highlights the important role that this telomeric protein plays in neuronal functions by blunting ATM activation. These results are also remarkable with respect to the possibility of developing new pharmacological interventions to counteract the memory decline associated to hippocampal aging.

At any rate, before getting to this final goal, many open questions still need to be answered.

First, ATM inhibition upon TRF2 downregulation leads to a partial memory rescue. Therefore, we wonder which other TRF2 functions might be responsible for memory safeguard, other than ATM inhibition. A possible uncanonical TRF2 function involved in this memory phenotype could be its ability to bind to interstitial telomeric sequences (ITS) throughout the whole genome, thereby influencing the expression of genes potentially involved in neuronal plasticity. TRF2-targeted ChIP-seq analyses, indeed, revealed that TRF2 binds in the proximity or to genes that are involved in the memory process (Simonet et al., 2011). gH2AX-targeted ChIP-seq, in turn, might reveal gene loci that

preferentially suffer DNA damage upon TRF2 downregulation, likely influencing their expression, thereby further elucidating the mechanisms driving memory decline upon TRF2 downregulation. Second, TRF2 downregulation causes a mild increase in telomere-specific DDR but no senescence. Thus, we ask ourselves what the mechanism by which ATM activity impairs memory upon TRF2 downregulation could be. ATM is involved in a multitude of processes of which any might affect memory if dysregulated when ATM activity is unleashed upon the downregulation of TRF2. For example, ATM phosphorylates or directly binds and activates proteins involved in DNA repair (*e.g.*, 53BP1 and BRCA1), cell cycle arrest (*e.g.*, CHK2), autophagy (*e.g.*, Parkin [direct binding] and Beclin-1 [via CHK2-mediated phosphorylation]), mitochondrial function (*e.g.*, NRF1), ROS regulation (*e.g.*, HSP27 [via G6PD and NADPH production]), and proteostasis (*e.g.*, FUS). Therefore, we consider that a deeper analysis of these pathways is worth an effort to more deeply understand the mechanism/s involved in the memory phenotype we discovered.

Third, we would like to assess whether other neuronal hallmarks of aging are involved in our memory phenotype. For example, autophagy, mitochondrial dysfunction and altered proteostasis, which are canonical hallmarks of postmitotic cell aging. Pursuing this goal, we performed *in vitro* experiments assessing the protein levels of several proteins involved in the above-mentioned phenomena and we found that the first steps of autophagy and those leading to autophagosome-lysosome fusion are both affected by TRF2 downregulation. Nonetheless, these experiments were performed with primary neurons downregulated for TRF2 at Day-in-vitro 1 (DIV1). Therefore, we cannot exclude that the results found are biased by an effect of TRF2 downregulation on neuronal development. Thus, analogous but differently designed experiments have been planned, where TRF2 will be downregulated only at DIV14 (when neurons are considered mature *in vitro*) thanks to an IPTG-inducible system.

Finally, we also wonder what mechanism is driving the hippocampal TRF2 decline during aging. Considering that Osteocalcin (Ocn), an osteoblast-produced neurotrophic hormone pivotal for brain development and endowed with rejuvenating functions, declines with age, much alike TRF2, we wondered whether Ocn decline could be upstream the age-related TRF2 decline that we observed. Interestingly, we found that Ocn treatment increases TRF2 levels in young animals, but not in already aged ones. Investigating the molecular dynamics by which Ocn modulates TRF2 expression might pave the way to exciting new research avenues. Other unexplored rejuvenating factors might also play a role in hippocampal TRF2 decline during aging (*e.g.*, GDF11).

With a far-sighted therapeutical perspective, we would like to know whether the AAV-mediated

hippocampal TRF2 overexpression can rescue the age-related memory decline in other *in vivo* models. We consider that its application to non-human primates would be the most relevant in view of a future application on humans.

Overall, we conceive that our findings concerning the age-related memory decline may have far reaching implications for medical interventions with evident societal relevance, thereby contributing to make healthy aging a goal hopefully accessible to anyone.

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## ***Dulcis in fundo***

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Best,

Marco