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# THÈSE DE DOCTORAT

La trajectoire non-télomérique de l'évolution de TRF2 chez le poisson zèbre révèle le rôle que joue cette protéine au cours du neurodéveloppement

**Yilin YING**

IRCAN - CNRS UMR 7284 - INSERM U1081

**Présentée en vue de l'obtention**

**du grade de docteur en** Sciences de la vie et de  
la santé

d'Université Côte d'Azur

**Dirigée par :** Eric Gilson, PU-PH

**Soutenue le :** 17 décembre 2020

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Unité de recherche : IRCAN - CNRS UMR 7284 - INSERM U1081

# THÈSE DE DOCTORAT

Présentée en vue de l'obtention du grade de Docteur en Sciences  
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**Yilin Ying**

**The non-telomeric evolutionary trajectory of TRF2 in zebrafish  
reveals its essential role in neurodevelopment and aging**

*La trajectoire non-télomérique de l'évolution de TRF2 chez le poisson zèbre révèle le  
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Mr. Lin Liu	Vice-president of NanKai University	Rapportrice
Mr. Songyang Zhou	Directeur of State Key laboratory for Biocontrol, SYSU	Examineur
Mme. Marina Shkreli	Professeur of Institute for Research on Cancer and Aging, Nice (IRCAN)	Examineur
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## **Abstract**

The shelterin protein complex is a key player of telomere protection, a process inextricably linked to ageing. Accordingly, decrease in shelterin expression is associated with increasing age in many organisms. Here we demonstrate that the zebrafish ortholog of the mammalian shelterin subunit TRF2 (zfTRF2 encoded by the TERFA gene) is expression higher in neural system and decreased during aging. Further investigation shows that zebrafish TRF2 is required to prevent ATM-dependent DNA damage signaling, but not specifically at the telomeres. Complete TERFA ablation induced severe embryonic neurodevelopmental failure and death, whereas TERFA haploinsufficiency led to premature aging in adult fishes. Remarkably, ATM inhibition completely prevented DNA damage signaling in terfa-compromised embryos but led to only partial recovery from embryonic neurodevelopmental failure, suggesting that zfTRF2 controls processes other than genome stability. Indeed, overexpressing genes that were downregulated in terfa-compromised embryos had an epistatic effect with ATM inhibition, resulting in recovery from neurodevelopmental failure. Furthermore, glial cell-specific restoration of zfTRF2 expression was sufficient to rescue the embryonic neurodevelopment phenotype, unveiling a key role of zfTRF2 in glial cells. Our results suggest that the key shelterin subunit TRF2 evolved in zebrafish as a general genome caretaker that is required for proper neurodevelopment by both preventing DNA damage signaling and regulating gene expression. Here, we propose a model of telomere evolution based on the redeployment of general factors involved in genome maintenance, coupling telomere status to development and aging.

**Key words:** telomere, TRF2, zebrafish, neuro-development, extra-telomeric function

## Résumé

Mon programme de doctorat était de comprendre la fonction de la protéine TRF2 de protéine shelterin (facteur de répétition de Telomere 2) dans le poisson zèbre. J'ai présenté mon travail en deux parties. Puisque très peu est connu quant à la façon dont l'expression des gènes sous-unités de shelterin est régulée pendant le développement et le vieillissement, j'ai d'abord utilisé deux modèles animaux différents et mesuré le niveau d'ARN et le niveau de protéine des gènes de shelterin dans divers tissus pendant le développement de souris et pendant la vie adulte chez le poisson zèbre. J'ai révélé que les sous-unités de shelterin présentent des expressions spatiales et temporelles distinctes qui ne sont pas corrélées avec le statut prolifératif des systèmes d'organes examinés. Par exemple, le gène *terfa* (gène TRF2 chez les poissons zèbres) est beaucoup plus élevé dans le cerveau que dans d'autres tissus au cours du développement et du stade adulte et a fortement diminué avec l'âge. Ceci suggère une fonction spécifique importante de TRF2 dans le cerveau pendant le développement et le vieillissement indépendamment de sa fonction à la protection de telomere. (Publication: Kay-Dietrich Wagner\*, Yilin Ying\* Aging 2017, Vol 9, No. 4 The différentielle spatiotemporal expression pattern of shelterin genes throughout lifesa non/authors contribute equally). Ainsi, dans la deuxième partie, je me suis concentré sur la façon dont TRF2 fonctionne pendant le développement du poisson zèbre, en particulier pour comprendre la fonction extra-téломérique de TRF2 dans le développement neuronal.

Ensuite, j'ai constaté que l'orthologue de poisson zèbre de la sous-unité de mammifères TRF2 (zfTRF2 codé par le gène TERFA) est intrinsèquement capable de lier les télomères et l'hétérochromatine pericentromérique et zfTRF2 joue un rôle général dans la protection du génome du poisson zèbre contre les dommages causés par l'ADN sans spécificité apparente pour la protection des télomères. Afin d'explorer le rôle de zfTRF2 in vivo, j'ai généré un modèle de poisson zèbre compromis par *terfa*. Je démontre que zfTRF2 est nécessaire pour empêcher la signalisation des dommages d'ADN dépendant des guichets automatiques, mais pas spécifiquement aux

télomères. Une ablation complète de *terfa* induit une insuffisance et une mort embryonnaires graves de neurodéveloppement tandis que son haploinsuffisance mène à un phénotype de vieillissement prématuré dans les poissons adultes. Fait remarquable, si l'inhibition des guichets automatiques empêche complètement les dommages de l'ADN à la signalisation chez les embryons compromis par la *terfa*, elle ne fournit qu'une récupération partielle de l'échec du neurodéveloppement embryonnaire, ce qui suggère que *zfTRF2* contrôle d'autres processus que la stabilité du génome. En effet, les gènes surexprimants qui sont réduits en embryons compromis par la *terfa* agissent d'une manière épistatique avec l'inhibition des guichets automatiques pour fournir une récupération de l'échec du neurodéveloppement. Une restauration spécifique de l'expression *zfTRF2* dans les cellules gliales est suffisante pour sauver le phénotype embryonnaire de neurodéveloppement, dévoilant un rôle clé de *zfTRF2* dans les cellules gliales pendant le neurodéveloppement. Ainsi, ces résultats révèlent qu'une sous-unité de shelterin clé a évolué dans le poisson zèbre en tant que gardien général de génome exigé pour un neurodéveloppement approprié en empêchant la signalisation de dommages d'ADN et en régulant l'expression spécifique de gène.

Il convient de noter que j'ai montré que *zfTRF2* conservait sa capacité intrinsèque à lier les télomères. La question de savoir si cette propriété de télomère conservée de *zfTRF2* joue un rôle dans le poisson zèbre est une question intéressante pour d'autres études. Inversement, il reste à déterminer la façon dont les télomères du poisson zèbre sont spécifiquement protégés. La différence frappante dans la liaison de télomère de *zfTRF2* entre les cellules humaines et de poisson zèbre suggère fortement une possibilité que *zfTRF2* est en concurrence dans les cellules de poissons avec d'autres protéines pour lier les télomères qui est une question intéressante à répondre dans l'étude plus approfondie.

Mots clés: télomère, TRF2, zèbre, neurodéveloppement, fonction extra-téломérique

## Introduction

Telomeres are key features of chromosome termini that preserve genome integrity, and recent reports have identified telomeres as cellular integrators of various stresses. Consequently, changes in their structure profoundly affect the ability of cells to proliferate and adapt to new environments (Blackburn, Epel and Lin 2015). Cells respond intrinsically to perceived telomere dysfunction by initiating the DNA damage response (DDR) cascade, which leads to senescence or apoptosis. The integrity of telomeres relies on particular chromatin organization, which protects chromosome ends from aberrant DDR signaling (Palm and de Lange 2008).

In many organisms, this chromatin-mediated pathway depends on the binding of shelterin proteins to telomeric DNA. Therefore, the disorder of shelterin components can lead to cell fate change and organism dysfunction. The most well-known disease in human is one type of the DKC (Congenital dyskeratosis) can triggered by TIN2 (shelterin component) mutation. Recently, more evidences show that shelterin are involved in the pathology process of disease, cancer and aging. Interestingly, these effect of shelterin components can be on or outside of telomere. **In the chapter 1**, the literature overview is a resume of the most recent and relevant findings in the field of shelterin related disorders in human. Also, the phenotypes of animal models with shelterin dysfunction, which may link back to human, will also be summarized and discussed.

**Chapter 2** represents the actual results of the thesis project. It is grouped into two articles. Article 1 is the published manuscript, which revealed distinct spatiotemporal regulation patterns of shelterin subunits during development and aging. In this manuscript, we use two different models, the mouse and zebrafish, and my work is focus on the expression pattern of zebrafish during development and tissue specific expression level. Interestingly, we also find an expectable role of *terfa* (zebrafish TERF2) in transcriptional regulation in neural system. Article 2 is the main work of my PhD study (in preparation for publication). In this article, we focus on the role of the function of *terfa* during neuro-development. Specially, we find that in

zebrafish, the *terfa* act as a general caretaker against ATM mediate DDR, instead of telomere specific protection. We also find *terfa* have an important role in glia cells (GFAP positive) during neuro-development which partially independent of ATM pathway.

**Chapter 3** is the final chapter of this dissertation. It discusses the main findings and suggests future work that is necessary to be done to better understand the mechanism of *terfa* in zebrafish neuro-development and questions remains to be solved.

## **CHAPTER 1. Shelterin related disorders: From human to animal model**

Telomeres are among the most important structural elements of eukaryotic chromosomes because they cap the chromosome ends and provide protection from endogenous and exogenous damage (Palm and de Lange 2008). Telomeric DNA consists of sequences of G-rich repetitive DNA nucleotides (e.g. TTAGGG in vertebrates). Telomere length varies among species and individual organisms, and it decreases in response to cell division and aging (Stewart and Weinberg 2006). Telomeric chromatin consists of a nuclear complex that includes a highly ordered arrangement of DNA, RNA, and proteins. This telomeric chromatin has many functions including protecting chromosome ends, cell proliferation, senescence, and tumor suppression (Gilson and Geli 2007). Telomeres also participate in the stress response, differentiation, and stem cell function (de Lange 2005, Ye et al. 2014). Despite the roles in molecular and cellular biology, how telomere dysfunction involved in human diseases are not fully understand.

### **1. Basics of telomere homeostasis and protection**

#### **1.1 The role of telomerase in telomere biology**

The DNA replication machinery is intrinsically incapable of completely replicating the extremities of parental DNA. Therefore, over time, cell division leads inevitably to telomere attrition (Shay 2016). After cells have divided a number of times, telomere attrition reaches a critical point, and the shortened telomeres mean that the

chromosome ends becoming unstable. The short telomeres trigger a DNA damage response (DDR) and activate checkpoint proteins such as p53, which induce cellular apoptosis or senescence (Stewart and Weinberg 2006). In cells with inactive cell-cycle checkpoints, for example due to p53 mutations, the shortened telomeres can induce tumorigenesis (Falandry et al. 2014).

In some situations, the inability to replicate chromosome ends can be resolved by a ribonucleoprotein telomerase (Blackburn et al. 2015). The telomerase holoenzyme consists of a telomerase reverse transcriptase (TERT) catalytic subunit, an associated telomerase RNA (TR) template, and the accessory proteins dyskerin, NHP2, and NOP 10, which stabilize TR in the nucleus. The catalytic TERT component adds telomeric repetitive sequences from the template region of the TR to the chromosome ends during each cell cycle. Therefore, telomerase activity is crucial for telomere elongation (Schmidt and Cech 2015). However, in human cells, telomerase expression and activation are strictly limited. Telomerase activity is not detectable in most human somatic cells and is very low in some types of stem cells, such as embryonic and hematological stem cells (Blackburn et al. 2015). Short telomeres, the DDR response in cells with inactivate cell-cycle checkpoints, and telomerase reactivation can cause chromosome instability, transcriptional reprogramming, and oncogenesis (Blanco et al. 2007, Armanios 2012, Cheung et al. 2014). Thus, understanding how telomerase is activated and recruited to elongate telomeres is very important.

## 1.2 Telomere protectors: The shelterin complex

The shelterin complex is a dynamic protein complex that binds specifically to telomeres. It consists of six protein components. Each of these proteins has a specific protective function. The shelterin complex can protect telomeres from an unwanted DDR and DNA repair, prevent telomere ends from becoming degraded, and preserve the three-dimensional structure of the telomeric nucleoprotein complex (Palm and de Lange 2008). Thus, shelterin is crucial for telomere homeostasis. The shelterin subunits can also associate to sites outside the telomeric regions, and this extratelomeric function of the shelterin components may provide new insights and opportunities for understanding the complex interplay between telomere and cell fate (Ye et al. 2014).

As described above, telomeres are multifunctional structures containing DNA and proteins. Six of these proteins form the shelterin complex, which binds to telomeres and plays an important role in maintaining telomere homeostasis (Palm and de Lange 2008). These proteins include the telomeric-repeat binding-factor (TRF)1 (also known as TERF1), TRF2 (also known as TERF2), repressor activator protein 1 (RAP1; also known as TRF2 interacting protein, TRF2IP), TRF1-interacting nuclear factor 2 (TIN2; also known as TINF2), TIN2-interacting protein 1 (TPP1), and protection of telomeres 1 (POT1). TRF1 and TRF2 contain a TRFH domain, which mediates their homodimerization and interacts with a set of proteins required for telomere protection, as well as the telo-box domain which recognizes duplex telomeric DNA sequences. POT1 binds to the 3' overhang of telomeres and interacts with TPP1. In

mammals, RAP1 cannot bind to telomeres directly. It is recruited to telomeres by interacting with TRF2. The TIN2 protein forms the core of the shelterin complex and binds to three shelterin components TRF1, TRF2, and TPP1 (Gilson and Geli 2007). Therefore, in mammals, TRF1, TRF2, and 3'-overhang-binding heterodimers are linked by a protein bridge, formed by TIN2, which interacts simultaneously with TRF1, TRF2, and TPP1 (Abreu et al. 2010, Wang et al. 2007). The expression of various mutant versions of the TIN2 protein, which abolish its capacity to bind to TRF1, TRF2, or TPP1 in human and mouse cells, results in telomere deprotection, confirming the importance of the shelterin bridge for telomere functionality (Yang et al. 2011, Abreu et al. 2010, Hoffman et al. 2016). The shelterin complex is involved in several telomeric functions, including preventing DDR activation, preserving chromosome stability and integrity, telomerase regulation, long- and short-range transcriptional regulation, and meiosis. It can act either as a full complex or as subcomplexes, which perform specific functions during the cell cycle and cellular differentiation. Therefore, the mechanism by which shelterin determines cell fates has become a focal point of telomere biology.

## **2. Multi-functional complex in telomere biology: Shelterin**

### **2.1 Shelterin forms part of a highly ordered chromatin structure at telomeres**

Telomeres in mammalian cells comprise G-rich repetitive DNA nucleotide sequences with 50–400 nucleotide 3' overhangs at the chromosome ends (Gilson and Geli 2007). Consequently, telomeres are fragile sites with free ends, which may activate DDR machinery. The G-cluster-rich telomeric DNA is particularly vulnerable to

intracellular or environmental stresses such as nucleases, chemical or physical damage, and oxidative or replicative stress (Armanios and Blackburn 2012). Therefore, telomere sequences are easily damaged if the cells are subject to stress or damage, and telomeric DNA cannot be repaired as easily as other parts of chromosomes (Cesare et al. 2013). This means that telomeres may act as sensitive detectors of DNA damage and chromosome instability. DNA damage may accumulate over time and telomeres may trigger DNA damage signals at a critical point.

One of the functions of the shelterin complex is to stabilize telomeres by forming a highly ordered chromatin structure : the telomere loop (T-loop) (Gilson and Geli 2007) . Indeed, TRF2 forms a complex in which the DNA wraps around itself to facilitate T-loop formation: TRF2 promotes supercoiling in telomeric DNA, induces strand invasion, and protects Holliday junctions *in vitro* (Gilson and Geli 2007). Importantly, TRF2 can generate T-loops *in vivo* and stabilize the Holliday junction-like structure: electron microscopy and super-resolution fluorescence microscopy showed that TRF2 can generate a T-loop lariat structure, which is formed by the invasion of the telomeric 3' overhang into the double-stranded telomeric repeat array (Benaroch Mol Cell). The T-loop architecture is essential for blocking DDR activation at telomeres because, this hides the accessible 3' DNA overhang of the telomere from the DDR proteins (Cesare et al. 2013).

Other studies have suggested that shelterin proteins can mediate the interaction between telomeres and several interstitial telomeric sequences (ITs) localized within the genome. Therefore, telomeres may be able to regulate the transcription of distant

genes without affecting the expression of nearby genes (Kim et al. 2016). Interestingly, telomere shortening may significantly affect such interactions, resulting in alterations in transcriptional regulation (Ye et al. 2014).

## **2.2 The shelterin complex inhibits the DDR and DNA repair at chromosome ends**

Shelterin protects the chromosome ends from an unwanted DDR. This is necessary because the free DNA at the ends of linear chromosomes may be mistaken for double-strand breaks. The shelterin proteins prevent all three types of DDR machinery from being activated at telomeres. These include the ataxia telangiectasia-mutated (ATM) kinase, the ataxia telangiectasia and Rad3-related (ATR) kinase, and DNA-dependent protein kinase (Palm and de Lange 2008). Different shelterin components repress different DDR signaling pathways. If shelterin components are depleted, then the ends of linear chromosomes are exposed to the DDR machinery. TRF2 suppresses ATM phosphorylation at telomeres. TPP1/POT1 heterodimers cap telomeres by binding to the G-rich 3' overhangs and repress the ATR-mediated DDR by preventing the recruitment of replication protein A. Therefore, inactivation of TPP1/POT1 activates the ATR signaling pathway (Jones et al. 2014, Wang, Shen and Chang 2011, Xin et al. 2007).

Shelterin-free telomeres are equivalent to unprotected DNA ends (Palm and de Lange 2008). They activate DDR signaling via the ATM and ATR kinase pathways and are also potential substrates for other DNA repair processes. These processes include classical Ku70/80- and DNA ligase 4-dependent nonhomologous end-joining (c-NHEJ),

micro-homology-dependent alternative-NHEJ (a-NHEJ) mediated by poly (ADP-ribose) polymerase 1 and DNA ligase 3, homology-directed repair, and CtIP-dependent 5' end resection (Hu et al. 2010, Palm and de Lange 2008, Celli, Denchi and de Lange 2006, Sfeir et al. 2009).

Deletion of TRF2 results in a specific dysfunctional telomere phenotype: activation of the ATM kinase cascade and c-NHEJ, which generates concatenated chromosomes fused at their telomeres (Sfeir et al. 2009, Denchi and de Lange 2007, Celli et al. 2006, Bae and Baumann 2007). TRF1 removal activates ATR kinase. RAP1 and TRF2 form a heterodimer and repress the homology-directed repair pathway (Chen, Liu and Songyang 2007, Rai et al. 2016). TIN2 deletion produces a complex response that includes ATM kinase signaling and c-NHEJ. However, these responses are partly due to the loss of TRF2 from telomeres (Takai et al. 2011)

### **2.3 Telomerase is recruited to telomeres by shelterin**

Shelterin proteins are extremely important for regulating telomere length. They play key roles in telomerase-induced telomere elongation. Telomerase is recruited to telomeres via a 'TEL' patch on TPP1. TPP1 interacts with TIN2 to facilitate the association of telomerase with telomeres (van Steensel and de Lange 1997, Wang et al. 2007, Schmidt, Zaug and Cech 2016). In addition, shelterin components also control how much telomerase can bind to the telomeres by occupying some of the binding sites. The presence of TRF1, TRF2, and POT1 can reduce the accessibility of telomeres

to telomerase, preventing telomere elongation (Schmidt et al. 2016, Abreu et al. 2010, Wang et al. 2007).

Moreover, shelterin components can regulate telomere length directly in the absence of telomerase. Overall, changes in shelterin dynamics at telomeres can facilitate telomeric DNA degradation or the recruitment and activation of telomerase (Abreu et al. 2010, Chiang et al. 2004). The resulting changes in telomere length can influence stem cell status, promote tissue repair, or repress cancer (Shay 2016).

### **2.3 Extratelomeric roles of shelterin**

Some shelterin components can bind to internal regions of chromosomes. Shelterin proteins located outside the telomeres are not randomly distributed throughout the genome. For example, TRF2 and RAP1 frequently bind to DNA regions in the vicinity of genes (Yeung et al. 2013, Zizza et al. 2019, Wood et al. 2014, Ovando-Roche et al. 2014). This suggests that shelterin proteins may have important extratelomeric functions, such as the regulation of gene expression. Interestingly, there are many shelterin-linked phenotypes that cannot be explained by telomere dysfunction, such as cases of obesity caused by RAP1 depletion or examples of neural cell dysfunction in TRF2-compromised cells.

#### **2.3.1 Telomere position effects (TPEs)**

The telomere can regulate gene expression by the so-called Telomere Position Effect or TPE. Yeast RAP1 can recruit the Sir protein complex and silence gene expression in sub-telomeric regions (Platt et al. 2013). In mammals, telomeres also silence gene expression in sub-telomeric regions (Biroccio et al. 2013). Clinical evidence has shown that some disorders may result from TPEs. One such disease is facioscapulohumeral muscular dystrophy (FSHD), which is characterized by progressive atrophy and weakness of the facial, scapular, and upper arm muscles. This disease is caused by upregulation of the double homeobox 4 gene, which is located near the telomere of chromosome 4q (Stuart et al. 2015).

### **2.3.2 Extratelomeric roles of RAP1**

An *in vitro* study showed that mouse embryonic fibroblast cells from RAP1-knockout mice did not exhibit significant telomere dysfunction or DDR activation, which indicates that RAP1 is unnecessary for telomere stabilization. Moreover, RAP1-knockout mice exhibit a form of early-onset obesity that is more severe in females than males (Martinez et al. 2010). RAP1-deficient mice did exhibit significant changes in gene expression. RAP1 binds to the promoter regions of peroxisome proliferator-activated receptor alpha and peroxisome proliferator-activated receptor gamma coactivator 1-alpha and regulates their expression (Martínez et al. 2013). Misregulation of these genes can cause metabolic disorders such as insulin resistance, diabetes, accumulation of fat, and hepatic steatosis (Martínez et al. 2013, Ye et al.

2014). RAP1 can also interact with IKK1 and IKK2 to activate the NF- $\kappa$ B signaling pathway (Teo et al. 2010).

### **2.3.3 Extratelomeric role of TRF2**

TRF2 also reportedly has extratelomeric functions. TRF2 binds to ITSs and, in cancer cells, regulates the expression of genes such as heparan sulfate glucosamine 3-O-sulfotransferase 4 (HS3ST4), which can inhibit the recruitment of natural killer (NK) cells (Biroccio et al. 2013). TRF2 is also involved in neuronal development by interacting with repressor element-1-silencing transcription factor (Ovando-Roche et al. 2014). These findings suggest that the relationship between shelterin and similar disorders should be investigated in future studies.

## **3. Human shelterin-associated disorders**

Telomere syndromes have different clinical outcomes but share the same etiology: abnormal shortening of telomeres. Patients with telomere syndromes usually develop age-associated disorders at a much younger age than the general population. These diseases include Hoyeraal-Hreidarsson syndrome (HHS), dyskeratosis congenita (DKC), pulmonary fibrosis, aplastic anemia, and liver fibrosis, and they can all be linked to mutations in genes involved in telomere elongation or maintenance. All of these diseases share common cellular dysfunctions, including declining proliferative capacity of stem cells and reduced tissue regeneration.

### **3.1 Shelterin in telomere syndromes**

#### **3.1.1 DKC**

One disease linked to telomere dysfunction is DKC (Heiss et al. 1998, Mitchell, Wood and Collins 1999, Vulliamy et al. 2001). The incidence of DKC is approximately one case per million individuals and its characteristic symptoms include skin hyperpigmentation, oral leukoplakia, and nail dystrophy (de la Fuente and Dokal 2007). DKC is usually diagnosed in a mild form at an early age. However, the symptoms are progressive and may soon lead to severe organ failure. The pattern of organ failure resembles that observed in many age-related diseases (Armanios 2009). In approximately 80% of DKC cases, organ failure begins in the bone marrow as aplastic anemia (de la Fuente and Dokal 2007).

#### **3.1.2 The etiology of DKC**

The genetic basis of DKC is complicated. X-chromosome-linked-recessive, autosomal-dominant, autosomal-recessive, and *de novo* cases of DKC have all been described clinically (Nelson and Bertuch 2012). Eight genes associated with DKC have been identified, and all of them are linked to telomere or telomerase function (Heiss et al. 1998, Armanios et al. 2005, Walne et al. 2007, Zhong et al. 2011). Two genes that are frequently linked to the most common form of DKC are TIN2 and the X-linked DKC1 gene, which encodes dyskerin. Germline mutations of TERT and TR account for less

than 10% of the autosomal-dominant cases of DKC (Vulliamy et al. 2001, Armanios et al. 2005). Mutations in genes encoding the telomerase accessory proteins NHP2, NOP10, and TCAB1 have been reported in rare autosomal-recessive cases (Yamaguchi et al. 2005, Walne et al. 2007, Zhong et al. 2011). All eight of these genes are involved in regulating telomerase activity and modulating telomere elongation. Thus, the etiology of DKC is telomerase dysfunction, which leads to shortening and dysfunction of telomeres (Mitchell et al. 1999, Vulliamy et al. 2001, Alter et al. 2007). Interestingly, mutations in TIN2 result in earlier clinical symptoms of DKC and organ failure at a younger age. This suggests that TIN2 may have particular roles in telomerase regulation that are critical for preventing the development of DKC.

### **3.1.3 Etiology of DKC cases resulting from TIN2 mutations**

TIN2 mutations are the second most common genetic cause of DKC and affect approximately 15% of DKC patients (Frescas and de Lange 2014, Yang et al. 2011). TIN2 dysfunction can impair telomerase function and activate the DDR at telomeres. This may explain the severe symptoms observed in DKC patients with TIN2 mutations. Telomeres from these patients are shorter than those from other patients with DKC, and the patients also develop clinical symptoms at a younger age and exhibit a higher rate of bone marrow failure (Frescas and de Lange 2014, Yang et al. 2011). Moreover, TIN2 mutations result in an autosomal dominant version of the disease. Therefore, heterozygous individuals will also have the disease, and most TIN2 DKC patients have *de novo* mutations.

Clinical data have shown that most TIN2 DKC patients have a mutation affecting a small region of 18 amino acids (K280–Q298) within the TIN2 protein. These mutations occur between the final amino acids of the TRF1-binding domain and an unidentified region of the TIN2 protein (Fig. 3). Interestingly, a mutation in the TRF1-binding domain only causes DKC, whereas mutations only a few amino acids away from the TRF1-binding domain can also lead to two other telomere syndromes: HHS and Revesz syndrome. Although DKC, HHS, and Revesz syndrome share most of the same symptoms and pathophysiology (i.e. extremely short telomeres), they have very different severities and prognostic characteristics (Glousker et al. 2015). These data indicate that this 18-amino-acid domain is very important for TIN2 function, and future studies using animal models may be useful to investigate its physiological and pathological functions. Another possibility is that this 18-amino-acid domain regulates separate interactions between TIN2 and both telomerase activity and telomere protection.

#### **3.1.4 Other telomere syndromes resulting from shelterin mutations**

With the exception of the TIN2 mutation in DKC, there are no clinical data showing the involvement of other mutated shelterin components in telomere syndromes such as IPF, aplastic anemia, or liver cirrhosis. Nevertheless, there is one report linking shelterin components with a pathological situation other than cancer: TRF2 and TPP1 mRNA levels are downregulated 12 out of 20 patients in idiopathic recurrent pregnancy loss compared to normal pregnancies. The telomeres from the

affected unborn infants were short. However, the mechanism underlying this TRF2- and TPP1-mediated decrease in telomere length is unclear and further studies are needed (Pirzada et al. 2019).

Importantly, although no mutations in TRF1, TRF2, RAP1, TPP1, or POT1 have been directly implicated in human diseases, it remains possible that these shelterin genes may be mutated in some still unidentified patients.

### **3.2 The role of shelterin in oncology**

One barrier to oncogenesis is replicative senescence (REF). Due to the end replication problem, pre-cancer cells must overcome telomere shortening and the DDR (Falandry et al. 2014). Because shelterin components can inhibit the DDR pathway and regulate telomere length, they may play a crucial role in preventing oncogenesis. Growing evidence has shown that the expression pattern of shelterin in oncogenic tissues varies significantly from that in adjacent normal tissues. Moreover, some evidence suggests that particular mutations in shelterin genes may be involved in the development of cancer.<sup>161</sup> Shelterin reportedly plays a role in immunosurveillance and angiogenesis by regulating gene expression (Falandry et al. 2014, Patel et al. 2015).

#### **3.2.1 The function of TRF1 in maintaining proliferation of cancer cells**

Clinical data show that many cancer cells have high levels of shelterin expression. For example, the mRNA levels of TRF1 and TRF2 were significantly increased in a study of 92 nephrotic carcinoma samples (Pal et al. 2015) and also in precancerous lesions (Hu et al. 2010). In addition, increased levels of TRF1 expression were found in high grade cancers. For example, TRF1 levels were increased in high-grade dysplastic liver nodules and increased further in hepatocellular carcinoma cells (Oh et al. 2005). The levels of TRF1 expression were also higher in high grade than in low grade hepatocellular carcinoma cells. These results suggest that TRF1 levels may be correlated with oncogenesis and cancer development because TRF1 can maintain telomere stability in cells undergoing proliferation. This hypothesis was supported by an *in vitro* study, which found that TRF1 is crucial for maintaining the proliferation of pluripotent stem cells (Schneider et al. 2013). In addition, the expression of TRF1 was increased in proliferative tissues such as those of the hematological system and increased further in hematogenous tumors such as chronic myeloid leukemia and acute lymphocytic leukemia (Campbell et al. 2006, Poncet et al. 2008, Ohyashiki et al. 2001). These results suggest that TRF1 may play a crucial role in cancer cell proliferation and may also be a suitable target for therapy. However, a separate study found that the expression of TRF1 was downregulated in acute myeloid leukemia (Ohyashiki et al. 2001). Therefore, further large cohort and multicenter clinical studies will be needed to clarify the association between TRF1 expression levels and cancer development. If a single shelterin protein can have opposing effects on different hematopoietic cancers, then it may have more than one relevant role.

### **3.2.2 The extratelomeric role of TRF2 in oncogenesis**

Clinical data show that TRF2 is upregulated in many human cancers such as lung, liver, gastric, and skin cancers. In addition, higher levels of TRF2 expression are associated with a poorer prognosis (Nakanishi et al. 2003, Diala et al. 2013, Benhamou et al. 2016, Biroccio et al. 2013). This suggests that TRF2 may have significant roles in oncogenesis. On one hand, TRF2 expression is regulated by several oncogenic pathways such as the p53, Wnt/b-catenin, and WT1 signaling pathways (El Mai et al. 2014). On the other hand, TRF2 can regulate multiple oncogenic pathways via transcription, independently of telomere dysfunction or significant changes in telomere length (Dong et al. 2009, Blanco et al. 2007, Zizza et al. 2019).

Overexpressed TRF2 boosts angiogenesis. In human endothelial cells, overexpressed TRF2 binds to the platelet-derived growth factor receptor  $\beta$  promoter and upregulates the expression of this gene. The platelet-derived growth factor receptor  $\beta$  plays an important role in angiogenesis and supports the angiogenic process during cancer growth. In this way, TRF2 may provide the vasculature necessary for tumor growth. TRF2 can also bind to the promoter of the sulfatase 2 gene, which encodes an endoglucosamine-6-sulfatase that may increase the level of vascular endothelial growth factor-A in the cancer cell secretome, enhancing angiogenesis (Zizza et al. 2019, El Mai et al. 2014, Cherfils-Vicini et al. 2019).

High levels of TRF2 in cancer cells also enable tumors to escape immune elimination. Indeed, upregulated TRF2 increases the expression of several genes

involved in the biosynthesis of heparan sulfate proteoglycan, leading to profound changes in glycocalyx length and stiffness, as revealed by atomic force microscopy. This TRF2-dependent regulation facilitated the recruitment of Myeloid-derived suppressor cells (MDSCs, their activation via the TLR2/MyD88/IL-6/STAT3 pathway leading to the inhibition of natural killer recruitment and cytotoxicity, and ultimately tumor progression and metastasis. The clinical relevance of these findings is supported by a correlation between high TRF2 expression and MDSC infiltration, which was inversely correlated with overall patient survival (Cherfils-Vicini et al. 2019).

### **3.2.3 The role of POT1 mutations in cancer susceptibility**

Reports of POT1 mutations in human diseases suggest these mutations are implicated in many heritable cancers including familial melanoma, familial glioma, Li-Fraumeni-like syndrome, mantle cell lymphoma, parathyroid adenoma, and chronic lymphocytic leukemia (Calvete et al. 2015b, Shi et al. 2014, Zhang et al. 2014a). This indicates that the POT1 gene may be involved in causing hereditary tumors such as melanomas and gliomas. Whole-exome sequencing of five unrelated melanoma-prone families identified several rare variants of the POT1 gene, which could be mutant versions that are responsible for melanomas. POT1 mutation carriers had fragile elongated telomeres. These results indicate that POT1 mutations may deprotect telomeres and compromise their function, resulting in familial melanomas (Shi et al. 2014). However, telomere dysfunction and fragility may also lead to changes in transcription, including increased oncogene expression and decreased tumor-

suppression-gene expression. Therefore, POT1 mutations may promote oncogenesis by several mechanisms.

Gliomas are another type of tumor that is linked to POT1 mutations. Whole exome sequencing of 90 individuals from 55 families was used to identify several POT1 mutations. These mutations were in the region of the POT1 protein involved in binding to TPP1 (Bainbridge et al. 2014). A recent study sequenced a TP53-negative Li-Fraumeni-like family exome and discovered that a POT1 mutation is responsible for cardiac angiosarcoma (Calvete et al. 2015a). *In vitro* studies showed that the number of POT1 proteins bound to telomeres decreased in proportion to telomere length and fragility. These three reports suggest that POT1 mutations are implicated in generating sarcomas and that the type of mutation can determine the tumor type (Akbay et al. 2013).

All of the tumors linked to POT1 mutations share a common molecular pathology involving elongated telomeres. Previous *in vitro* studies have shown that POT1 regulates telomere length, which can determine whether cancer cells can bypass replication senescence (Pinzaru et al. 2016). These observations suggest that POT1 mutations promote tumorigenesis by elongating telomeres. POT1 mutations in tumor cells may inhibit the CST complex and alter telomere length, triggering the activation of the ATR pathway. A recent study is consistent with the hypothesis that POT1 mutation-induced tumorigenesis is associated with a compromised ATR signaling pathway (Pinzaru et al. 2016). An analysis of somatic samples from 10 patients with cardiac angiosarcomas showed that loss function of the POT1 protein can trigger the

ATR-mediated DDR pathway, leading to genome instability and mutations in proteins such as vascular endothelial growth factor.

In conclusion, POT1 mutations may gradually promote cancers by several mechanisms. POT1 mutations contribute to tumor maintenance by elongating telomeres and causing other mutations (Calvete et al. 2019). The elongation of telomeres enables cancer cells to bypass cell-cycle checkpoints. POT1 mutations also cause telomere dysfunction and fragility increasing the probability of other mutations occurring in proteins such as ATR or P53. This might explain why POT1-mutation-induced cancers are heritable. Further studies using animal models with POT1 mutations may improve our understanding of the role of POT1 in cancer development.

#### **3.2.4 Roles of other shelterin subunits in tumorigenesis**

Mutations in TPP1 and RAP1 are also associated with familial melanomas (Aoude et al. 2015). Moreover, families of individuals with TPP1 or RAP1 mutations exhibit many types of tumors, suggesting that TPP1 and RAP1 mutations can result in a predisposition toward cancer (Kocak et al. 2014). Several mutations of the TPP1 gene occur in its POT1-binding domain, suggesting that the relationship between TPP1 mutations and cancer may depend on POT1 dysfunction (Liu et al. 2004).

#### **4. The role of shelterin in normal aging**

Normal aging is an irreversible and inescapable process, associated with physical degeneration, that leads to an increased risk of disease and death.<sup>127</sup> Biologists have identified nine critical processes as hallmarks of aging. These are genome instability, telomere attrition, epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, cellular senescence, deregulated nutrient sensing, stem cell exhaustion, and altered intercellular communication. These processes can interact and overlap with signaling pathways, forming a network of aging mechanisms. Therefore, the pathology of aging may be different according to the type of tissue. For example, in tissues that are rapidly turned over, such as the skin or intestines, stem cell exhaustion can disrupt aging, whereas in tissues that show little turnover, such as the brain, genome instability and cellular senescence are more likely to cause age-related disorders.

Studies on animal models have shown that the shelterin subunits exhibit a different spatiotemporal expression pattern in different tissues during aging (Wagner et al. 2017). Overall, it emerges that shelterin expression decreases during aging. Here, I discuss the functions of shelterin in specific tissues and its potential interactions with different diseases associated with aging.

## **5. A decrease in TRF2 levels is associated with pathological aging**

Cardiovascular disease is the most common severe disease among older adults. Shelterin expression levels are reportedly altered in these patients, and telomere shortening is associated with cardiovascular disease (Wang et al. 2016, Xu et al. 2016).

The senescence of vascular smooth muscle cells decreases cell proliferation and leads to plaques forming, which accelerates the development of cardiovascular diseases such as atherogenesis. These processes correlate with telomere shortening and the DDR, as well as shelterin deficiency in the plaques that form in smooth muscle cells. *In vitro* experiments have shown that TRF2 deficiency is associated with telomere dysfunction and vascular cell senescence (Fujita et al. 2010).

TRF2 expression decreased during aging in animal models (Wagner et al. 2017). For example, in mouse and zebrafish models, the expression of TRF2 was significantly greater in brain than in other tissues and decreased with age. In addition, TRF2 expression was negatively correlated with patient age in muscle tissues (Wang et al. 2015). TRF2 expression levels also decreased in chronic age-related diseases such as diabetes, cardiovascular diseases, depression, and cognitive decline (Wang et al. 2016, Cai, Yan and Ratka 2012, Liu, Bloom and Donato 2019).

TRF2 reportedly regulates neurological function. Among the six shelterin proteins studied in animal models, only TRF2 expression was significantly higher in brain tissue, and only TRF2 expression levels decreased with the age (Wagner et al. 2017). This suggests that TRF2 may play a crucial role in neurodegenerative disorders such as Alzheimer's disease, depression, and cognitive decline. Moreover, the expression levels of TRF1 and TRF2 in peripheral blood are significantly lower in Alzheimer patients compared to healthy individuals of the same age (De Felice and Lourenco 2015, Weissman et al. 2007, Wu et al. 2019, Cai et al. 2012). Nonetheless, there is limited clinical information regarding shelterin expression in neurodegenerative diseases, and

further studies using animal models with TRF2 dysfunction may improve our understanding of how TRF2 functions in the nervous system.

Second, TRF2 levels decline with age in human skeletal muscle. In cultured human myotubes, TRF2 downregulation did not trigger telomere dysfunction but did suppress expression of the mitochondrial Sirtuin 3 gene, inhibiting mitochondrial respiration and increasing the levels of reactive oxygen species (Robin et al. 2020).

Other clinical findings have highlighted the relationship between TRF2 and aging. Research shows that association between TRF2 and ITSs is stabilized by A-type lamins (Wood et al. 2014). Knockdown of lamin A/C or overexpression of a mutated lamin A/C domain that binds to TRF2 can result in premature aging and a particular disorder called Hutchinson-Gilford progeria syndrome. A decrease in lamin A/C levels or impairment of the interaction between TRF2 and lamin A/C can lead to 3D-structure change and telomere dysfunction. These results suggest that TRF2 may be implicated in Hutchinson-Gilford progeria syndrome via its interaction with lamin A/C and modification of heterochromatin structure (Wood et al. 2014).

In conclusion, decreases in TRF2 levels are linked to different aging-associated pathologies and mechanisms in different tissues. These include telomere protection and transcriptional reprogramming of genes linked to aging.

## **6. Shelterin disorders in animal models**

Various types of conditional gene knockouts have been used in shelterin research studies allowing researchers to better understand the etiology and pathology of shelterin associated disorders.

### **6.1 TRF1 knockouts in the hematological system**

TRF1-knockout mouse models are embryo lethal (mouse: E7.5d), indicating the importance of TRF1 in embryonic development (Karlseder et al. 2003). Many apoptotic cells can be observed in these embryos. TRF1 is implicated in stem cell maintenance and proliferation, and TRF1 dysregulation is a characteristic of several human hematological diseases. Blood cells are constantly turned over throughout an individual's life. Interestingly, acute *Trf1*-conditional-deletion mice (*Trf1*<sup>Tg:Mx1-Cre/Tg:Mx1-Cre</sup>) exhibited pancytopenia and bone marrow failure after a few days, together with an increased number of telomere dysfunction-induced foci but no telomere shortening (Beier et al. 2012). The hematopoietic progenitor cells in these mice showed strong senescence signatures via induction of the p53-p21 pathway but did not become apoptotic. If the *Trf1* conditional deletion is produced progressively, these mice also develop bone marrow failure and telomere shortening and have a comparatively short life span (Beier et al. 2012). These results show that acute deletion of TRF1 leads to stem-cell senescence without telomere shortening. Therefore, TRF1 is important for stem-cell maintenance and proliferation and the DDR signaling pathway may be blocked by inactivating p53 or ATM to determine whether stem-cell senescence can be rescued. The progressive deletion of TRF1 likely mimics

human hematological disorders. As with research showing the effect of the conditional knockout mutation  $Trf1^{Tg:K5-Cre/Tg:K5-Cre}$ , stem cell defects are also produced by long-term deficiency of TRF1 in epithelial tissue. These defects include severe hyperkeratosis and dysplasia in the digestive system, severe skin atrophy, and skin hyperpigmentation (Martinez et al. 2009). All of these phenotypes are characteristic of progressive and long-term TRF1 deficiencies and are reminiscent of telomere syndromes and DKC. As described above, one of the mutations that causes DKC is found in the TRF1-binding domain of TIN2. Long-term deficiency of TRF1 *in vitro* led to telomere deprotection and shortening. However, there was no evidence that the interaction between TIN2 and TRF1 had been disrupted in DKC caused by TIN2 mutations. There was also no evidence to indicate that telomere shortening was due to inhibition of telomerase. Therefore, further studies will be needed to understand how TRF1 affects cell fate, tissue regeneration, and aging *in vivo*.

## 6.2 A dual role for TRF1 in tumorigenesis

Another obvious phenotype of TRF1-deficient mice is oncogenesis. Tissue-specific conditional deletion of TRF1 in a p53-null background can lead to tumorigenesis.  $Trf1^{lox/lox}$  K5-cre p53<sup>-/-</sup> mice lacking TRF1 in stratified epithelia spontaneously developed invasive and genetically unstable squamous cell carcinomas in the tail and ear skin (Martinez et al. 2009). However, a separate study showed that deletion of TRF1 can prevent cancer.  $Trf1^{lox/lox}$  p53<sup>-/-</sup> K-RasLSLG12V mice exhibited impaired lung carcinoma growth and increased survival, regardless of telomere length

(Garcia-Beccaria et al. 2015). These two studies are based on depletion of TRF1 in different tissues and suggest that TRF1 exhibits tissue-specific responses to oncogenesis. Further studies will be needed to determine whether tumorigenesis is due to the DDR surveillance machinery or tissue-specific transcriptional reprogramming. However, all of these findings suggest that deficiencies in TRF1 produce genome instability and the DDR.

### **6.3 Conditional models of TRF2 deficiencies**

TRF2 knockout mice are embryo lethal (E13.5d), thus it is mandatory to use conditional mouse models to study its function in different tissues (Munoz et al. 2005). Overexpression of TRF2 in epithelial tissues ( $Trf2^{+/+}$  Tg:KRT5-TRF2) induced severe phenotypes in mice, including atrophy of sebaceous glands and accumulation of melanin deposits (Celli and de Lange 2005b). Overexpressing TRF2 caused critical telomere shortening which could not be rescued by telomerase. The characteristic skin phenotypes were apparently due to shortened telomeres and telomere dysfunction induced by TRF2 overexpression. UV-treatment led to a high incidence of skin carcinomas in these mice, and this was due to misregulation of the XPF/ERCC1 nuclease involved in UV-damage repair. Another work showed that KRT5-promotor-induced TRF2 deletion also stimulated tumorigenesis. These TRF2-tissue-specific knockout mice exhibited severe DNA damage, telomere fusion, and chromosome instability (Bojovic et al. 2013). Conditional-knockout studies of TRF2 in liver cells

showed that the DDR was induced, but the cells exhibited no functional impairment (Lazzerini Denchi, Celli and de Lange 2006).

A conditional KO model of TRF2 in the lung leads to the senescence of type 2 alveolar epithelial cells (AEC2s) (Alder et al. 2015). In addition, telomere dysfunction in AEC2s triggered an immune response and was associated with AEC2-derived upregulation of cytokine signaling pathways that are known to provoke inflammatory responses in the lungs.<sup>159</sup> These TRF2-deficient mice are also vulnerable to bleomycin treatment, suggesting that telomeres may play an important role in alveolar repair in AEC2s. Therefore, the shelterin component TRF2 may be critical in preventing IPF (Alder et al. 2015). Interestingly, conditional knockout of the TRF1 gene in AEC2s has similar effects and can induce IPF in mice. The onset of IPF and emphysema usually occur during aging, suggesting multiple factors may be involved in both conditions. These may include environmental factors, smoking, and tissue damage caused by stress and dust (Sanders and Newman 2013, Hoffman et al. 2016).

Since TRF2 indicate to have specific roles in nervous system, a conditional knockout mouse model has been construct to better understand the function of TRF2 in nervous system (Lobanova et al. 2017). This study shows that TRF2 deficiency may have different outcome due to the different cell types (including progenitor cells, immature neurons and differentiated neurons). The data show that the deficiency of TRF2 in all three cell types, telomere dysfunction can be seen including TIF and end to end joint. But the phenotypes (pathology or behavior decline) can not be seen in differentiated neurons (Lobanova et al. 2017). This indicate that TRF2 is dispensable

in post-mitotic cells like differentiated neurons. According to this study, it seems that the main function of TRF2 in nervous system is to protect the telomere. It is predictable that loss TRF2 can lead to proliferation disable due to the telomere dysfunction. But the differentiation is a process, the function of TRF2 in nervous system can not be valued separately. On the other hand, loss function of TRF2 in progenitor cells show strong phenotype in DNA damage and may cover the effects such as transcriptional regulation.

#### **6.4 TIN2 mouse models of DKC**

A recent study showed that mice with the TIN2 K280E DKC allele (TIN2DKC), which is analogous to the TIN2 mutation in patients with DKC, were healthy and fertile in the first generation (Frescas and de Lange 2014). However, by the third generation, telomeres had become shortened and the mice exhibited DKC symptoms such as bone marrow failure. Interestingly, this process was accelerated in the absence of telomerase activation (i.e., in an  $mTR^{-/-}$  mutant background). A weak ATR kinase signal at the telomeres and a fragile telomere phenotype were also observed (Frescas and de Lange 2014). This indicates that the TIN2 mutation induces DKC via telomerase dysfunction and also via telomere deprotection and DDR activation.

#### **6.5 POT1 mouse models**

In contrast to humans, mice have two POT1 transcripts, known as Pot1a and Pot1b. Deletion of Pot1a leads to embryo lethality (E6.5d) and a massive DDR signal at telomeres (Wu et al. 2006). This indicates that POT1 is crucial in the early stages of development due to its role in telomere protection. Deletion of Pot1a in adult mice increases telomeric DDR signaling and apoptosis and prevents bone marrow reconstitution (Hosokawa et al. 2017). These observations show that the *in vivo* functions of Pot1a include telomere protection and cell proliferation. Recent findings suggested that exogenous Pot1a treatment can repress the telomeric DDR and restore HSC function in aging mouse models. In addition to telomere protection, there was also some evidence that exogenous Pot1a treatment could alter metabolism via mTOR signaling. Exogenous Pot1a applied to human HSCs prolonged stem-cell activity in cell culture. However, further research will be needed to confirm this extratelomeric function of Pot1a (Wang et al. 2011).

In contrast, Pot1a<sup>lox/lox</sup> p53<sup>lox/lox</sup> hCD2-iCre mice developed severe T-cell lymphomas and Pot1a<sup>lox/lox</sup> p53<sup>lox/lox</sup> Sprr2f-Cre mice developed invasive metastatic endometrial adenocarcinomas with nuclear atypia and tetraploid genomes (Pinzaru et al. 2016, Akbay et al. 2013). These tissue-specific conditional-knockout mouse models show severe telomere dysfunction including telomeric DNA damage and fragility. These changes may lead to chromosome instability and changes in transcriptional regulation, which may result in tumorigenesis.

Surprisingly, Pot1a also has roles in post-mitotic neurons. Transgenic mice with Pot1a deletions in mature neurons (Pot1a<sup>F/F</sup> Tg:Nes-Cre) exhibited premature

senescence of astrocytes and loss of neurons, including Stellate, basket, and granule cells, as well as Golgi I neurons (Hosokawa et al. 2017). These phenotypes were completely rescued by p53 inactivation. Furthermore, damage to telomeres could be observed in these cells (Hosokawa et al. 2017). This protective role of Pot1a provides a basis for new studies to understand telomere protection. It remains unclear whether telomere deprotection, resulting from shelterin dysfunction, produces an observable phenotype in post-mitotic cells.

Deletion of Pot1b did not cause death at an early stage. However, mice with these mutations did show a series of adverse phenotypes within 15 months including germ cell apoptosis, a decrease in body size, crypt epithelia atrophy, leucopenia, anemia, and thrombocytopenia (Wang et al. 2011). Interestingly, crossing Pot1b-deletion mice with *Terc*<sup>+/-</sup> induced severe phenotypes such as early bone marrow failure, testis atrophy, and premature death (Wang et al. 2011). Telomere shortening and the DDR were also observed. These features are similar to characteristics of DKC and telomere syndromes. The mutant phenotypes observed indicate that Pot1b plays a role in proliferation, which may be linked to telomerase function. Therefore, Pot1b mutations combined with telomerase dysfunction may cause symptoms similar to those of DKC and telomere syndromes. Telomerase inactivation combined with Pot1b deletion leads to embryo lethality and telomere fusion.

Deletion of both POT1b and TERC produces some characteristic features of DKC, including hyperpigmentation and bone marrow failure (Hockemeyer et al. 2008, He et al. 2009).

## **6.6 A p53 progeroid model associated to shelterin dysregulation**

The p53<sup>Δ31/Δ31</sup> mutant mice express a truncated p53 protein lacking the C-terminal domain and suffer from aplastic anemia and IPF, as well as other problems associated with DKC and its more severe variant, HHS (Simeonova et al. 2013). In addition, these p53 mutants may be affected by heart failure, hyperpigmentation, nail dystrophy, oral leukoplakia, hypogonadism, and cerebellar hypoplasia. Importantly, this p53 mutation affects the expression of several telomere-related genes such as DKC1, regulator of telomere elongation helicase 1, TIN2, and TRF1.

## **6.7 Rap1 mouse models**

RAP1 knockout mouse models are the only shelterin-deficient models that show a normal birth rate (Yeung et al. 2013, Martínez et al. 2010). Telomere dysfunction was not observed in RAP1 knockout mice (Martínez et al. 2013, Teo et al. 2010). However, these mice did exhibit metabolic disorders including diabetes, increased blood glucose, insulin resistance, and obesity. These studies indicate that RAP1 deficiency causes phenotypes that are unrelated to telomere dysfunction. They also suggest that shelterin components can have effects in low-turnover tissues, such as key metabolic tissues. These observations provide a basis for new studies to investigate the function of shelterin proteins during aging in low-turnover tissues (Teo et al. 2010, Martínez et al. 2010, Martínez et al. 2013).

## **6.8 Mouse model of TPP1**

TPP1/Acd is a key protein in telomerase recruitment, and deletions in the corresponding gene are embryo lethal (E5.5d) (Beamer et al. 1994). Adult mice with deletions in TPP1/Acd exhibited acute chromosome instability, upregulation of p53 target genes, and G2/M cell-cycle arrest. In addition, the supply of hematopoietic stem cells (HSCs) was immediately exhausted. Therefore, impairment of telomerase recruitment leads to HSC exhaustion. However, p53 deletion does not rescue TPP1/Acd-deficient HSC function. This indicates that TPP1/Acd maintains the homeostasis of the hematopoietic system via p53-dependent and -independent pathways (Tejera et al. 2010, Takai et al. 2011). A separate study showed that a hypomorphic mutation in TPP1 together with p53 inactivation in mice can increase the incidence of carcinomas. However, complete loss of TPP1 function in epithelial cells of the  $Tpp1^{lox/lox}$  K5-cre  $p53^{-/-}$  mouse model did not lead to epithelial carcinomas. Presumably, loss of function of TPP1 and failure to recruit telomerases leads to telomere shortening and resistance to cancer development (Else et al. 2009). Therefore, the phenotype of TPP1 mutants is linked to telomerase recruitment. The loss of TPP1 leads to telomere shortening and dysfunction, which can cause stem cell exhaustion in both a p53-dependent and -independent manner.

## **7. shelterin subunits as therapeutic targets**

### **7.1 Shelterin therapy for age-related diseases**

The first purpose of targeting shelterin in age-related disease is to prevent telomere shortening and the DDR. However, any shelterin therapy must be carefully planned to avoid side effects such as chromosome instability or oncogenesis. Consequently, shelterin therapy is still at the experimental stage. The most advanced research on shelterin-associated treatments involves using the AAV9 virus to exogenously express the TRF1 gene in mice and prevent the aging process (Garcia-Beccaria et al. 2015). A non-integrative vector was used to overexpress TRF1 in adult mice, but constitutive TRF1 overexpression in transgenic mice led to telomere shortening due to cleavage by the XPF nuclease (Muñoz et al. 2009). Expression of the TRF1 gene using AAV9 did not lead to telomere cleavage. This work showed that the incidence of cancer did not increase in these mice, which may also indicate that exogenous expression of shelterin does not lead to carcinogenesis. However, the exogenous expression of TRF1 only rescued the aging phenotype in older mice (2 years old) and did not extend the lifespan of younger adult mice (1 year old), suggesting that TRF1 overexpression may only be effective as a treatment, and not as a preventive measure, for aging (Garcia-Beccaria et al. 2015). Interestingly, these studies showed that TRF1-overexpressing mice did not have longer telomeres than control mice, which suggests that overexpressing shelterin components may not elongate telomeres. However, overexpressed TRF1 may have stimulated telomere cleavage by the XPF nuclease, which would also account for this observation. In addition, it is unclear whether TRF1 overexpression would be effective for extremely short telomeres

because overexpression did not elongate the telomeres and may not inhibit the DDR or enable the cells to bypass cell-cycle checkpoints.

## **7.2 Shelterin and cancer treatment**

The fact that several shelterin subunits are overexpressed in cancer cells, contribute to oncogenesis and are involved in anti-cancer therapy resistance suggests that targeting shelterin expression is an interesting therapeutic strategy in oncology. For example, high expression levels of TRF1 and TPP1 enhanced the resistance to radiotherapy of telomerase-positive breast-cancer cells, and suppression of TPP1 increased the sensitivity to radiotherapy of a telomerase-negative osteosarcoma cell line (Li et al. 2014, Qiang et al. 2014).

Deletion of TRF1 can impede the progression of lung cancer and increase survival in mouse models with a p53-null background. This deletion can trigger telomere dysfunction, regardless of telomerase activity or telomere length (Garcia-Beccaria et al. 2015). Small-molecule TRF1 inhibitors have also been used in mouse cancer models. These small-molecule TRF1 inhibitors can induce a rapid DDR without decreasing survival rates, which indicates that acute depletion of shelterin levels may be a promising way to treat cancers such as lung cancer (Lin et al. 2006). Although TRF1-inhibition therapy has shown some efficacy in mouse models, it is unlikely to be ready for clinical testing in the near future. Small molecules which target the TRFH domain and block the DNA binding domain in TRF2 have also been tested in tumorigenic cell lines. In the HeLa cell line, inhibition of TRF2 can stimulate the DDR, end-to-end fusion,

and cell death via telomere dysfunction. However, whether such inhibition is effective *in vivo* and whether it produces adverse effects remain unknown.

Although these inhibitors will need to be studied extensively before they are ready for clinical use, focusing on shelterin components is a promising strategy for human cancer treatment because these proteins can be targeted independently of telomere length and telomerases.

**Table1. Examples for mouse transgenic model in shelterin deficiency (general or conditional) phenotypes**

Gene name	Experiment manner	System/Organ	Phenotype/disorder	REF
<b>Trf1</b>	Terf1 <sup>Δexon1/Δexon1</sup>	embryo	homozygotes E5-E5.5 litters with normal appearing and die before E7.5	(Karlseder et al. 2003)
		cellular	Cell apoptosis, no degradation of telomeric DNA or telomere fusions, cells from E3.5 fail to grow in vitro culture	
	Terf1 <sup>E/F</sup> Tg(K5-Cre)	embryo	Born in mendelian ratio; more than 92% of homozygotes die before P3	(Martinez et al. 2009)
		cellular	1. dramatic increase of γH2AX and 53BP1 and colocalized with telomeric DNA 2. increased p53, p21, and p16 cell cycle inhibitors and G2/M cell cycle arrest	
		Digestive system	Severe hyperkeratosis and dysplasia	
		skin	1. severe skin atrophy and skin hyperpigmentation 2. Severe epidermal stem cell defects, such as lack of mature hair follicles and of sebaceous glands 3. dermis inflammation hyperpigmentation and severe hyperkeratosis and dysplasia	
	Trf1 <sup>E/F</sup> Tg(Mx1-Cre)	Hematopoietic system	Acute TRF1 deletion: 1. Pancytopenia and BMF. 2. No telomere shortening. 3. TIFs increased. 4. Aplastic anemia Cellular senescence via p21 in progenitor cells Chronic TRF1 deletion: 1. Massive impaired overall survival because of BMF. 2. Massive telomere shortening	(Beier et al. 2012)
<b>Trf2</b>	Trf2 <sup>Δexon1-2/Δexon1-2</sup>	embryo	Embryonic lethality before E13.5 days	(Munoz et al. 2005)
		mortality	born at lower frequency	
	Trf2 <sup>+/+</sup> Tg(KRT5-TRF2)	aging	premature deterioration of the skin	(Celli and de Lange 2005a)

		skin	Atrophy of sebaceous glands, accumulation of melanin deposits, venerable to the stimulation and higher incidence of skin tumors	
	Terf2 <sup>EF</sup> Tg(Mx-Cre)	Liver	Increased DDR without senescence or apoptosis, no function impairment in liver	(Martinez et al. 2014)
	Terf2 <sup>EF</sup> Tg(Nestin-Cre)	embryo	Embryonic lethal	(Lobanova et al. 2017)
		cellular	DDR and apoptosis	
	Terf2 <sup>EF</sup> Tg(Dcx-Cre)	mortality	normal	
		cellular	Telomere fusion	
		Nervous system	DG area shrinkage (p53 <sup>-/-</sup> partial rescue)	
	Terf2 <sup>EF</sup> Tg(Nex-Cre)	Nervous system	Normal (no DG shrinkage)	
<b>Rap1</b>	Rap1 <sup>Δexon1-2/Δexon1-2</sup>	cellular	Normal proliferation and no telomere dysfunction	(Martinez et al. 2010)
		Growth/weight	Adult onset obese, especially female, independent of food intake	(Yeung et al. 2013)
		metabolism	Significant increase of blood glucose, insulin, cholesterol; impaired glucose tolerance and insulin resistance; accumulation of fat	
<b>Tinf2</b>	Tinf2 <sup>Mu-K267E/Mu-K267E</sup>	embryo	Embryonic lethality before E5.5 days	(Chiang et al. 2004)
	Tinf2 <sup>Mu-K267E/+</sup> -F3	cellular	Progressive shortening and fragile telomere over 3 generation	(Frescas and de Lange 2014)
		Reproductive system	Progressive seminiferous tubules and testicular atrophy (2/10)	
		Nervous system	Seizures (2/14); hydrocephaly (1/14); gliosis (1/14)	
		skin	skin dermatitis with neutrophilic serocellular crusting (2/14)	
		Hematopoietic system	Myeloid hyperplasia (1/14); lymphoid hyperplasia (2/14); pancytopenia in F3	
<b>Trp53(Acd)</b>	Trp53(Acd) hypomorphic mutant	mortality	Die before 6 months	(Beamer et al. 1994)
		Hematopoietic system	Fetal liver HSCs: 1. Loss of cell cycle quiescence/G2/M arrest. 2. Completely loss of long-term BM reconstitution activity	
		skin	heavy pigmentation of ears, snout, body, feet, and tail	
		metabolism	adrenals are highly dysplastic (enlarged nuclei)	
	Trp53 <sup>Tg:exon3-8/Tg:exon3-8</sup>	embryo	Die before E14.5	
	Trp53 <sup>EF</sup> Tg(K5-Cre)	mortality	Die before 2 weeks	(Tejera et al. 2010)
		cellular	Telomere shortening, increased polyploidy, cycle arrest G2 to M in keratinocytes (can be partially rescued by inactivation of Trp53)	(Kronenberg et al. 2017)
		skin	Heavy pigmentation, epithelial abnormalities especially oral mucosa (unable to feed, can be partially rescued by inactivation of Trp53)	

	Tpp1 <sup>F/-</sup> Tg(Mx1-Cre)	Hematopoietic system	Adult HSPCs: 1. Deplete HSPCs 5 in days. 2. No telomere shortening or acute chromosomal instability	(Jones et al. 2014)
<b>Pot1a</b>	Pot1a <sup>Δexon8/Δexon8</sup>	embryo	Die before E6.5	(Wu et al. 2006)
		cellular	cultured E1.5-3.5 embryos fail to form an inner cell mass	
	Pot1a <sup>Δexon3/Δexon3</sup>	embryo	Die before E5.5	
	Pot1a knockdown	Hematopoietic system	Adult: Increase telomeric DDR signaling and apoptosis Loss of BM reconstitution ability	(Hosokawa et al. 2017)
	Pot1a overexpression	Hematopoietic system	Adult: 1. Inhibit telomeric DDR signaling. 2. Prevent the activation of mTOR signaling and mitochondrial ROS production	
	Pot1a <sup>FF</sup> Tg(Nes-Cre)	embryo	Born in mendelian ratio	
		mortality	Shorter life span	
		Wight/growth	Decreased weight and body size since born	
		Nervous system	Astrocytes premature senescence (p53 dependent) Loss of all kinds of neurons (Stellate, Basket, Golgi neurons and granule neurons) through apoptosis both p53 and ATM dependent	
<b>Pot1b</b>	Pot1b <sup>Δexon3-4/Δexon3-4</sup>	Reproductive system	20-fold increase in apoptosis in seminiferous tubules by 220 days; testes size is reduced by about 2-fold; no spermatogenesis in 60% of seminiferous tubules; sterile after producing 3 to 4 litters	(He et al. 2009)
		Hematopoietic system	15 months: Leucopenia, anemia and thrombocytopenia	
		growth	Decreased body size in both sex	
		Digestive system	about a 15-fold increase in apoptotic in intestinal crypt epithelia	
		skin	hyperpigmentation in tails, legs, paws, and snout	
	Pot1b <sup>Δexon3-4/Δexon3-4</sup> mTERC <sup>+/-</sup>	Hematopoietic system	1. Dyskeratosis congenita (DC) like BMF 2. loss population of HPSCs in 6 months	(Wang et al. 2011)
		mortality	Premature death	
		cellular	1. telomere shortening. 2. Increase DDR, telomere fusion and apoptosis in p53 dependent manner	
		Reproductive system	Atrophy testes	
		Digestive system	Atrophy intestinal epithelia	
	Pot1b <sup>Δexon3-4/Δexon3-4</sup> mTERC <sup>-/-</sup>	embryo	Embryonic lethal	
		cellular	Dramatic DDR, telomere fusion and apoptosis	
	Pot1b <sup>Δexon3-4/Δexon3-4</sup> p16 <sup>-/-</sup>	Mortality	Premature death	(Wang, Sharpless and Chang 2013)
		cellular	Stronger telomere dysfunction than Pot1b <sup>Δexon3-4/Δexon3-4</sup>	
		Hematopoietic system	1. Declined repopulation ability compare to Pot1b <sup>Δexon3-4/Δexon3-4</sup> . 2. Loss HSPCs completely earlier than Pot1b <sup>Δexon3-4/Δexon3-4</sup> in p21 dependent manner	
		Reproductive system	Atrophy testes	
		Digestive system	Atrophy intestinal epithelia	

Pot1b <sup>Δexon3-4/Δexon3-4</sup>	embryo	Embryonic lethal
p16 <sup>-/-</sup> mTERC <sup>-/-</sup>		

**Table2. Examples for mouse transgenic model in shelterin deficiency/overexpression tumorigenesis susceptibility**

Genotype	Tumorigenesis	Publish time
Trf1F/F K5-cre p53 <sup>-/-</sup>	Squamous cell carcinoma	Martínez et al., 2009b
Trf2 <sup>+/+</sup> Tg(KRT5-TRF2)	Skin carcinoma	Munoz P et al. 2005
Pot1a <sup>F/F</sup> p53 <sup>F/F</sup> hCD2-iCre	T cell lymphomas	Pinzaru et al., 2016
Pot1a <sup>F/F</sup> p53 <sup>F/F</sup> Sprr2f-Cre	Endometrial adenocarcinomas	Akbay et al
Tpp1Acd p53 <sup>-/-</sup>	Carcinomas	Else et al., 2009
Tpp1 <sup>F/F</sup> K5-cre p53 <sup>-/-</sup>	Cancer suppressor	Tejera et al., 2010
Trf1 <sup>F/F</sup> p53 <sup>-/-</sup> K-RasLSLG12V	Growth impairment of lung carcinoma	García-Beccaria et al., 2015

## Telomere protection

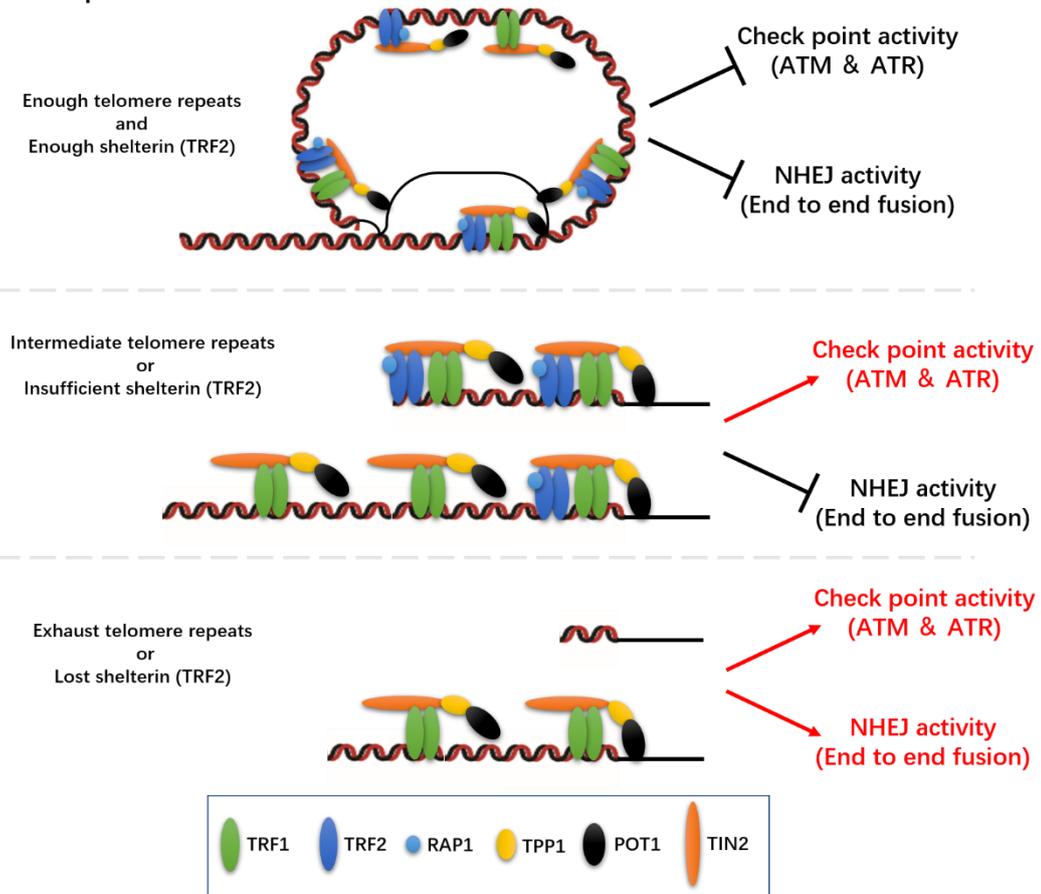


Fig1. The different state of telomere protection. Chromosome ends were protected by shelterin complex (including TRF1, TRF2, RAP1, TPP1, POT1, TIN2). Tandem repeats (TTAGGG) and shelterin complex formed a "T-loop" to hide and protect the 3' overhang from unwanted DNA damage response (including ATM and ATR pathway). The shortened telomere (not enough to form the T-loop) or the insufficient of shelterin complex (For example: TRF2) can lead to unwanted DNA damage response and activation of check point (p53 or ChK). If the telomere is critically short or lose the function of shelterin (For example: TRF2), it will not only activate the DDR but the NHEJ pathway, which lead to chromosomes end to end fusion.

## Telomere damage response

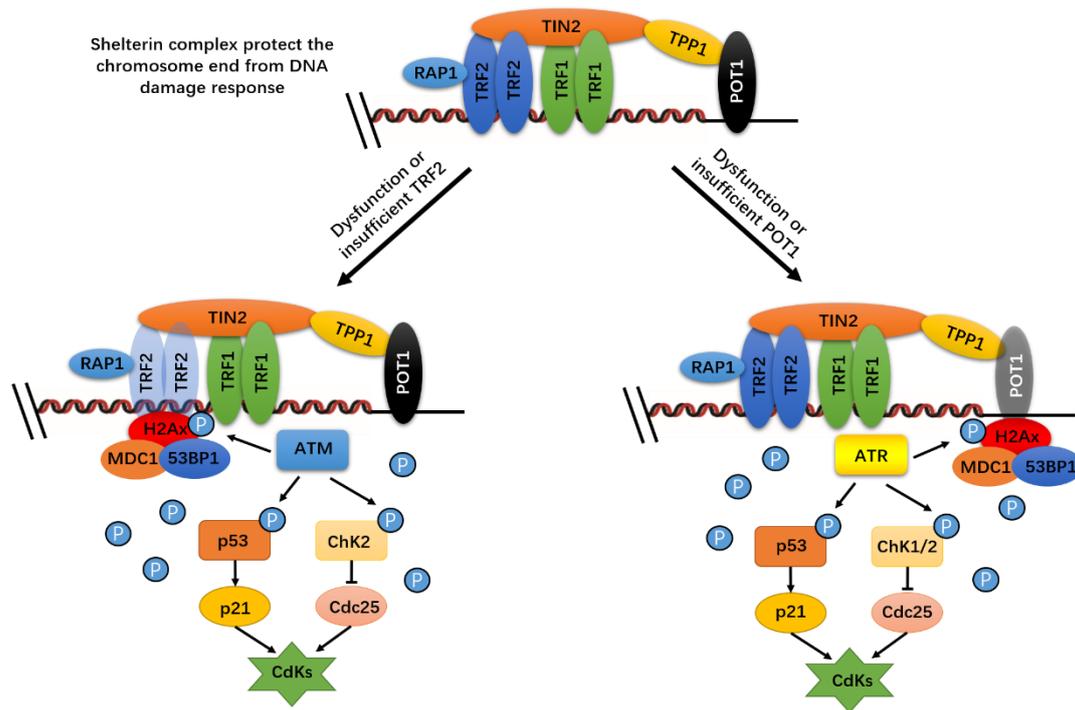


Fig2. The structure of shelterin complex and the DNA damage pathway triggered by dysfunction or insufficient of shelterin components (For example: TRF2 and POT1). The dysfunction of TRF2 activate the ATM, phosphorylate the check point protein p53 and ChK. While the dysfunction of POT1 activate the ATR pathway. The activation of both pathway (ATM and ATR) can lead to cell cycle arrest, induce cellular senescence or apoptosis.

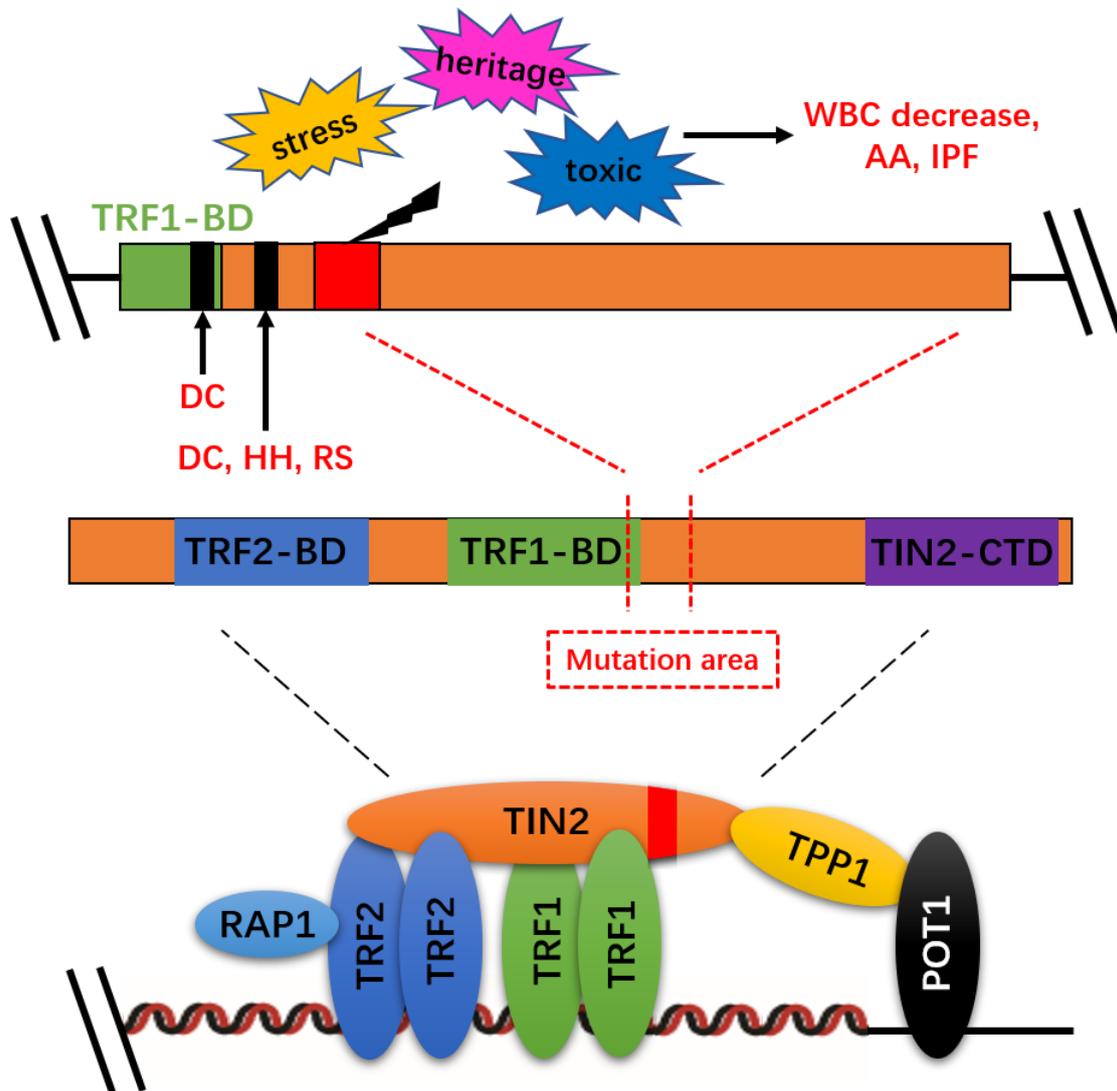


Fig3. Structure organization of TIN2 with disease-associated mutation locations. As TIN2 mutation lead to DC is the most well-known shelterin defected disease, TIN2 was used as an example to show the characteristics of shelterin defected disease. 1. One mutation can be associated with multiple pathways, as TIN2 mutation affected telomerase binding, telomere protection, sister chromosome cohesion. 2. One mutation can show different phenotypes and clinical features, as TIN2 mutation can lead to DC, HHS or RS. 3. Some diseases related to shelterin mutations may not show disorders in early life or first generation, but it can accumulate through inheritance or induced by stress or toxic environment. (DC, Dyskeratosis congenita; AA, Aplastic anemia; HHS, Hoyeraal-Hreidarsson syndrome; RS, Revesz syndrome; BD, binding domain; CTD; C-terminal domain)

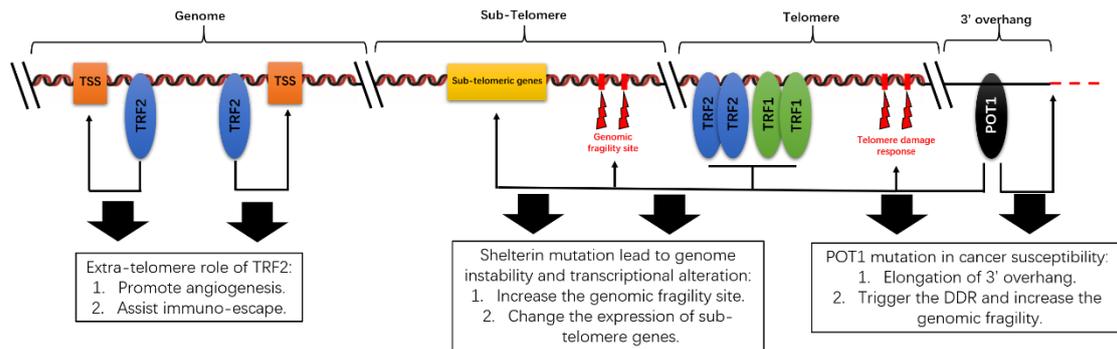


Fig4. The roles of shelterin in tumorigenesis. Depending on the mouse model research and in vitro study, TRF2 may have roles of regulating gene expression, involved in angiogenesis and tumor cell immune-escape. Most of the research show that the mutation or the abnormal expression of shelterin in cancer are related with genomic instability and transcriptional alteration. The POT1 mutations have been found in several family heritable cancers including melanoma, glioma and leukemia. According to the mouse model and in vitro research, POT1 mutation can lead to 3' overhang elongation and activation of ATR pathway. It will also increase the genomic instability and fragility sites, which can lead to transcriptional alteration and tumorigenesis.

## CHAPTER 2. Research project

### 2 Objective of study

#### 2.1 Article 1

## Differential spatio-temporal expression pattern of Shelterin genes throughout life span

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### Summary

The shelterin is the core complex of telomere proteins, which plays a critical role in telomere protection against unwanted activation of DNA damage response and in telomere length homeostasis. Although shelterin is believed to be ubiquitously expressed to provide chromosome end stability, some evidence exists that at least some of the shelterin subunits play tissue-specific functions (Ye et al. 2014). Notably the shelterin component TRF2 has been reported to be upregulated in neuronal tissues during brain maturation (Cheng et al.) (Zhang et al. 2008) (Zhang et al. 2011) (Ovando-Roche et al. 2014). However, very little is known on the regulation of shelterin gene expression during development and aging. Using zebrafish model, we reveal here that shelterin subunits have each distinct spatio-and temporal expression pattern, which are not correlated to proliferative state in the organ systems investigated. Interestingly, a higher expression level of *terfa* (zebrafish homology of TERF2) in zebrafish brain. We

also find a dysregulation of several selected neuro-development genes by interference with expression of *terfa* using morpholino (Biroccio et al. 2013, Simonet et al. 2011). Taken together, we demonstrate a highly dynamic expression pattern of the different shelterin components and a specific transcriptional function of *terfa* in neuro-system, which suggests important tissue-specific functions of shelterin during development and life span.

## **Results**

### **1. Shelterin genes are differentially expressed in zebrafish tissues throughout lifespan**

The subunit composition of zebrafish shelterin is similar to that of humans; the complex is composed of the six subunits TRF1, TRF2 (termed TRFA in zebrafish), RAP1, TIN2, TPP1, and POT1. We determined the relevant mRNA levels in various tissues of 6 female fishes, from the young adult stage (3 months) to aged fish (36 months) (Figure 4). We confirmed tissue identities using specific markers (Supplementary Figure 2). Thus, the relative levels of TERFA mRNA were highest in brain and muscle and lowest in liver; TPP1 mRNA showed highest expression in the heart and lowest expression in the intestine and ovaries (Figure 4, Table 1). During aging, we observed a trend toward general downregulation of shelterin gene expression (Figure 4, Table 2); this was particularly marked in the brain and ovaries. The relative shelterin gene expression pattern was usually preserved, with the exception of the RAP1, which decreased in mRNA expression more rapidly than did the other shelterin genes in the intestine and

the gill. We performed whole-mount in situ hybridization of zebrafish embryos for TERFA mRNA using an RNA-probe (Figure 5). The signal corresponding to TERFA mRNA was present throughout the entire embryo from the blastula (4 hpf) to the gastrula (8 hpf) stage. In contrast, the neuronal marker, Neurog1(Fode et al.), was detected only in neuronal tissues and only from 12 hpf to hatching at 72 hpf, whereas the hematopoiesis factor c-MYB was detected only in hematopoietic tissue and then only during the late stages of development (Zhang et al.) (Figure 5). Interestingly, high expression of TERFA mRNA in the nervous system was maintained from the beginning of the somite stage to the time of hindbrain formation (20 hpf) and thereafter (Figure 5). This was also true for Neurog1, at 20 hpf, TERFA mRNA appeared to be expressed prominently in the dorsal root ganglion and midbrain boundary, in the regions of the neural tube that give rise to the neocortex, midbrain, and hindbrain, in the dorsal and ventral spinal cord, and in regions of the peripheral nervous system. These results are in agreement with the quantitative reverse transcription PCR (qRT-PCR) data on zebrafish tissues (Figure 4) and the specific neuronal staining of TRF2 during mouse development (Figures 2, 3 and Supplementary Figure 2). In summary, TRFA expression appears to be ubiquitous during early development but becomes progressively more restricted to neuronal tissues during later stages of development and into young adulthood.

## **2. In zebrafish, terfa specifically regulates the expression of neuronal genes**

The above results suggest that *terfa* has specific roles in zebrafish nervous system. Indeed, *terfa*-compromised zebrafish show early-onset neurodegenerative phenotypes(Kishi et al. 2008). Moreover, TRF2 downregulation in mammalian neural progenitor and tumor cells alters neuronal differentiation by mechanisms that are distinct from those regulating telomere protection(Ovando-Roche et al. 2014). The ability of TRF2 to bind at or close to neuronal genes and to activate their transcription is an attractive mechanism to explain the extratelomeric roles of TRF2 (Biroccio et al. 2013, Ye et al. 2014). We tested this hypothesis by designing a morpholino antisense oligonucleotide (MO) targeting the *terfa* gene and injected this MO into zebrafish embryos at the 1–2-cell stage. Then we tested in the MO-treated embryos the expression by RT-qPCR of eight neuronal genes that are bound by TRF2 in human cells(Simonet et al. 2011). The expression of four of them was decreased upon TRFA inhibition (Figure 6a). Among them, PPP2R2C encodes a neuronal isoform of the regulatory subunit of Protein Phosphatase 2A (PP2A). We ruled out an off target of the MO against *terfa* by showing a rescue of *ppp2r2c* mRNA expression upon co-injection of *terfa* mRNA (Figure 6b). Importantly, the expression of other, not neuronal specific, PP2A subunit genes is not TRFA dependent (Figure 6c), highlighting further the neuronal specificity of genes whose expression is regulated by TRFA in zebrafish. Interestingly, these results suggest that the network of neuronal genes regulated by TRF2 is conserved between human and fish.

## **Discussion**

This work reveals an unexpected role of the key shelterin subunit TRF2 in neuro-development. Firstly, we unveil a tissue-specific shelterin gene expression pattern of zebrafish in aging (Table 1). With the exception of a study on tissue-specific expression of human shelterin genes in response to physiological stress(Laye et al. 2012), very few study shows the shelterin gene expression level change in a tissue-specific manner during development and aging. To the best of our knowledge, our study is the first to show the shelterin expression patten in zebrafish, which may give an evolutionary angle to see the function of each shelterin components. In particular, the terfa mRNA levels were specifically elevated in the neural system, a tissue of low proliferative activity, may indicate a specific role of terfa independent of telomere protection but neuro-development. This hypothesis also coincides with the transcriptional function of TRF2 in human neuro-cell lines(Zhang et al. 2014b, Zhang et al. 2011). We also found that in zebrafish, the terfa involved in transcriptional regulation during neuro-development, which indicate an extratelomeric role of terfa in zebrafish.

This study had certain limitations too. Firstly, the tissues evaluated are composed of many different cell types, and thus we cannot clearly conclude whether the changes noted suggest that the more proliferative cell types do not significantly express shelterin genes. Second, most of our significant findings were detected at the mRNA level. Overall, our results suggest that important tissue specific shelterin subcomplexes exist. This is consistent with previous studies on the tissue-specific roles played by TERF2 (Ovando-Roche et al. 2014, Zhang et al. 2011)and RAP1 (Martínez et al. 2013, Platt et al. 2013). Consequently, the existence of tissue-specific shelterin gene

expression may explain the broad contributions made by telomeres and telomerase to normal development and aging, as well as the roles played by dysregulation of telomeres and telomerase in cancer and various other tissue-specific pathologies (Armanios and Blackburn 2012). These data of shelterin expression pattern also form a solid foundation and raise several questions for future studies exploring the role of zebrafish TRF2 (*terfa*) during neuro-development and aging.

### Figure legend

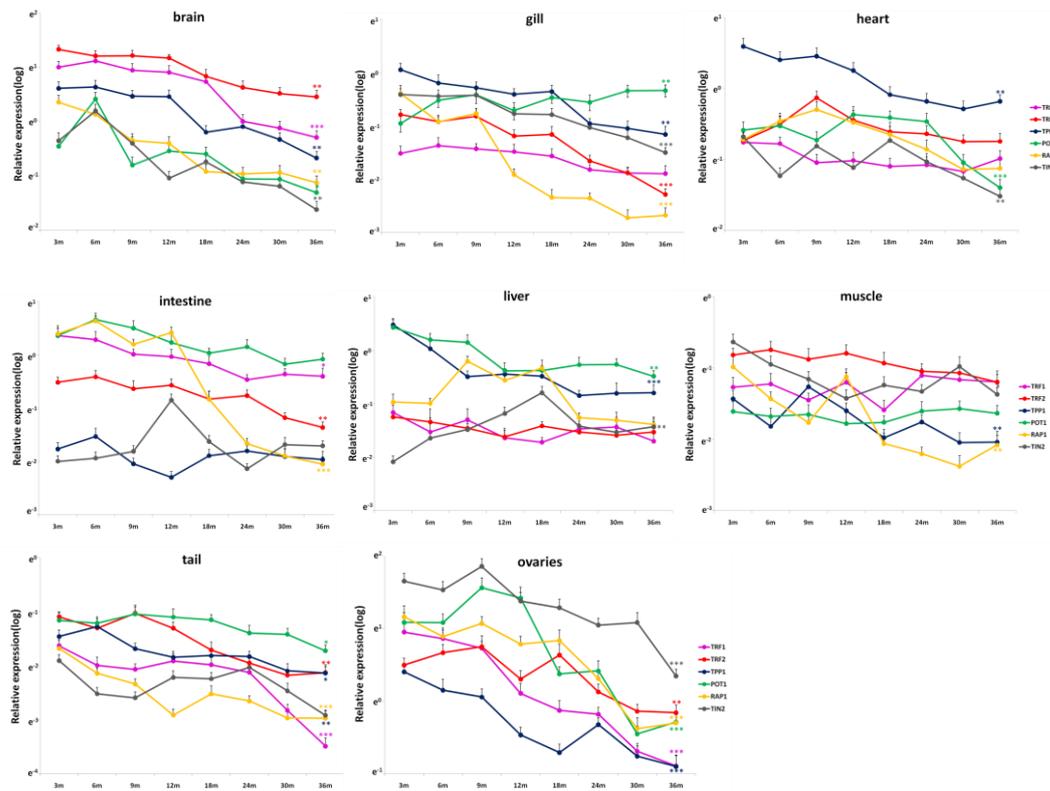
**Figure 1. Shelterin genes are differentially expressed during zebrafish life span.** Quantitative RT-qPCRs for shelterin components TRF1, TRF2(*TERFA*), RAP1, TPP1, TIN2 and POT1 in zebrafish's brain, heart, liver, intestine, muscle, gill, tail and ovary at different time-points of life. Span from 3 month to 36 month (n=6 each). Significance was tested between 3month and 36 month. Data are mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

**Figure 2. Expression of tissues specific markers in the dissected organs from zebrafishes at different ages.** Quantitative RT-qPCRs for different zebrafish organs was tested by different tissues specific primers, including brain, heart, liver, intestine muscle, gill, tail and ovary from 3 month to 36 month (n=6 each). Data are presented at mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

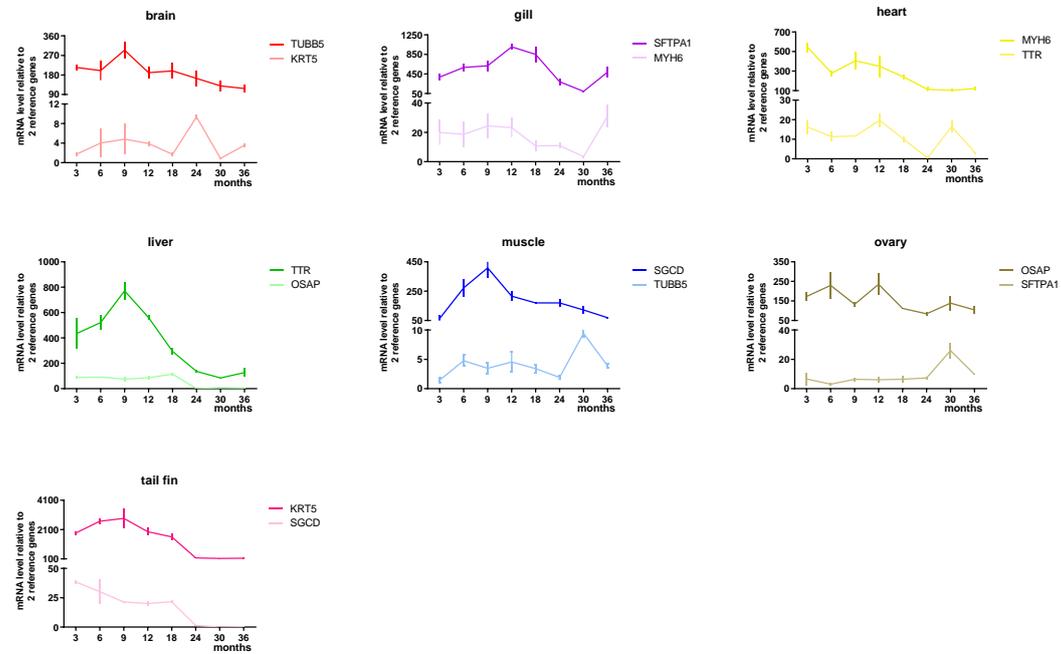
**Figure 3. TERFA expression increase since neural development at embryonic stage and remains high in the brain during larval stage development.** Representative photomicrographs of whole-mount in situ hybridization of *terfa*, *neurog1* and *c-myb* mRNA. The RNA probe labelled with DIG was stained in dark blue. The green arrow indicates the dorsal root ganglion. The black arrow in 24hpf indicates the midbrain boundary. The red arrow indicates the *c-myb* signal marked hematopoietic tissue. (scale bar, 500 $\mu$ m)

**Figure 4. TERFA activates the expression of neuronal gene in zebrafish embryos.** (A) Gene expression measured by RT-qPCRs in zebrafish embryos 24h after microinjection of MO targeting TERFA gene. (B) PPP2R2C expression upon co-injection of TERFA mRNA or PPP2R2C mRNA in zebrafish embryos after microinjection of MO targeting TERFA gene. (C) Expression of PP2A subunits genes in zebrafish embryos 24h after microinjection of MO targeting TERFA gene. Each qRT-PCR was repeated three times and the mRNA level of PP2A genes was expressed relative to  $\beta$ -actin and normalized to the Morpholino-control. Data are mean  $\pm$ SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ ,

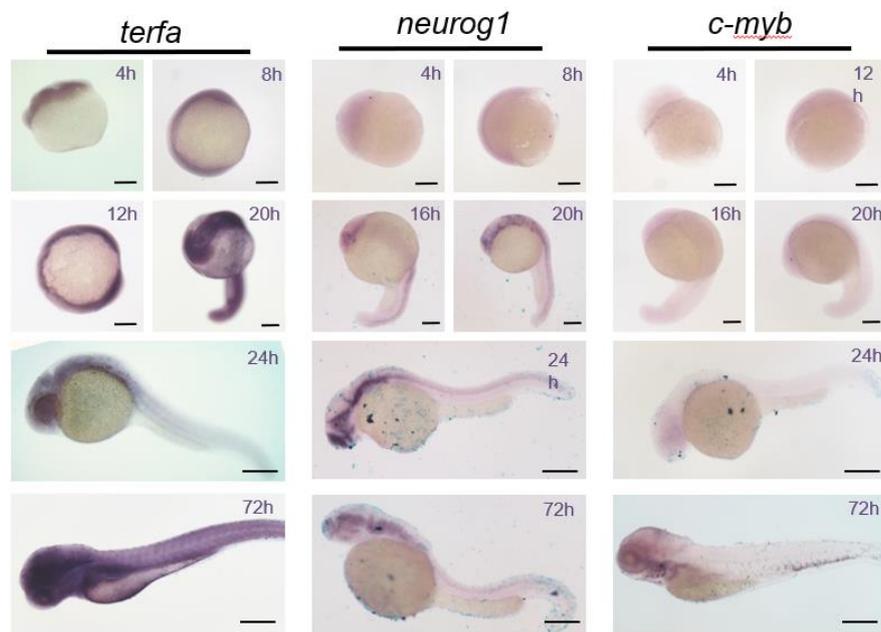
**Figure 1**



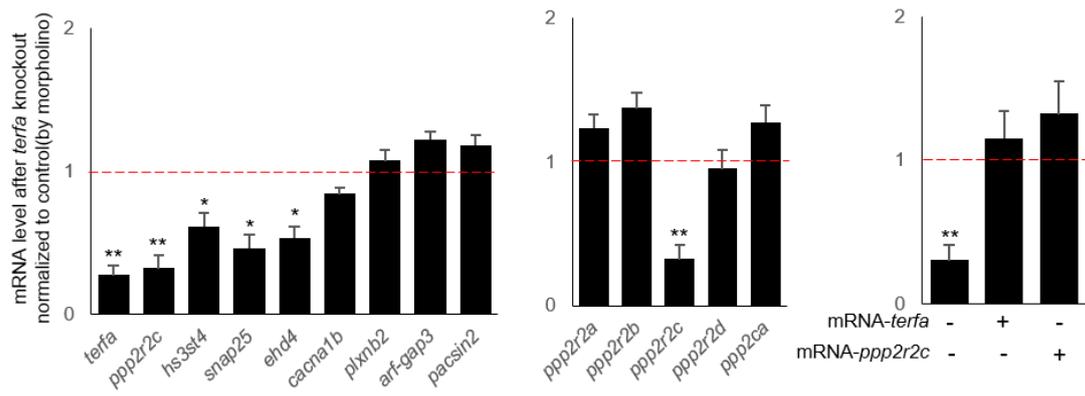
**Figure 2**



**Figure 3**



**Figure 4**



**Table 1**

Shelterin	brain	gill	heart	intestine	liver	muscle	Tail	ovaries
terf1	2	6	6	6	5	4	4	4
terfa	1	5	2	4	4	1	2	5
rap1	4	4	3	2	3	6	5	3
tin2	5	3	5	6	6	2	6	1
tpp1	3	1	1	5	2	3	3	6
pot1	6	2	4	1	1	5	1	2

Shelterin	brain	gill	heart	intestine	liver	muscle	Tail	ovaries
terf1	DOWN	DOWN	S	DOWN	S	S	DOWN	DOWN
terfa	DOWN	DOWN	S	DOWN	S	DOWN	DOWN	DOWN
rap1	DOWN	DOWN	S	DOWN	DOWN	DOWN	DOWN	DOWN
tin2	DOWN	DOWN	DOWN	S	UP	DOWN	DOWN	DOWN
tpp1	DOWN	DOWN	DOWN	S	DOWN	DOWN	DOWN	DOWN
pot1	DOWN	S	DOWN	DOWN	DOWN	S	DOWN	DOWN

## 2.2 Article 2

### The non-telomeric evolutionary trajectory of TRF2 in zebrafish reveals its essential role in neurodevelopment and aging

#### Summary

In many organisms, the chromatin-mediated pathway depends on the binding of shelterin proteins to telomeric DNA. The organization and composition of shelterins vary between organisms, from the unique RAP1 protein in budding yeast to a complex of six proteins in humans (TRF1, TRF2, RAP1, TIN2, TPP1, and POT1). Various combinations of shelterin components are found in eukaryotes; for instance, ciliate telomeres only contain orthologs of POT1 and TPP1, and no TRF1 and TRF2 orthologs are present in the worm *Caenorhabditis elegans*. Notably, some metazoans do not possess shelterin subunits; for example, chromosomal terminal protection in *Drosophila* is mediated by terminin, a recently evolved protein complex (Cacchione, Cenci and Raffa 2020).

In mammals, the binding of shelterin to telomeric DNA is mediated by TRF1, TRF2, and POT1; POT1 binds to the single-stranded overhang, whereas TRF1 and TRF2 recognize the duplex regions of telomeric DNA. The TIN2 protein bridges TRF1 and TRF2 to the TPP1 - POT1 heterodimer, thus linking the duplex component of telomeres to the single-stranded tail. In mouse and human cells, TRF2 plays a central role in telomere protection by preventing ATM-dependent DDR activation and telomere end-to-end fusions (Gilson and Geli 2007). The expression of TRF2 is downregulated during normal and pathological aging in several vertebrates, whereas it is upregulated in several cancers of different origins (Wu et al. 2019, Wagner et al. 2017, Robin et al. 2020, Wang et al. 2015).

Previous studies using mouse models of TRF2 dysregulation suggest a pivotal role of TRF2 in aging and cancer. Cre-inducible whole-body *terf2* deletion in mice led to pro-oxidative and premature-aging vascular phenotypes (Morgan et al. 2019). In a

conditional TRF2-knockout mouse lung model, senescence in type 2 alveolar epithelial cells and increased inflammation were observed (Alder et al. 2015). Interestingly, the targeted deletion of TRF2 in skeletal muscle cells did not lead to obvious telomere damage but instead, to mitochondrial dysfunction and oxidative stress (Robin et al. 2020); this indicates that TRF2 may exhibit telomere-independent functions in certain cell types. Indeed, TRF2 can also bind to non-telomeric DNA, where it serves various functions involved in DNA repair, heterochromatin replication, mitochondrial metabolism, neuronal differentiation, and oncogenesis. This raises questions about the telomeric and extra-telomeric properties of TRF2, and their contribution to the phenotypes of TRF2-compromised animals.

Our knowledge regarding the role of shelterin in fish is very limited. Zebrafishes that carry a heterozygous insertional mutation in the TERFA gene (encoding the TRF2 homolog zfTRF2) exhibit an accelerated aging phenotype, including shorter lifespans and progressive retinal cell degeneration (Kishi et al. 2008). In this work, we investigated the role of zfTRF2 in zebrafish.

## **Results**

### **1. zfTRF2 does not primarily bind to telomeres in zebrafish cells**

First, we investigated whether zfTRF2 associates with zebrafish telomeres. We raised polyclonal antibodies against purified zfTRF2, which we validated using terfa-compromised ZF4 cells; ZF4 was a zebrafish cell line established from 1-day-old zebrafish embryos (Fig. 1a). In ZF4 cells, zfTRF2 staining was mostly localized in the nucleus. However, only 5% of telomeres, as visualized using a telomere-specific peptide nucleic acid (PNA) probe, exhibited colocalization with zfTRF2 (Fig. 1b). We also examined zfTRF2 colocalization with various nuclear structures using probes that specifically recognize zebrafish pericentromeric and centromeric satellite DNA sequences. Similar to humans, in which TRF2 binds to pericentromeres as well as to telomeres (Mendez-Bermudez et al. 2018), 20% of pericentromeric foci and 5% of centromeric foci were associated with zfTRF2 in ZF4 cells (Fig. 1b).

Because our results suggested that zfTRF2 binds only rarely to telomeres in zebrafish cells, we investigated the intrinsic capacity of zfTRF2 to bind to telomeres in a heterologous cellular system. We expressed a Myc-tagged version of zfTRF2 in human 293T cells (Extended Data Fig. 1a) and monitored its colocalization with human telomeres using a telomere-specific PNA probe. In contrast to observations in ZF4 cells, zfTRF2 predominantly associated with telomeres in 293T cells, as visualized using both anti-zfTRF2 (Fig. 1c) and anti-Myc (Extended Data Fig. 1b) antibodies; zfTRF2 colocalized with more than 70% of the human telomeres. Notably, zfTRF2 also colocalized with the pericentromeric satellite III DNA probe, similar to endogenous human TRF2 (Extended Data Fig. 1c). Upon hTRF2 gene downregulation (but not that of hTRF1), the association of zfTRF2 with telomeres and satellite III was partially reduced (Fig. 1c, Extended Data Fig. 1b and c).

From these results, we inferred that zfTRF2 was able to dimerize with endogenous human TRF2, implying that the residual binding of zfTRF2 in human TRF2-compromised cells reflects its independent ability to bind to telomeres or satellite III even in the absence of human TRF2. Thus, we concluded that in human cells, zfTRF2 is intrinsically capable of binding telomeres and pericentromeric heterochromatin. This suggested that, in zebrafish cells, zfTRF2 competes with other factors for telomere-specific binding.

## **2. zfTRF2 prevents global DDR, but not specifically for telomeres**

Next, we explored the involvement of zfTRF2 in telomere protection in fish cells. The downregulation of each of the six shelterin subunits by RNA interference in ZF4 cells triggered a potent DDR; the DDR was quantified by counting the total number of  $\gamma$ H2AX foci (Fig. 1d, Extended Data Fig. 1d). The percentage of damaged telomeres increased following the simultaneous downregulation of all shelterin subunits. When each shelterin subunit was downregulated separately, only zfPot1 downregulation elicited a specific increase in the percentage of damaged telomeres (Fig. 1d). This highlighted a specific telomere-protection role for zfPOT1, but not for zfTRF2. Furthermore, we did not find any specific protective functions of zfTRF2 for

pericentromeric or centromeric DNA (Extended Data Fig. 1e and f). Overall, these results demonstrated that zfTRF2 plays a general role in protecting the zebrafish genome against DNA damage, but with no apparent specificity for telomere protection.

### **3. zfTRF2 is required for brain development**

To explore the role of zfTRF2 in vivo, we generated a *terfa*-compromised zebrafish model by introducing a frameshift mutation into exon 2 at the center of the TRFH domain (Extended Data Fig. 2a). This led to reduced TERFA mRNA expression probably due to the presence of a premature stop codon and subsequent nonsense-mediated mRNA decay (Extended Data Fig. 2b), which consequently reduced zfTRF2 expression (Fig. 2a). Homozygous (*terfa*<sup>-/-</sup>) and heterozygous (*terfa*<sup>+/-</sup>) fishes, collectively named *terfa*-compromised fishes, exhibited detrimental phenotypes; most homozygous fishes died at the larval stage (Fig. 2b), and only approximately 50% of heterozygous fishes continued to develop to the adult stage (Extended Data Fig. 2c). The adult heterozygous fishes exhibited a reduced lifespan and a premature-aging phenotype, as well as cardiac dysfunction and a marked reduction in brain development (Extended Data Fig. 2c–f).

In *terfa*-compromised fishes, the first prominent phenotype that appeared during development was brain edema, followed by heart edema and intestinal dystrophy (Fig. 2c and d). Brain development failure in *terfa*-compromised fishes was confirmed by decreased expression of the pro-neural Neurogenin 1 gene, a neurodevelopment marker, at 2 days post-fertilization (dpf). At the same development stage (2 dpf), the expression levels of heart (*cmcl2*) and hematopoiesis (*c-myb*) development markers did not change (Fig. 2e). The neurodevelopmental failure was likely caused by the *terfa*- mutation; the percentage of fishes with brain edema was higher for homozygous fishes compared to heterozygous fishes, and the detrimental phenotype was fully rescued following injection with TERFA mRNA (Extended Data Fig. 2g). The brains of the *terfa*-compromised fishes were also strongly stained by senescence-associated beta-galactosidase (SA-β-gal) (Fig. 2f) and acridine orange (Fig.

2g), indicating a rapid burst of cellular senescence and apoptosis, respectively. The partial or complete loss of TERFA also resulted in increased DDR in fish brains and eyes at 3 dpf, as measured by calculating the percentage of  $\gamma$ H2AX-positive cells (Fig. 2h). This was accompanied by a decrease in cell proliferation (Extended Data Fig. 2h) as well as decreases in the number of glial fibrillary acidic protein (GFAP)-positive glial cells, HuC-positive neurons, and SOX2-positive pluripotent stem cells (Extended Data Fig. 2i and j). Notably, the increases in  $\gamma$ H2AX-positive and apoptotic cells were notably more pronounced in GFAP-positive glial cells than in HuC-positive neurons or SOX2-positive pluripotent cells (Fig. 2i, Extended Data Fig. 2k).

Similar to observations in ZF4 cells (Fig. 1), the DNA damage in *terfa*-compromised brain cells was not restricted to telomeres (Extended Data Fig. 2l). Moreover, no significant increase in telomere abnormalities was detected in metaphase spreads obtained from embryos (Extended Data Fig. 2m). Thus, *zfTRF2* appears to exhibit a general caretaker function without the telomere-specific capping functions displayed by mammalian TRF2.

#### **4. Neurodevelopmental failure in *terfa*-compromised fishes is partially, but not fully, dependent on DDR activation**

To investigate whether the DNA damage induced by TERFA deficiency is responsible for neurodevelopmental abnormalities, we introduced null alleles of ATM, a key DDR kinase blunted by TRF2 in mammals, and of P53, a central effector of DDR signaling. In *atm*<sup>-/-</sup> fishes, but not *p53*<sup>-/-</sup> fishes, the loss of TERFA did not lead to an increase in  $\gamma$ H2AX-positive cells in the brain, even in homozygous fishes (Fig. 3a). Therefore, *zfTRF2* inhibited ATM-dependent DDR activation, similar to its ortholog in mammals. Both *atm*<sup>-/-</sup> and *p53*<sup>-/-</sup> *terfa*-compromised fishes displayed reduced rates of apoptosis and senescence; this effect was more pronounced in *atm*<sup>-/-</sup> fishes, supporting the generally accepted model in which ATM is upstream of P53 in the DDR signaling pathway (Fig. 3b and c, Extended Data Fig. 3a and b). However, embryonic death rate, brain edema, and neuron loss in *terfa*-compromised fishes were not fully prevented by ATM and P53 gene disruption (Fig. 3d and e, Extended Data Fig. 3c). We concluded

that ATM-dependent activation of the DDR contributed to the developmental failure of terfa-compromised fishes, but that other factors were also at play. This suggests that zfTRF2 performs additional DDR-independent functions during zebrafish development.

### **5. In addition to DDR, transcriptional dysregulation of neural genes contributes to neurodevelopmental failure in terfa-compromised fishes**

To explore the neurodevelopmental functions of zfTRF2, we examined the transcriptomic changes induced by TERFA deficiency. First, we applied reverse transcription-quantitative PCR (RT-qPCR) to quantify the mRNA levels of genes previously shown to be regulated by zfTRF2 or its human ortholog (Wagner et al. 2017, Biroccio et al. 2013, Ovando-Roche et al. 2014, Robin et al. 2020). The terfa-compromised embryos exhibited decreased expression of the following genes: PPP2R2C, which encodes a neural-specific regulatory subunit of protein phosphatase 2; SNAP25, which encodes a key protein of vesicular transport in neurons; and EHD4, which encodes an endosomal trafficking factor (Extended Data Fig. 3d). These results correspond to those of a previous study, in which TERFA was downregulated in zebrafish embryos using morpholino (Wagner et al. 2017). Similar to results in human and mouse cells, zfTRF2 loss led to decreased expression of the mitochondrial sirtuin SIRT3 gene (Robin et al. 2020).

To investigate whether the reduced expression of the above genes contributed to neurodevelopmental failure in terfa-compromised fishes, we injected PPP2R2C, SNAP25, EHD4, and SIRT3 mRNA into terfa-compromised zygotes. The restoration of PPP2R2C expression decreased the rate of brain edema in both homozygous and heterozygous fishes, whereas SNAP25 and SIRT3 restoration only partially rescued heterozygous and homozygous fishes from brain edema (Fig. 3f, Extended Data Fig. 3e). The combined restoration of PPP2R2C and SNAP25 expression had an additive effect on brain edema reduction in heterozygous fishes (Fig. 3f), thus indicating that genes regulated by zfTRF2 contribute individually to brain development. In *atm*<sup>-/-</sup>

and p53<sup>-/-</sup> terfa-compromised fishes, the restoration of PPP2R2C and SNAP25 further decreased the incidence of brain edema (Fig. 3f). Overall, these results showed that the loss of TERFA affected neurodevelopment in a DDR-independent manner through the downregulation of neural gene transcription.

## **6. Neurodevelopmental failure in terfa-compromised fishes is mediated by glial dysfunction**

To identify which neural cells were responsible for neurodevelopmental failure in terfa-compromised fishes, we selectively restored the expression of zfTRF2 in three neural cell types in terfa-compromised fishes. This was achieved by injecting a construct containing the TERFA gene fused to mCherry by a P2A peptide linker. The construct was placed under the control of the GFAP, HuC, or Nestin promoter, which drives gene expression specifically in glial, neuron, or progenitor cells, respectively (Fig. 4a, Extended Data Fig. 4a and b). Recovery from neurodevelopmental failure, which was determined by a reduction in brain edema and an increase in neuron and glial cells, was only observed when zfTRF2 expression was driven by the GFAP promoter (Fig. 4b and c, Extended Data Fig. 4c). The restoration of zfTRF2 expression was sufficient to rescue DDR activation in glial cells (Fig. 4d) but not in HuC-positive or Nestin-positive neurons (Extended Data Fig. 4d). The gene downregulation observed in terfa-compromised embryos was rescued by restoring the expression of zfTRF2 specifically in glial GFAP-positive cells (Fig. 4e). These results show that zfTRF2 plays a protective role in neural cells during development, primarily in GFAP-positive glial cells.

## **Discussion**

This study has revealed that a key shelterin subunit, TRF2, plays a non-specific role in telomere protection in zebrafish and acts as a general caretaker against ATM-dependent DDR signaling. Zebrafish that partially or completely lack the terfa gene can initiate seemingly normal embryogenesis; however, a selective neurodevelopmental defect is triggered before the transition to the larval stage (at 3 dpf). This

neurodevelopmental failure is predominantly mediated by zfTRF2 in GFAP-positive glial cells; the detrimental phenotype was rescued when zfTRF2 expression was restored in GFAP-positive cells but not in HuC-positive or Nestin-positive cells. An explanation for this could be that zfTRF2 controls the fate of GFAP-positive radial glial cells, which are the progenitors for several types of neural cells during development (Götz and Barde 2005). Another important conclusion of our study is that haploinsufficiency of TRF2 triggers premature aging in adult fishes; this suggests that zfTRF2 is involved in both neurodevelopment and aging in a manner unrelated to its canonical protective function at telomeres.

The limited binding of zfTRF2 to telomeres in zebrafish cells, as well as the absence of telomere-specific uncapping during zfTRF2 inhibition in both cultured ZF4 cells and in terfa-compromised fishes, demonstrates that zfTRF2 is only marginally involved in telomere protection. Noteworthy, zfTRF2 has conserved its intrinsic ability to bind to mammalian telomeres, as shown in this work by its association with human telomeres. Whether this intrinsic telomere binding property of zfTRF2, revealed here in human cells, plays a specific role in zebrafishes is an interesting question for further studies.

In both mouse and zebrafish models, the complete loss of TRF2 was not lethal during early embryogenesis (Munoz et al. 2005, Kishi et al. 2008), suggesting that TRF2 carries out conserved functions during later developmental stages. Here, we identified a prominent role of zfTRF2 in neurodevelopment by blunting the DDR pathway and controlling the transcription of neural genes. The results showed that zfTRF2 appeared to exhibit distinct and independent functions, in part because ATM ablation added to the phenotype-rescuing effect resulting from the restoration of TRF2-dependent neural gene expression in terfa-compromised fishes. Our results support previous reports in which the extra-telomeric localization and function of TRF2 in human and mouse cells were characterized, and which highlighted direct and indirect roles of TRF2 in DNA repair, mitochondrial metabolism, and neuronal differentiation (Ye et al. 2014, Mao et al. 2007); these functions were shown to be unrelated to telomeric damage and TRF2 dosage-dependent in human cells (Biroccio et al. 2013, Cherfils-Vicini et al. 2019, Mukherjee et al. 2019). Our work suggests that the TRF2 mechanisms in

zebrafish are similar, as zfTRF2 inhibition, even to a half dosage, triggered neurodevelopmental failure and premature aging in the absence of specific telomere uncapping.

The question still remains: how does zfTRF2 dosage regulate the transcription of neural genes? A possible mechanism could arise from the reported interaction of TRF2 with repressor element 1 silencing transcription factor (REST), a master repressor of neuronal gene networks (Zhang et al. 2008, Zhang et al. 2011, Ovando-Roche et al. 2014). The SNAP25 gene is a known target of REST and TRF2 (Zhang et al. 2011), thus supporting this hypothesis. However, overexpressing REST mRNA in terfa-compromised fishes did not rescue the neurodevelopmental phenotype (Extended Data Fig. 3e). Alternatively, zfTRF2 could mediate long-range chromatin interactions required for effective gene expression, similar to the regulation of the sub-telomeric SIRT3 gene in human muscle cells (Robin et al. 2020). Another question raised by our studies is how zebrafish telomeres are specifically protected. Our provide evidence for a conserved role for POT1 in telomere protection, but other proteins may also be involved, as suggested by proteomic studies of telomeric DNA-binding proteins in zebrafish nuclear extracts.

TRF1 and TRF2 originated from a common TRF ancestor and duplicated during genomic replication at the root of the chordate lineage circa 500 million years ago (Poulet et al. 2012). TRF proteins with demonstrated telomere function are quite widespread among eukaryotes such as Taz1 in the fission yeast or tbTRF in *Trypanosoma brucei*. In the mammalian lineage, TRF2 exerts both telomeric and extra-telomeric functions. Overall, we conclude that TRF2 lost its telomeric specific function in the fish lineage, while preserving other extra-telomeric functions that we demonstrate here to be involved in neurodevelopment. The preservation of the genomic caretaker role of zfTRF2 despite its non-telomeric evolutionary trajectory supports the model in which telomeres evolved from general components involved in genome maintenance having acquired telomere specific protective functions during their evolution. Our results further suggest that some telomere factors can subsequently lose their telomere specific protective functions while conserving their

non-telomeric properties. Such a toggle evolution could explain the enigmatically variety of telomere protective factors found throughout the eukaryotic domain, despite the fact that telomere protection is an essential and conserved process.

Our results revealed that the zebrafish is an invaluable model for understanding the evolution and multifunctionality of shelterin subunits, in particular, their extra-telomeric roles during development and aging. Research in this area could aid the development of drugs with non-telomeric targets, such as TRF2, to fight against age-related diseases, thus avoiding the potential pro-oncogenic effects of reinforced telomere protection.

## Figure legends

Fig. 1 | Zebrafish TRF2 does not specifically bind to telomeres and acts as a general protecting factor against the DNA damage response. a, Immunofluorescence and Western blotting of ZF4 fish cells following TERFA downregulation by siRNA. The antibodies used were specifically raised against zfTRF2. Scale bars, 20  $\mu\text{m}$ . b, Representative images and signal quantification of telomeres, pericentromeres and centromeres colocalizing with zfTRF2 in ZF4 fish cells. c, Representative images and quantification of human telomeres colocalizing with zfTRF2 in zfTRF2-expressing human 293T cells. Scale bars, 7  $\mu\text{m}$ . d, Representative images and signal quantification of  $\gamma\text{H2AX}$  foci (global and telomere-associated) in ZF4 fish cells upon shelterin subunit downregulation. Scale bars, 7  $\mu\text{m}$ . Data are shown as the mean  $\pm$  standard error of the mean (SEM) of three biological replicates. Statistical analyses were performed using unpaired two-sided t tests (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

Fig. 2 | *terfa*-compromised zebrafishes exhibit neurodevelopmental failure. a, Western blot analysis of zfTRF2 expression in *terfa*-compromised embryos at 7 dpf (left) and fish brains at 6 months post-fertilization (mpf) (right). b, Survival curves of *terfa*-compromised larvae (*terfa*<sup>+/-</sup> and *terfa*<sup>-/-</sup>) from generations F3 to F6. c, Percentages of *terfa*-compromised embryos with indicated abnormal phenotypes during

development (0–7 dpf). The dotted line indicates the dates at which the brain edema phenotype first presented and peaked. d, Representative images of the indicated abnormal phenotypes in *terfa*-compromised embryos from 2–4 dpf. Scale bar, 0.6 mm. Quantification of the abnormalities is shown in (c) with enlarged side views of the abnormal phenotypes (right) (brain edema at 3 dpf; heart edema at 4 dpf; intestinal dystrophy at 4 dpf). e, Representative images (left) and signal quantification (right) from RNA fluorescence in situ hybridization (RNA-FISH) using tissue specific markers (neural tissue, *neurog1*; cardiovascular tissue, *cmcl2*; hemopoietic tissue, *c-myb*). Quantification was performed using ImageJ software (NIH). *Neurog1* and *cmcl2* signals were quantified by analyzing positive signals in the same area ( $10^{-2}$  mm<sup>2</sup>), and *c-myb* was quantified by counting foci (scale bar, 250  $\mu$ m). f, Representative images (left) of SA- $\beta$ -gal staining in *terfa*-compromised brains at 3 dpf and signal quantification (right). Scale bar, 250  $\mu$ m. g, Representative images (left) of acridine orange staining in *terfa*<sup>+/+</sup>, *terfa*<sup>+/-</sup>, and *terfa*<sup>-/-</sup> embryos at 3 dpf. The number of acridine foci in the whole brain was calculated (scale bar, 0.6 mm). h, Representative images (left) showing immunohistochemical staining of  $\gamma$ H2AX in brains (top) and eyes (bottom) of *terfa*-compromised embryos at 3 dpf. The percentage of neural cells with  $\gamma$ H2AX signals in the brain (right) was calculated (scale bars, 30  $\mu$ m). i, Colocalization of  $\gamma$ H2AX with the indicated neural markers in the brain of *terfa*-compromised embryos at 3 dpf. Left: representative confocal section images showing immunofluorescent signals of  $\gamma$ H2AX (red) colocalizing with GFP-positive cells (green); GFP marks glial cells in Tg(GFAP:GFP) brains. Right: the percentages of the indicated neural cells that colocalized with  $\gamma$ H2AX foci or TUNEL signals in *terfa*<sup>+/-</sup> or *terfa*<sup>-/-</sup> embryos at 3 dpf. Neurons and glial cells were marked with GFP in Tg(HuC:GFP) and Tg(GFAP:GFP) embryos, respectively. Progenitor cells were labeled with anti-SOX2 antibody via immunofluorescence staining (scale bars, 20  $\mu$ m). Data are shown as the mean  $\pm$  SEM of three biological replicates. The number inside or below each column indicates the number of embryos detected for each condition. Statistical analyses were performed using unpaired two-sided t tests, and survival curves were plotted using a log-rank test (\*P < 0.05, \*\*P < 0.01, \*\*\* P < 0.001).

Fig. 3 | Transcriptional role of zfTRF2 during neurodevelopment. a, Left: representative images of  $\gamma$ H2AX foci (red) in the brains of terfa-compromised p53<sup>-/-</sup> or atm<sup>-/-</sup> embryos at 3 dpf (scale bars, 30  $\mu$ m). Right: percentages of neural cells with  $\gamma$ H2AX foci in brains. b, Percentages of neural cells with TUNEL signals in the brains of terfa-compromised p53<sup>-/-</sup> or atm<sup>-/-</sup> embryos at 3 dpf. c, Number of SA- $\beta$ -gal foci in the brains of terfa-compromised p53<sup>-/-</sup> or atm<sup>-/-</sup> embryos at 3 dpf. d, Survival curve of terfa-compromised p53<sup>-/-</sup> or atm<sup>-/-</sup> embryos during development. e, Percentages of embryos with brain edema phenotypes in terfa-compromised p53<sup>-/-</sup> or atm<sup>-/-</sup> embryos at 3 dpf. f, Percentages of embryos at 3 dpf with brain edema phenotypes after microinjection with the indicated mRNA (ppp2r2c, snap25 or ppp2r2c+snap25) in terfa<sup>+/-</sup>:p53<sup>-/-</sup> or terfa<sup>+/-</sup>:atm<sup>-/-</sup> (left) and terfa<sup>-/-</sup>:p53<sup>-/-</sup> or terfa<sup>-/-</sup>:atm<sup>-/-</sup> (right) embryos. All data are shown as the means  $\pm$  SEM of three biological replicates. Statistical analyses were performed using unpaired two-sided t tests; survival curves were plotted using the log-rank test (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001). The number below the graph indicates the total number of tested individual embryos. Asterisks directly above columns indicate a significant difference between the indicated treatment and the control group (left). Asterisks above two columns indicate a significant difference between the two columns.

Fig. 4 | Glial functions of zfTRF2 during neurodevelopment. a, Schematic diagram showing plasmid construction for zfTRF2- and mCherry-overexpression in specific neural cells. b, Percentages of embryos exhibiting brain edema phenotypes at 3 dpf in terfa-compromised embryos following microinjection with the plasmids described in (a). c, Top: representative three-dimensional (3-D) computationally-reconstructed brain images of living embryos. Images were taken via light-sheet microscopy. Green signals indicate neurons of terfa-compromised Tg(HuC:GFP) embryos at 3 dpf after microinjection with the plasmids described in (a). Bottom: GFP-positive cells in whole brains were quantified using Imaris software (Bitplane). d, Left: representative confocal section images showing immunofluorescent detection of  $\gamma$ H2AX foci, and

their colocalization with GFP-positive or mCherry-positive cells in the brains of *terfa*-compromised Tg(GFAP:GFP) embryos at 3 dpf. Embryos were microinjected with the plasmids described in (a). Right: percentages of GFP-positive cells that colocalized with  $\gamma$ H2AX foci. e, Transcript levels of *ppp2r2c*, *snap25*, *ehd4* and *sirt3* genes in *terfa*<sup>+/+</sup>, *terfa*<sup>+/-</sup>, and *terfa*<sup>-/-</sup> embryos at 3 dpf. Embryos were microinjected with the plasmids described in (a). Transcript levels were measured using RT-qPCR. All data are shown as the mean  $\pm$  SEM of three biological replicates. The number below each column indicates the number of embryos detected for each condition. Statistical analysis was performed using the unpaired two-sided t test, and survival curves were plotted using the log-rank test (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001). Asterisks directly above columns indicate a significant difference between the indicated treatment and the control group (left). Asterisks above two columns indicate a significant difference between the two columns.

**Extended Data Fig. 1 | Zebrafish TRF2 does not specifically bind to telomeres and acts as a general protective factor against the DDR.** a, Immunoblots demonstrating the affinity of the purified anti-zfTRF2 antibodies for zfTRF2. The immunoblot assays were performed with antibodies against proteins extracted from human 293T cells overexpressing exogenous zfTRF2 and zfTRF1. Human 293T cells were subjected to lentiviral transduction with pWPIR vectors containing *terfa*-Myc and *terf1*-Myc. b, Representative confocal section images (left) and signal quantification (right) from a PNA-FISH assay of human 293T cells. The percentages of telomeres (red) that colocalized with Myc (magenta) in transduced 293T cells overexpressing exogenous zfTRF2-Myc are shown. Human TRF2 (hTRF2) and hTRF1 were knocked down by siRNA for 72 h (scale bars, 7  $\mu$ m). c, Representative confocal section images (left) and signal quantification (right) from PNA-FISH. The percentages of SATIII (green) colocalizing with zfTRF2 (top) or Myc (bottom) (magenta) in the 293T cells described in (b) are shown (scale bars, 7  $\mu$ m). d, left: the efficiencies of shelterin gene siRNA knockdowns in ZF4 cells were calculated using RT-qPCR. Right: representative confocal section images of a PNA-FISH assay performed using telomeric PNA probes in ZF4 cells

subjected to shelterin knockdown by siRNA. Telomere-induced dysfunction foci (TIFs) and their colocalization with  $\gamma$ H2AX (green) are shown (scale bars, 7  $\mu$ m). Quantitative results are shown in Fig. 1d. e, Representative confocal section images (top) and signal quantification (bottom) from a PNA-FISH assay performed using pericentromere-specific PNA probes in ZF4 cells subjected to shelterin knockdown by siRNA. Pericentromere-induced dysfunction foci (PIFs) are highlighted, and the colocalization of  $\gamma$ H2AX (green) and pericentromeres is shown (scale bars, 7  $\mu$ m). f, Representative confocal section images (top) and signal quantification (bottom) from a PNA-FISH assay using centromere-specific PNA probes to detect centromere-induced dysfunction foci (CIFs) and the colocalization of  $\gamma$ H2AX (green) with centromeres in ZF4 cells. ZF4 cells were subjected to shelterin knockdown by siRNA (scale bars, 7  $\mu$ m). For all PNA-FISH experiments, approximately 30 nuclei were analyzed per replicate. All data are shown as the mean  $\pm$  SEM of three biological replicates. Statistical analyses were performed using unpaired two-sided t tests (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001). Asterisks directly above columns indicate a significant difference between the indicated treatment and the control group (left). Asterisks above two columns indicate a significant difference between the two columns.

**Extended Data Fig. 2 | terfa-compromised zebrafishes exhibit neurodevelopmental failure.** a, Schematic diagram showing the deletion of the zebrafish *terfa* gene using the CRISPR-Cas9 system. The diagram shows the sgRNA design and construction of *terfa*-mutated zebrafishes. Yellow and red boxes indicate the targeted exon and TRFH domain, respectively. The red dotted line indicates the nucleotide deletion in *terfa*. b, Schematic diagram (top) showing primer design for the amplification of *terfa* mRNA. Primer-1 was designed to target the mutation site, and primer-2 and primer-3 were designed to target the regions before and after the mutation site, respectively. The *terfa* mRNA in *terfa*<sup>+/+</sup>, *terfa*<sup>+/-</sup>, and *terfa*<sup>-/-</sup> embryos at 3 dpf and adult brains at 6 mpf was quantified (bottom) using three primer pairs. c, Survival curves of generations F3 to F6 of *terfa*<sup>+/+</sup> and *terfa*<sup>+/-</sup> zebrafish strains. d, Representative photographs of adult

terfa+/+ and terfa+/- fishes at 6 mpf. Left, male; right, female. e, Representative computed tomography (CT) images (left) and quantification (right) of zebrafish at 6 mpf. The images show 3-D reconstructions of whole fish bodies developed using the CT images. The middle column show extracted two-dimensional (2-D) images of the sagittal and coronal sections of the brain collected via CT. The graph (right) shows brain capacity following 3-D reconstruction of the brain via CT. f, Representative ultrasonic cardiogram (UCG) images (left) and measurements (right) of terfa+/+ and terfa+/- zebrafish at 6 mpf. Volume indices from the UCGs are shown, including the end systolic volume index (ESVi), end diastolic volume index (EDVi), and systolic volume index (SVi). g, Percentages of terfa+/+, terfa+/- and terfa-/- embryos with brain edema phenotypes at 3 dpf. Embryos were microinjected with terfa mRNA. h, Representative images (right) and quantitative results (left) from an immunohistochemical assay showing the percentages of terfa+/+, terfa+/-, and terfa-/- embryos exhibiting PCNA signals in the brain (top) and eye (bottom) at 3 dpf (scale bars, 30  $\mu$ m). i, The neural cell types (neurons, glial cells, and progenitor cells) present in terfa-compromised embryo brains at 3 dpf were quantified using flow cytometry. Left: the percentage of SOX2-positive (progenitor) cells was calculated by labeling embryos with anti-SOX2 antibody. Middle: percentage of GFP-positive cells (neurons) in terfa-compromised Tg(HuC:GFP) embryos. Right: percentage of GFP-positive (glial) cells in terfa-compromised Tg(GFAP:GFP) embryos. j, Representative 3-D computational reconstructions (left) from brain images of living embryos taken using light-sheet microscopy. Green signals indicate neurons at 3 dpf in terfa-compromised Tg(Huc:GFP) embryos. Quantification (right) of GFP-positive cells in whole brains of terfa+/+, terfa+/-, and terfa-/- embryos. The terms terfa+/-Low or terfa+/-High denote embryos exhibiting weak or strong GFP signals, respectively (scale bars, 150  $\mu$ m). k, Representative confocal section images showing TUNEL signals (left) and  $\gamma$ H2AX foci (right) colocalized with GFP-positive cells (green) in terfa+/- or terfa-/- embryos at 3 dpf. GFP marked neurons in Tg(HuC:GFP) brains and glial cells in Tg(GFAP:GFP) brains. SOX2-positive cells represent progenitor cells (middle). Scale bars, 20  $\mu$ m. Quantitative results are shown in Fig. 2i. l, Representative delta-vision microscope images and

quantitative results from PNA-FISH assays showing the colocalization of telomeric PNA probes and  $\gamma$ H2AX foci, and their respective percentages (scale bars, 3  $\mu$ m). m, Representative images and quantification of metaphase spreads visualized using PNA-FISH. Spreads were acquired using fluorescence microscopy of embryos stained with a telomeric PNA probe (TTAGGG, green) at 1 dpf. Strains were generated by intercrossing *terfa*<sup>+/-</sup> fish. The number at each symbol indicates the number of chromosomes analyzed for each condition. Solid lines highlight chromosome abnormalities, including multiple telomere signals (MTS), telomere loss (TL), and telomere fusion (TF). Scale bars, 10  $\mu$ m. For all FISH experiments, approximately 30 nuclei were analyzed per replicate. All data are shown as the mean  $\pm$  SEM of three biological replicates. Statistical analyses were performed using unpaired two-sided t tests, and survival curves were plotted using the log-rank test (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001). Asterisks directly above columns indicate a significant difference between the indicated treatment and the control group (left). Asterisks above two columns indicate a significant difference between the two columns.

Extended Data Fig. 3 | Transcriptional role of zfTRF2 during neurodevelopment. a, Representative confocal section images showing TUNEL signals (red) in the brains of *terfa*-compromised *p53*<sup>-/-</sup> or *atm*<sup>-/-</sup> embryos at 3 dpf (scale bars, 30  $\mu$ m). Quantitative results are shown in Fig. 3b. b, Representative light microscopy images showing SA- $\beta$ -gal staining in brains of *terfa*-compromised *p53*<sup>-/-</sup> or *atm*<sup>-/-</sup> embryos at 3 dpf (scale bars, 250  $\mu$ m). Quantitative results are shown in Fig. 3c. c, Representative 3-D computationally reconstructed light-sheet microscope images of the brains of living embryos (left) and results of quantitative analysis (right). Neurons of *terfa*-compromised *Tg(HuC:GFP);atm*<sup>-/-</sup> embryos at 3 dpf are indicated in green. The number of GFP-positive cells in whole brains was calculated using Imaris imaging processing software (Bitplane). Scale bars, 150  $\mu$ m. d, Transcript levels of indicated genes in *terfa*-compromised embryos (*terfa*<sup>+/+</sup>, *terfa*<sup>+/-</sup>, and *terfa*<sup>-/-</sup>) at 3 dpf as measured by RT-qPCR. e, The percentages of *terfa*-compromised embryos exhibiting brain edema phenotypes at 3 dpf. Embryos were microinjected with the indicated

mRNAs. f, mRNA levels of indicated genes in *terfa*-compromised embryos microinjected with the indicated mRNAs at 3 dpf, as measured by RT-qPCR. The relative mRNA levels of each gene were calculated based on the level in the corresponding *terfa*<sup>+/+</sup> control group. All data are shown as the mean  $\pm$  SEM of three biological replicates. Statistical analyses were performed using unpaired two-sided t tests, and survival curves were plotted using the log-rank test (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001). The number in each column indicates the number of embryos assayed for each condition.

Extended Data Fig. 4 | The function of zfTRF2 in glial cells during neurodevelopment. a, Representative confocal section images showing the cellular-specific expression of exogenous zfTRF2 (magenta) foci or mCherry signals (see the scheme of vector construction in Fig. 4); zfTRF2 expression was indicated by GFP in Tg(HuC:GFP)/Tg(GFAP:GFP) embryos or by the progenitor cell-specific marker SOX2 (green) after microinjection with vectors for *terfa* expression driven by neural-specific promoters. b, Results of quantitative analysis for (a). The percentages of zfTRF2 foci or mCherry signals that colocalized with GFP in Tg(HuC:GFP)/Tg(GFAP:GFP) embryos or the progenitor cell-specific marker SOX2 (green) after microinjection with the corresponding plasmids shown in Fig. 4a. c, The percentages of GFP-positive cells (glial cells) in *terfa*-compromised Tg(GFAP:GFP) embryo brains expressing zfTRF2 with the GFAP promoter at 3 dpf, as measured by flow cytometry. d, Top: representative confocal section images showing  $\gamma$ H2AX foci colocalized with GFP-positive or mCherry-positive cells in the brains of *terfa*<sup>+/+</sup> embryos at 3 dpf, following microinjection of the plasmids described in Fig. 4a. The *terfa*-compromised Tg(GFAP:GFP) embryos and percentages of GFP-positive cells that colocalized with  $\gamma$ H2AX foci are shown in Fig. 4d. Middle: *terfa*-compromised Tg(HuC:GFP) embryos after plasmid microinjection; the percentages of GFP-positive cells colocalizing with  $\gamma$ H2AX foci are shown. Bottom: results of the same experiments with *terfa*-compromised embryos after microinjection with plasmids that elicit TRF2 expression driven by the Nestin promoter; progenitor cells were marked by anti-SOX2 antibodies. The percentages of SOX2 foci that

colocalized with  $\gamma$ H2AX foci are shown. All data are shown as the mean  $\pm$  SEM of three biological replicates. Statistical analyses were performed using unpaired two-sided t tests, and survival curves were plotted using the log-rank test (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001). Asterisks directly above columns indicate a significant difference between the indicated treatment and the control group (left). Asterisks above two columns indicate a significant difference between the two columns.

## Materials and methods

**Zebrafish maintenance.** The experiments were performed using the zebrafish Tübingen strain. Zebrafishes were maintained under standard conditions as described previously (Kimmel et al. 1995). All experiments were performed according to the guidelines of the Animal Care and Use Committee of Shanghai Jiao Tong University. The buffer for embryo maintenance was freshly prepared in phosphate buffered saline (PBS). The zebrafishes were fed once per day, and the tank water was replaced once per week.

**Preparation of antibodies against the ZfTRF2 protein.** The cDNA of the TERFA gene of zebrafish was synthesized following codon optimization for Escherichia coli expression, then cloned into a modified version of the pTrcHisB vector. The modified zfTRF2 contained a fused C-terminal His-tag for downstream purification. This plasmid was transformed into competent E. coli BL21 cells grown in lysogeny broth medium containing 100  $\mu$ g/mL ampicillin at 37°C. When the cells reached an optical density (OD) of 0.7 at 600 nm, 0.1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside was added, and the culture was incubated at 25°C for 16 h. Cells were harvested by centrifugation at 4,500  $\times$  g at 4°C for 15 min, and the pellets were used for protein extraction. The C-terminally His-tagged zfTRF2 was purified using nickel affinity chromatography (His-Trap HP; GE Healthcare); 900  $\mu$ g of zfTRF2 protein was sent for antibody production (Covalab).

Six control serum samples were collected from six different rabbits and tested for pre-immune responses using a 67-day protocol. Serum samples were tested by Western blotting against recombinant zfTRF2 protein; three rabbits were selected for protein production. Three inoculations were performed intradermally with 0.5 mL of antigen and 0.5 mL of Freund's complete adjuvant on days 1, 14, and 28, respectively. The final immunization was performed subcutaneously with 0.5 mL of antigen and 0.5 mL of Freund's incomplete adjuvant on day 42. On day 67, final serum samples were collected and supplemented with sodium azide to a final concentration of 0.02%, then aliquoted and stored at  $-80^{\circ}\text{C}$ . The antibodies used in this study were affinity purified from serum using purified zfTRF2 protein.

**Cell culture and treatment.** Human 293T cells were grown in GlutaMAX-containing Dulbecco's modified Eagle medium (DMEM) (#11995-065; Gibco) supplemented with 10% fetal bovine serum (FBS) (#S11550-500; Biosera) at  $37^{\circ}\text{C}$  under 5%  $\text{CO}_2$ . ZF4 cells were isolated from a line of zebrafish embryos and grown in GlutaMAX-containing DMEM/F12 (1:1) (#11330-032; Gibco) supplemented with 10% FBS at  $30^{\circ}\text{C}$  under 5%  $\text{CO}_2$ .

**Plasmid construction.** The *terfa* and *terf1* sequences were synthesized (Genescript) according to their mRNA sequences (NCBI) with an N-terminal Myc-tag. The neural promoter sequence was synthesized according to previously published sequences (Andrae et al. 2001, Park et al. 2000, Kaslin et al. 2007) and fused to the mRNA sequences of *terfa* and mCherry. The mRNAs of *terfa* and mCherry were joined by the P2A oligopeptide. The neural promoter plasmid pTol2-mCherry was kindly donated by the Gang Peng laboratory and used as the vector backbone.

**Plasmid transductions and microinjection.** Human 293T cells were transfected with 4.3  $\mu\text{g}$  pCMV-dR8.91, 1.4  $\mu\text{g}$  pCMV-VSVG, and 4.3  $\mu\text{g}$  pWPIR plasmids using the ProFection<sup>®</sup> Mammalian Transfection System (Promega E1200). Supernatants were collected after 48 h and 72 h and filtered for cell transduction. The transduction

efficiency was determined for pWPIR vectors using qPCR and Western blot analyses (see Fig. 1) at 3 days after infection. Plasmid microinjections were performed using 1-cell-stage embryos, with 50 pg plasmid injected per embryo, together with the Tol2 transduction enzyme mRNA (100 pg per embryo) which was transcribed in vitro using an SP6 mRNA transcriptional kit (Thermo). Each step was verified by agarose gel electrophoresis. The efficiency of Tol2 transduction was measured by observing mCherry-positive embryos every 24 h after injection. Samples were collected at 3 dpf for downstream experiments.

**RT-qPCR.** Total RNA from cell lines (ZF4, 293T) or zebrafish (embryos and tissues) was extracted using TRIzol reagent (Ambion) and reverse transcribed using a PrimeScript RT reagent kit (Takara) according to the manufacturers' protocols. RT-qPCR analysis was performed using a QuantStudio Dx Real-Time PCR Instrument (ABI) with SuperRealPreMix Plus (TianGen). The primers used in this study are shown in Extended Data Table 3.

**Western blot.** Protein lysates of cells (ZF4, 293T) or zebrafish (embryo and tissues) were prepared using ice-cold radioimmunoprecipitation assay buffer buffer supplemented with dimethylformamide (Beyotime Biotechnology), phosphatase, and protease inhibitors (Roche). Protein concentrations were determined using a Pierce<sup>TM</sup> BCA Protein Assay kit (Thermo). Protein samples were loaded into SurePAGE 4-20% Bis-Tris gradient gels (Genscript). Samples were transferred onto Immobilon-P PVDF 0.45- $\mu$ m membranes (Millipore) using a Trans-Blot Turbo Transfer System (BioRad). Membranes were blocked in 5% non-fat powdered milk (Sangon) with 0.1% Tween-20 in tris buffered saline (TBST) for 1 h at room temperature. Hybridization with primary antibodies (listed in Extended Data Table 2) was performed at 4°C overnight, followed by washing with TBST three times. Hybridization with the corresponding secondary antibodies was performed at 37°C for 2 h, followed by washing with TBST three times. Images were taken using an AI600 gel imaging system (GE Healthcare) and processed using ImageJ software (NIH).

**Generation of terfa-knockout zebrafish.** We generated terfa-knockout zebrafish using the CRISPR-Cas9 system. The guide RNA (sgRNA), which targeted exon 2 of terfa (sgRNA: 5' -GTGTGGTGGTCAGGCCGG-3'), was designed using ZiFIT Targeter software (<http://zifit.partners.org/ZiFiT>). The sgRNA was generated by cloning annealed oligonucleotides into the sgRNA vector as described previously (Xiao et al. 2013). The founder embryos (F0 generation) were raised to 3 months of age and outcrossed with wild-type zebrafishes to obtain potential F1 indel mutations. PCR amplification and sequencing were performed using genomic DNA isolated from the tails of F1 zebrafish to identify terfa mutants (primers for genotyping: forward 5' -TTAACCCGCGTTATCTTCAG-3' ; reverse 5' -CGTCTCCGACATTCACTCAC-3' ).

**Immunofluorescence of embryo slices.** Embryo sections were fixed in 4% paraformaldehyde (PFA) for 30 min, then permeabilized and blocked with 1× PBS, 0.5% Triton X-100, and 2% FBS for 1 h at room temperature. Hybridization with primary antibodies was performed at 4°C overnight, followed by washing three times with 0.1% Tween-20 in PBS. Hybridization with the corresponding secondary antibodies (Invitrogen) was performed at 37°C for at least 2 h. Brain slices were incubated with 1× 4',6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Imaging was performed using a confocal laser scanning microscope (SP8; Leica). The primary antibodies used in this study are listed in Extended Data Table 4.

**Immunohistochemical analysis of embryo slices.** Embryo sections were fixed in 4% PFA for 30 min, and samples were stained using an immunohistochemical kit (Cell Signaling Technology) following the manufacturer's instructions. Signals were observed at 5-min intervals. Slices were incubated with hematoxylin for 3 min for nuclear staining.

**PNA-FISH.** Cells were grown on glass coverslips and fixed for 15 min with 3.7% formaldehyde. Cells were then permeabilized with 0.5% Triton X-100 for 8 min and

dehydrated in increasing concentrations of ethanol (50%, 75%, and 100%) for 5 min each. Samples were denatured in 70% formamide, 10 mM Tris (pH 7.2), and 1% blocking solution (Roche) at 85°C for 3 min. The denatured samples were then incubated with PNA probes for at least 2 h at room temperature. The cells were then washed with 70% formamide, 10 mM Tris (pH 7.2) solution for 30 min, and subsequently washed with 150 mM NaCl and 50 mM Tris (pH 7.5) for 15 min. Next, the cells were incubated with blocking buffer (1% Triton X-100, 1% bovine serum albumin [BSA], and 5% serum) and incubated overnight at 4°C with the desired antibody. The cells were washed with 1× PBS and incubated for 2 h with the corresponding secondary antibody, then incubated for 10 min with DAPI. Finally, the cells were preserved in mounting solution (Vector Laboratories).

**Metaphase analysis.** Embryos were arrested at 10 h post-fertilization (hpf) after being treated with colchicine (final concentration, 0.4 mg/ml) for 8 h. Embryos were dissociated into single cells, then incubated with 1.1% sodium citrate for 15 min at 28.5°C, followed by methanol/glacial acetic acid (3:1) fixation. Samples were incubated with telomere-specific PNA probes in 70% formamide, 10 mM Tris (pH 7.2), and 1% blocking solution buffer (Roche) for 2 h. Images were obtained using a fluorescence microscope (Zeiss).

**Whole-mount in situ hybridization.** Full-length cDNA fragments of *terfa*, *neurog1*, and *c-myb* were cloned into the pCS2 vector. The EcoRI and XhoI enzymatic sites were incorporated into the ends of the cDNA sequences to facilitate directional cloning. Digoxigenin-labeled antisense mRNA probes were transcribed using T3 polymerase from an EcoRI linearized plasmid according to the manufacturer's instructions (Roche). The embryos were collected at 24, 48 and 72 hpf and fixed with 4% PFA overnight at 4°C. Samples were dehydrated and rehydrated using increasing concentrations of formaldehyde (30%, 70%, and 100%) for 10 min each. Samples were fixed again with 4% PFA for 30 min at 4°C. Embryos were then digested with proteinase K according to the hpf at which they were collected (24 hpf, 10 µg/ml for 5 min; 48 hpf, 20 µg/ml for

15 min; 72 hpf, 50  $\mu\text{g}/\text{ml}$  for 20 min). Embryos were fixed again with 4% PFA for 2 h at 4°C. Embryos were then pre-heated at 68°C in Hyb buffer (50% formamide, 5 $\times$  saline-sodium citrate [SSC], 0.1% Tween-20, 5 mg/ml yeast RNA, 50  $\mu\text{g}/\text{ml}$  heparin) for 30 min. At least 50 ng/ml of each probe was added to the embryos, which were incubated at 68°C overnight with gentle rocking. Embryos were then washed with pre-heated SSCT buffer (1 $\times$  SSC and 0.1% Tween-20) at 68°C for 15 min three times. Following this, embryos were washed with MABT buffer (100 mM maleic acid, 150 mM NaCl, and 0.1% Tween-20, pH 7.5) at room temperature for 5 min three times. Samples were blocked using blocking buffer (MABT, 2% BSA, and 1% FBS) for 1 h at room temperature, then anti-Dig antibody (1:5000) was added, and the samples were rocked gently at 4°C overnight. Embryos were stained using a BCIP/NBP kit following the manufacturer's instructions (Vector Laboratories). Embryos were observed every 10 min following staining.

The primers used for cDNA cloning are shown in Extended Data Table 3.

**Whole-mount light sheet fluorescence microscopy and image processing.** Transgenic zebrafish Tg(HuC:GFP) were crossed with *terfa*-compromised zebrafish to generate the *terfa*<sup>+/−</sup>Tg(HuC:GFP) and *terfa*<sup>−/−</sup>Tg(HuC:GFP) strains. Embryos were collected at 3 dpf and anesthetized with 1 $\times$  Tricaine (Sigma-Aldrich). Each embryo was transferred to 1.5% low melting point agarose with Tricaine and maintained in liquid form below 35°C. Each individual embryo was placed vertically in a transparent tube prior to light sheet scanning and incubated for 5 min at room temperature for the agarose to solidify. Images were taken using a Zeiss Z.1 system, and results were represented as 3-D reconstructions using Imaris (version 8.1) software. The Tg(HuC:GFP) zebrafish strain was kindly donated by the Du Jilin laboratory.

**Apoptosis assay.** Samples were fixed in 4% PFA for 20 min, then blocked in 3% BSA for 30 min. The apoptosis assay was performed using a TMR red in Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions. Samples were

subsequently incubated with DAPI for 10 min. Images were taken using a confocal laser scanning microscope (Leica SP8).

**Flow cytometry.** Embryos were collected for flow cytometry at 3 dpf. Anesthetization of the embryos was performed using 1× Tricaine (Sigma-Aldrich), after which they were incubated with 0.5% trypsin for 30 min at 30°C. The reaction was stopped by adding DMEM followed by gentle rocking for 30 min at room temperature until the embryos separated into individual cells. Embryos were washed with 1× PBS three times, then analyzed by flow cytometry. For stem cell detection, embryos were fixed in 0.1% formaldehyde for 10 min at room temperature, washed with ice-cold PBS for 10 min three times, then incubated in 0.5% Triton X-100 for 10 min. Embryos were incubated in blocking buffer comprising 1% BSA for 30 min on ice, then incubated with primary antibody plus anti-SOX2 antibody for 2 h in antibody buffer (3% BSA, 0.1% Tween-20, and 1:100 antibody). After washing with ice-cold PBS, embryos were fixed with 0.1% formaldehyde for 10 min at room temperature. Embryos were then incubated with secondary antibody, washed three times with 1× PBS and 1% BSA for 10 min, and subjected to flow cytometry analysis. A blank control (no antibodies) and a compensation control (no primary antibodies) were included in the analysis.

**Whole-mount acridine orange staining.** The embryos were collected at four time points (24, 48, 72, and 96 hpf) and anesthetized with 1× Tricaine (Sigma-Aldrich). Embryos were then incubated in 3 µg/ml acridine orange (Sigma-Aldrich) in the dark. Following this, embryos were washed for 10 min four times, then embedded in 1% low-melting-point agarose with 1× Tricaine. Images were taken using a Zeiss A2 microscope.

**SA-β-gal senescence detection assay.** Zebrafish slices were fixed in 4% PFA for 30 min and washed with 1× PBS for 10 min three times. Whole embryos were collected and fixed in 4% PFA at 4°C overnight. Embryos were dehydrated and rehydrated in increasing concentrations of formaldehyde (30%, 70%, and 100%) for 10 min per

concentration, then incubated with proteinase K (as described above; see methods for RNA-FISH). SA- $\beta$ -gal staining was performed using a Senescence  $\beta$ -galactosidase Staining Kit (Cell Signaling Technology) according to the manufacturer's instructions. Images were taken with a Zeiss A2 microscope.

**CT and ultrasonic cardiography.** Both CT and ultrasonic cardiography of zebrafishes at 6 mpf were performed using the X-med platform at Shanghai Jiaotong University. Adult fishes were collected at 6 mpf and anesthetized with 1 $\times$  Tricaine (Sigma-Aldrich) before scanning. Fishes were wrapped with a wet cloth, and each scan was performed for less than 8 min to ensure the survival of the fishes. CT was performed using an Inveon PET/CT scanner (Siemens), and ultrasonic cardiography was performed using a Vevo2100 imaging system (Fujifilm VisualSonics).

Figure 1

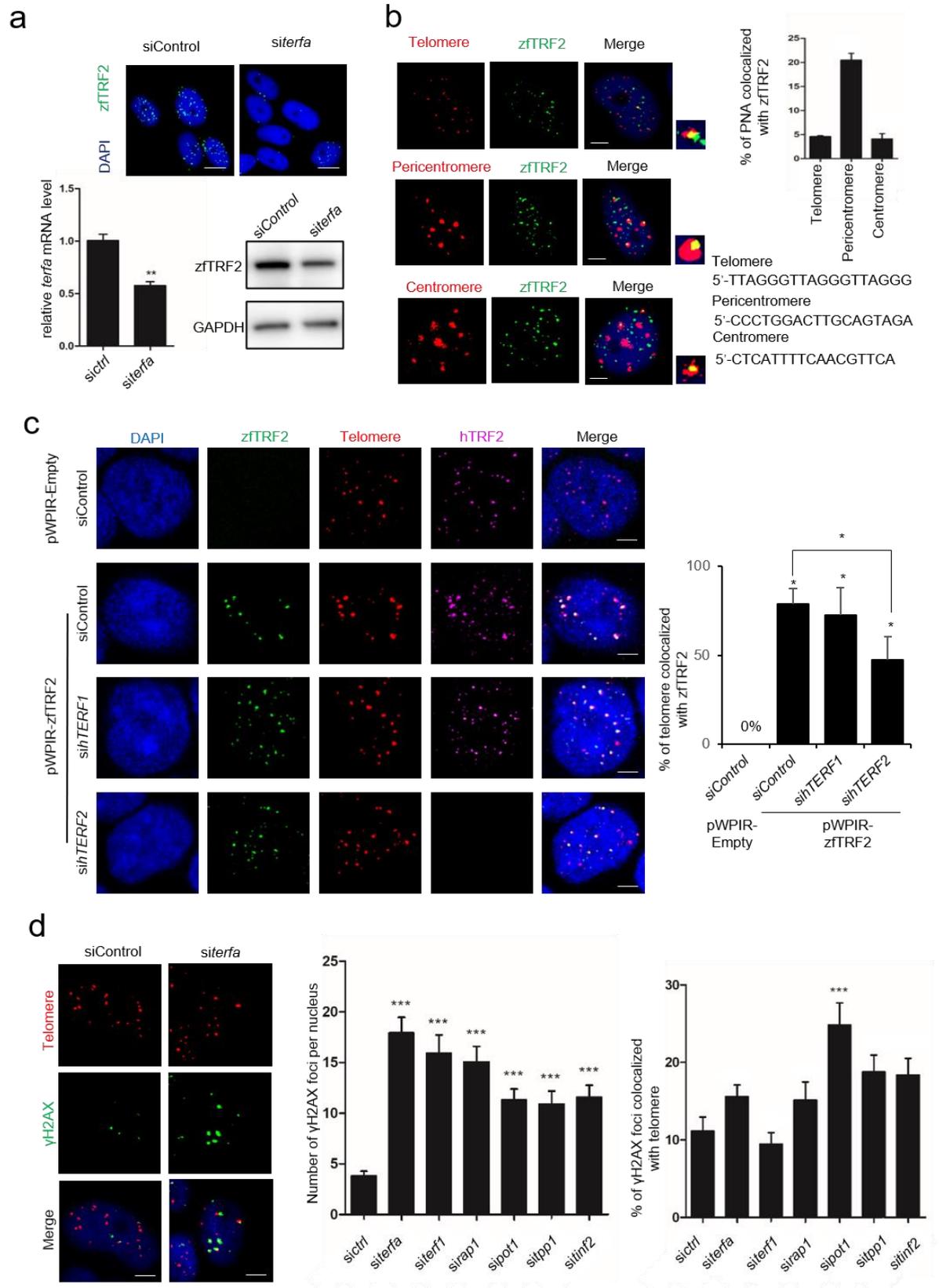
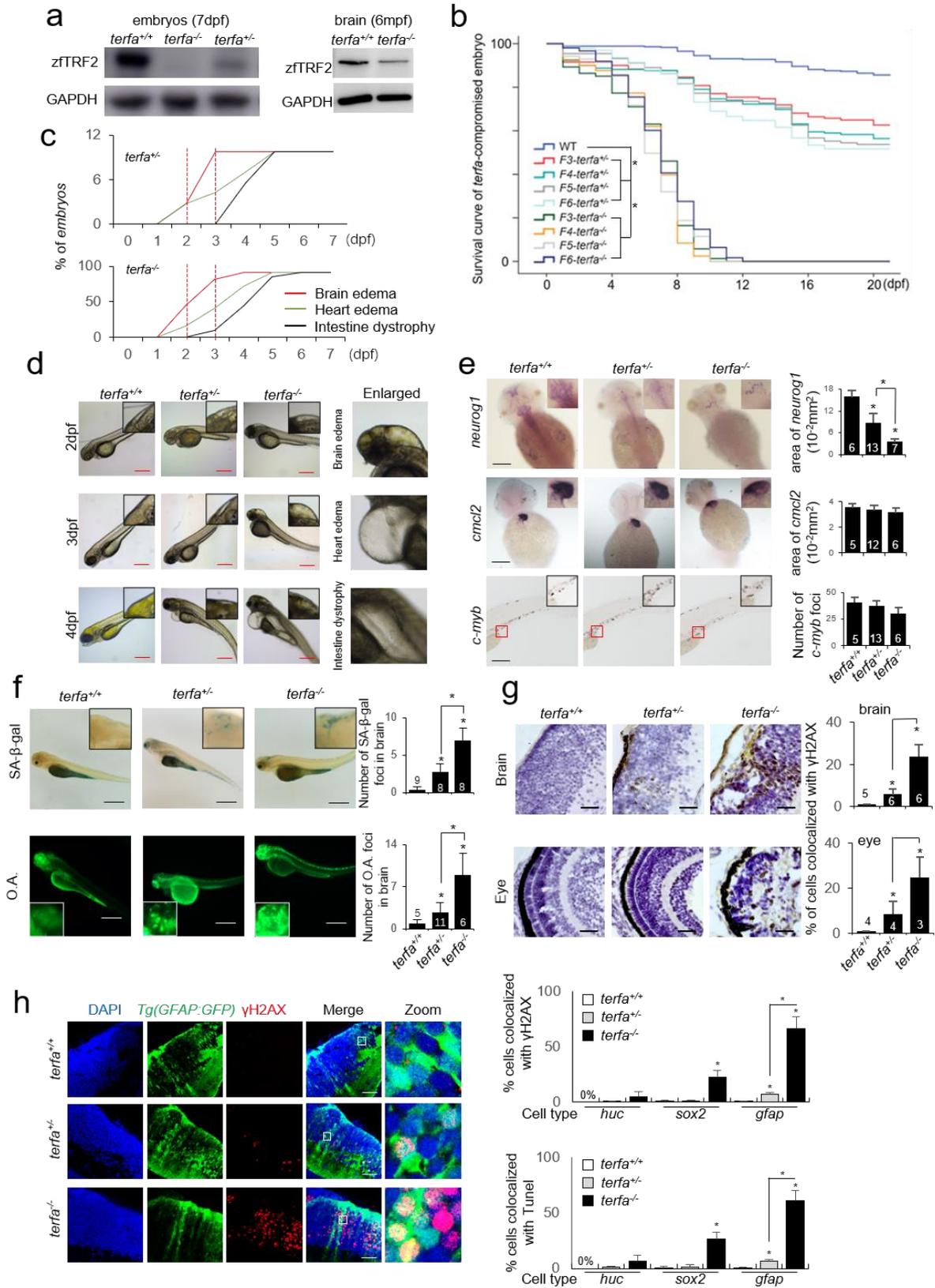


Figure 2



**Figure 3**

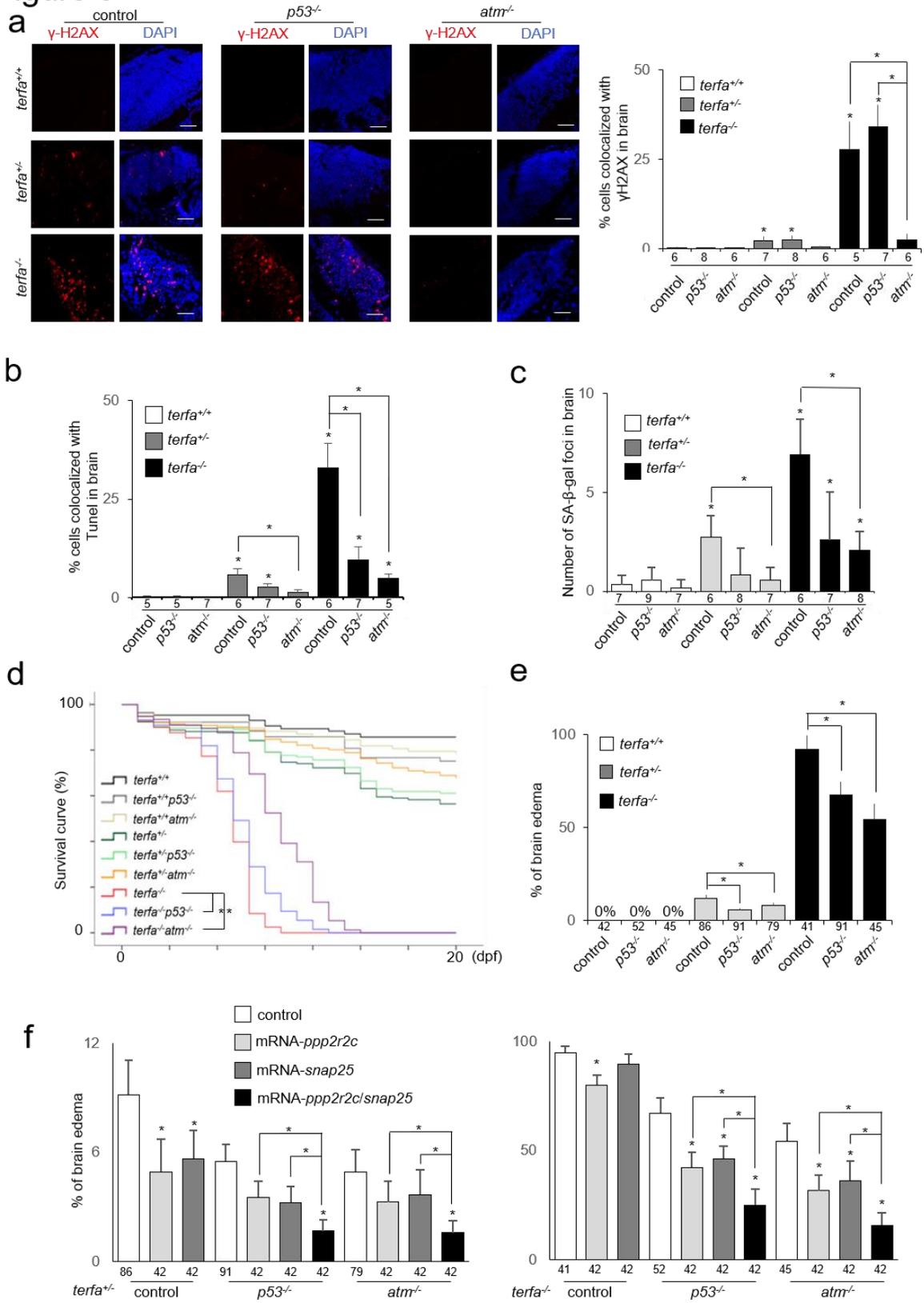
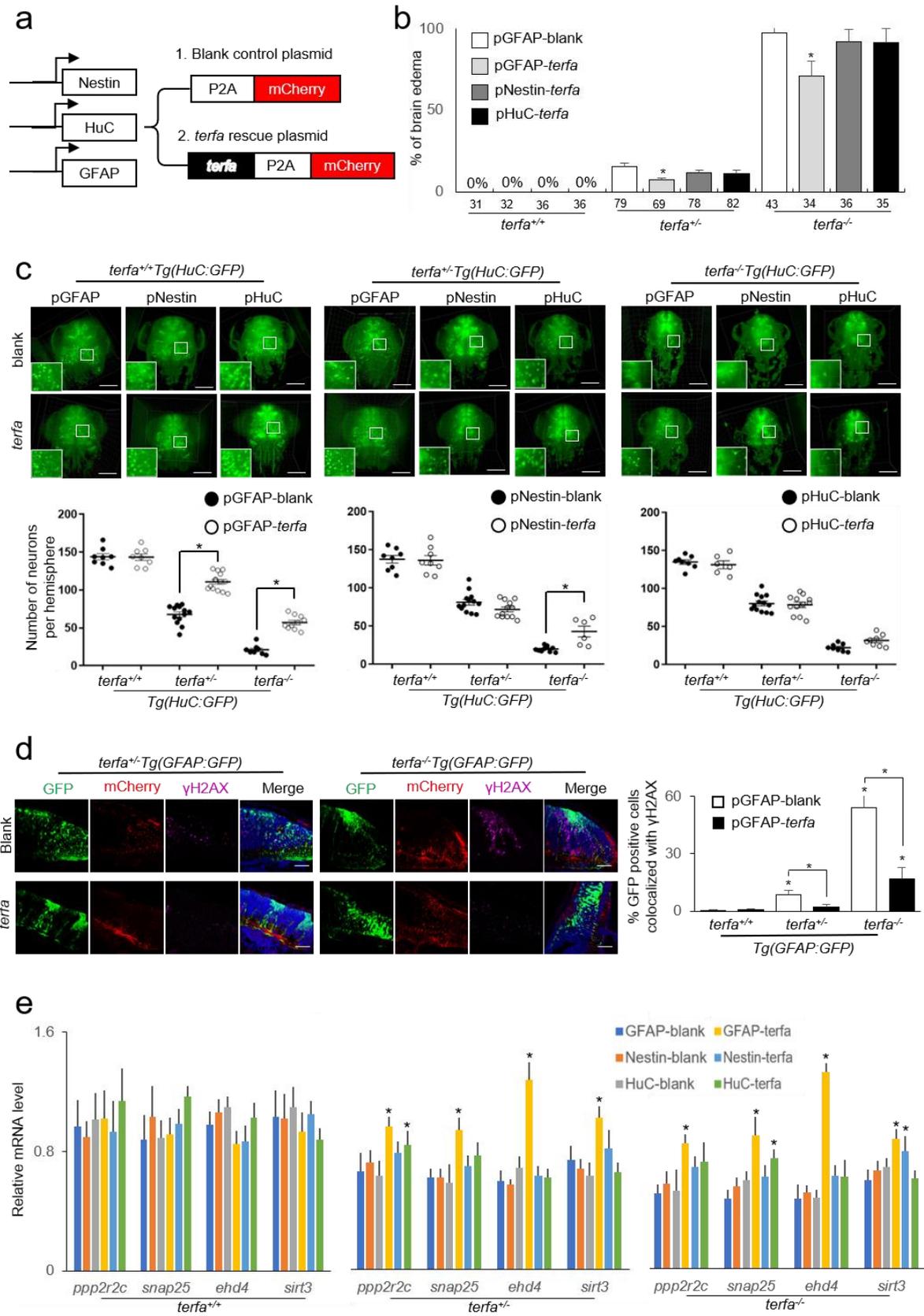
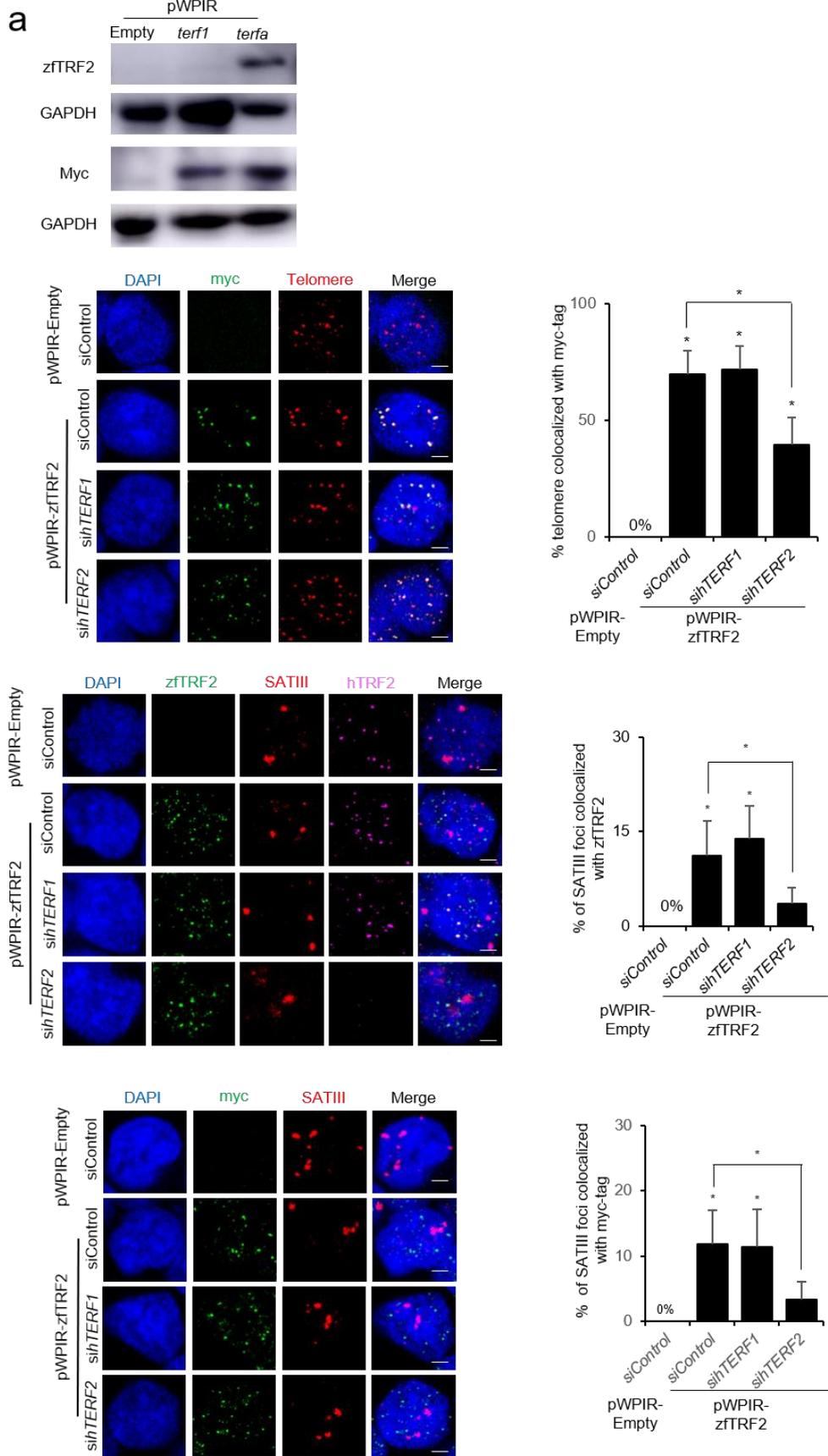
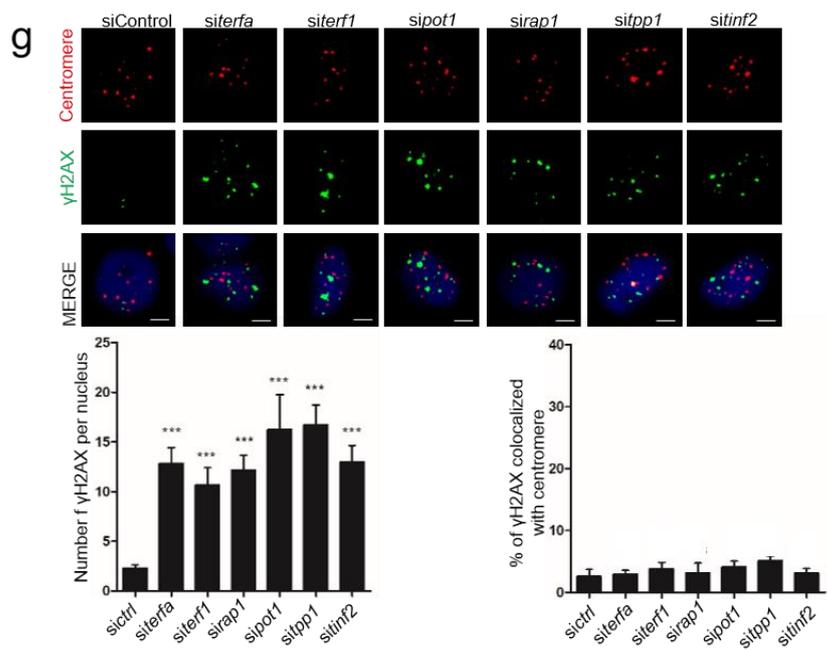
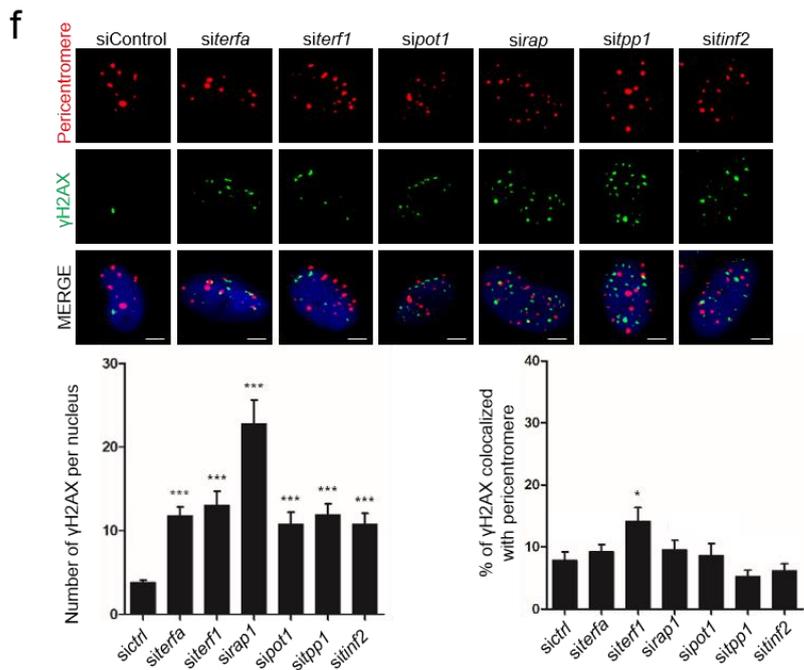
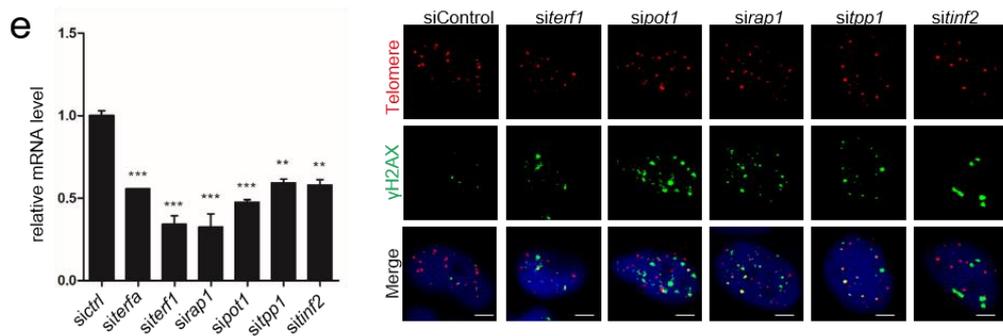


Figure 4

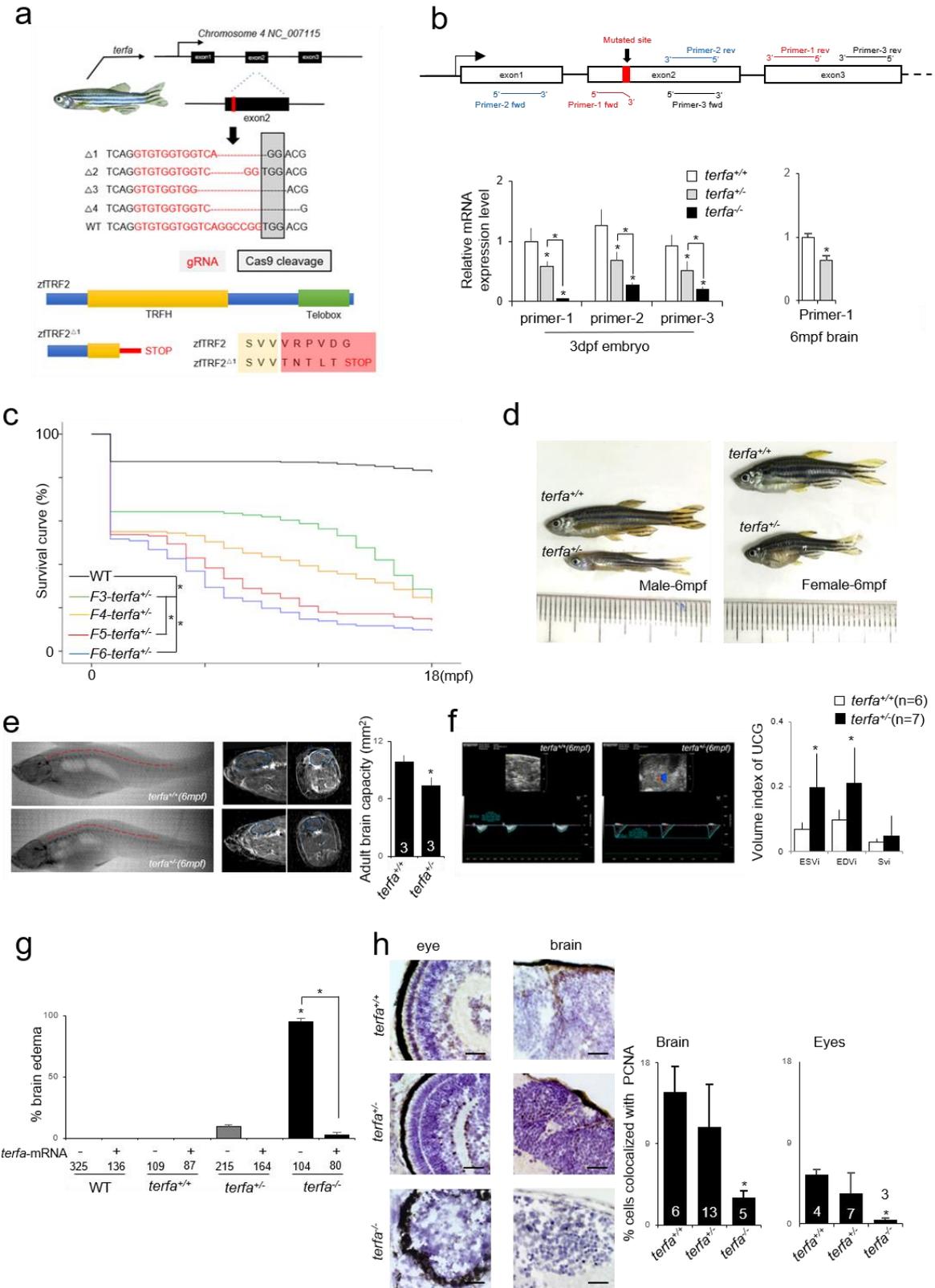


# Extended Figure 1

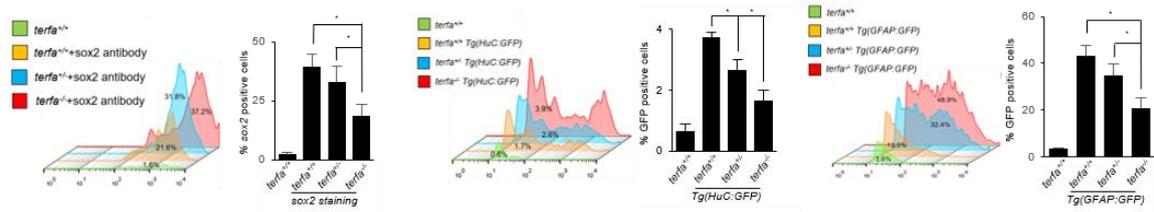




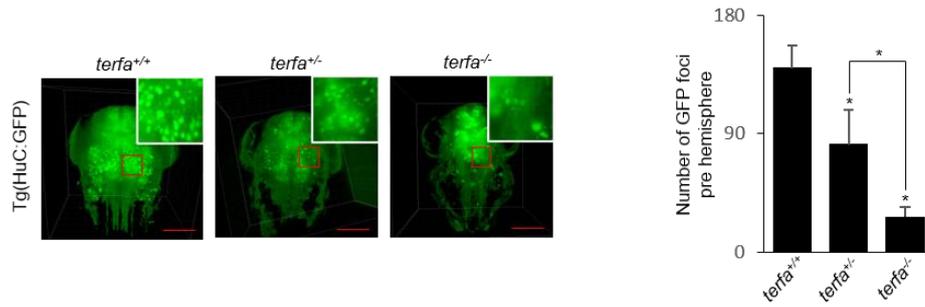
## Extended Figure 2



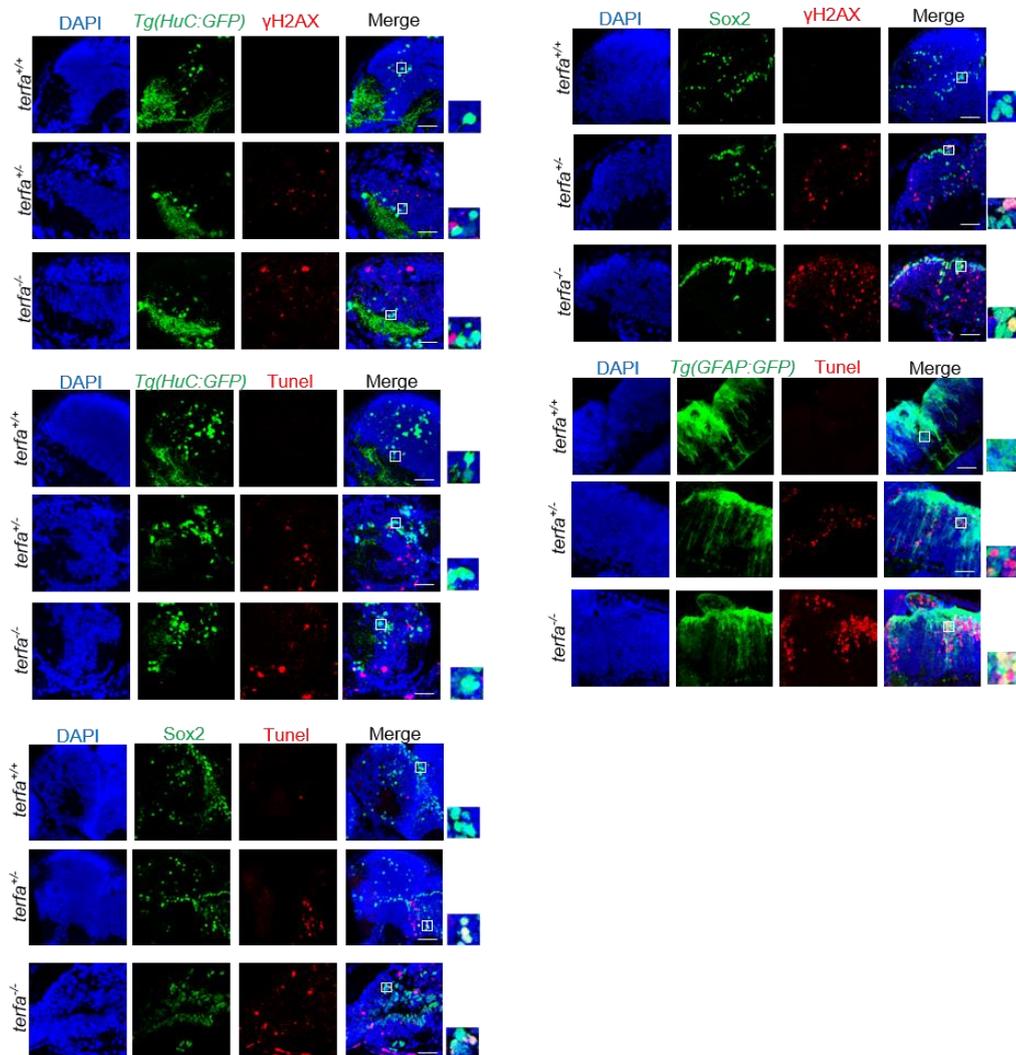
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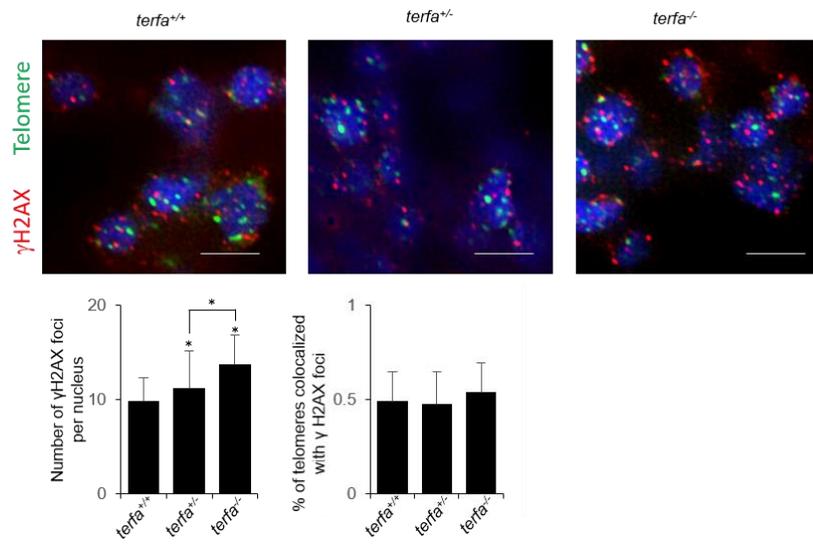
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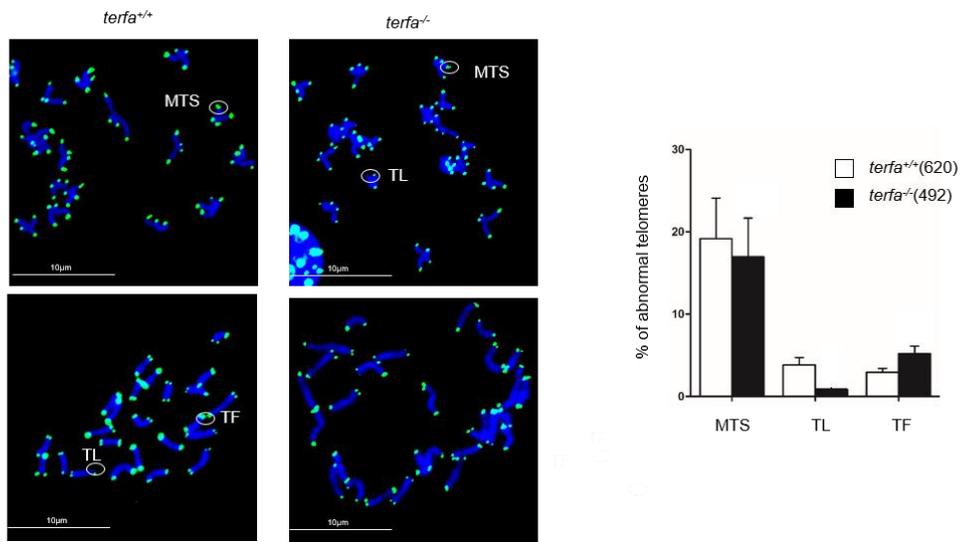
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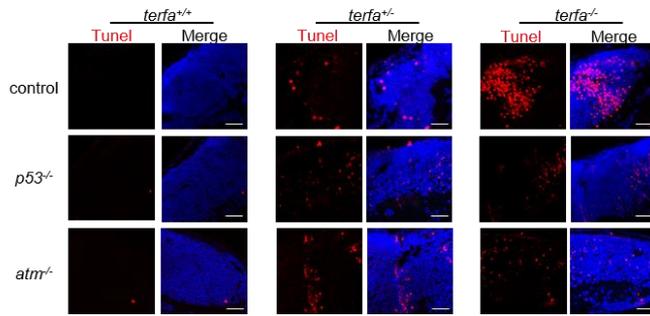


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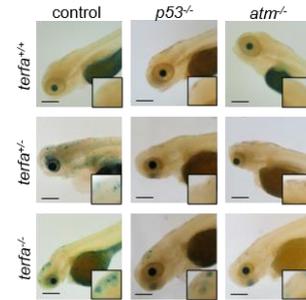


# Extended Figure 3

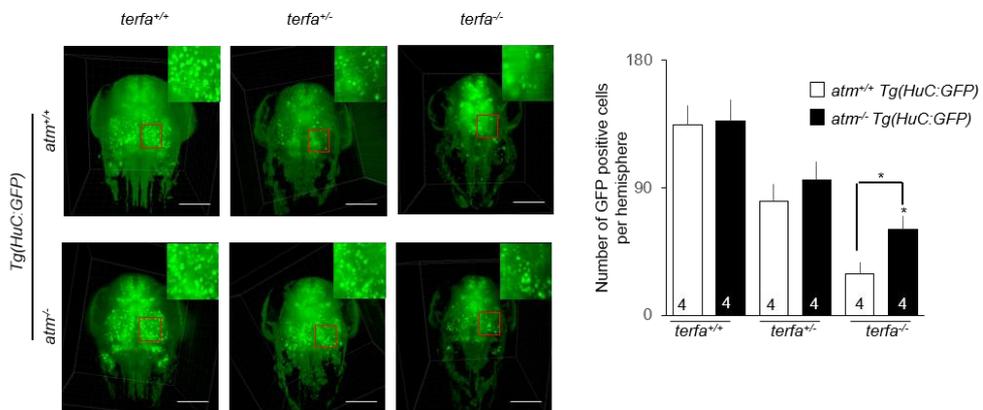
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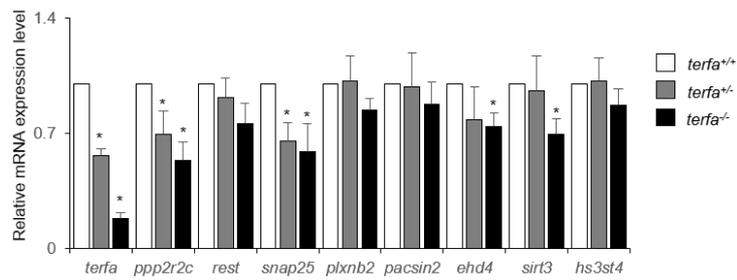
**b**



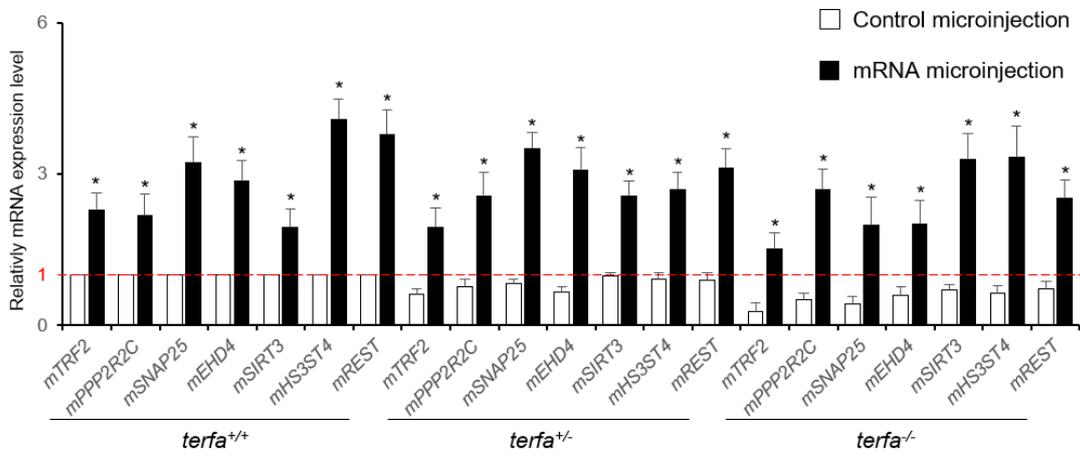
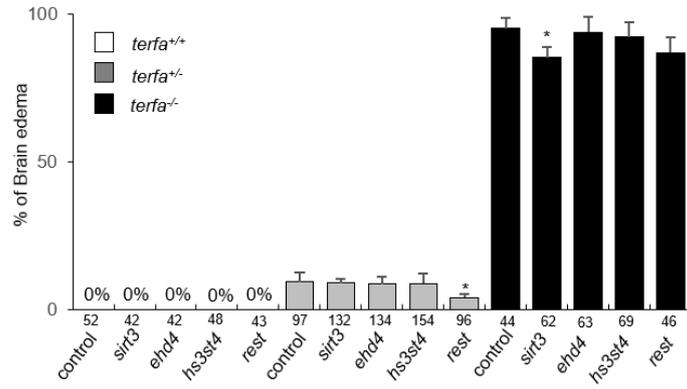
**c**



**d**

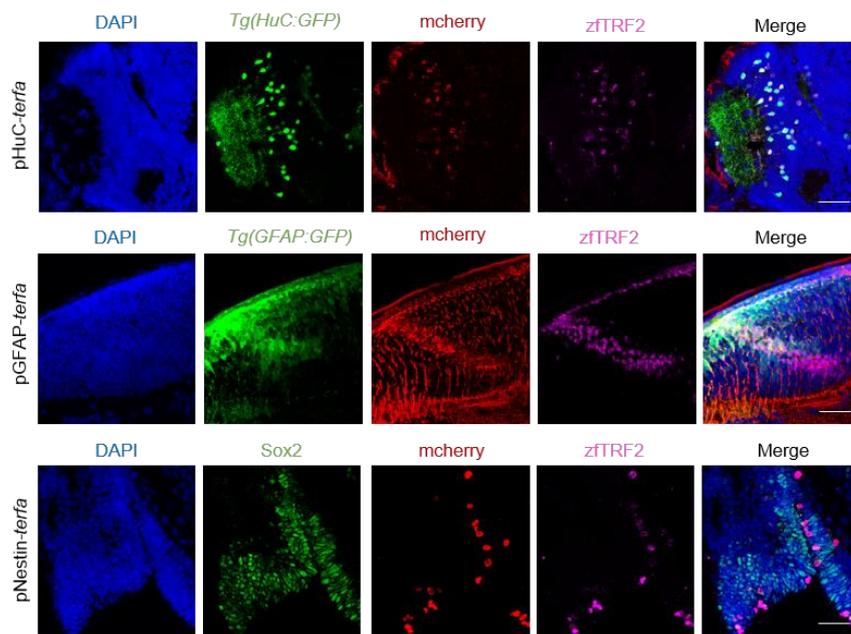


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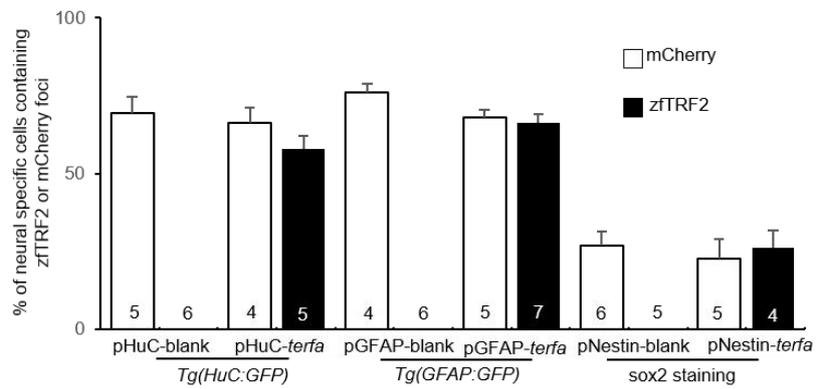


# Extended Figure 4

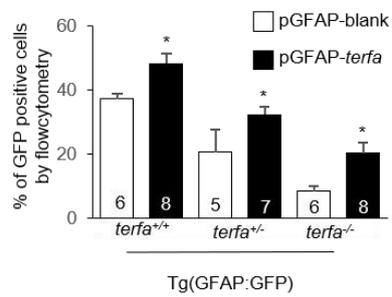
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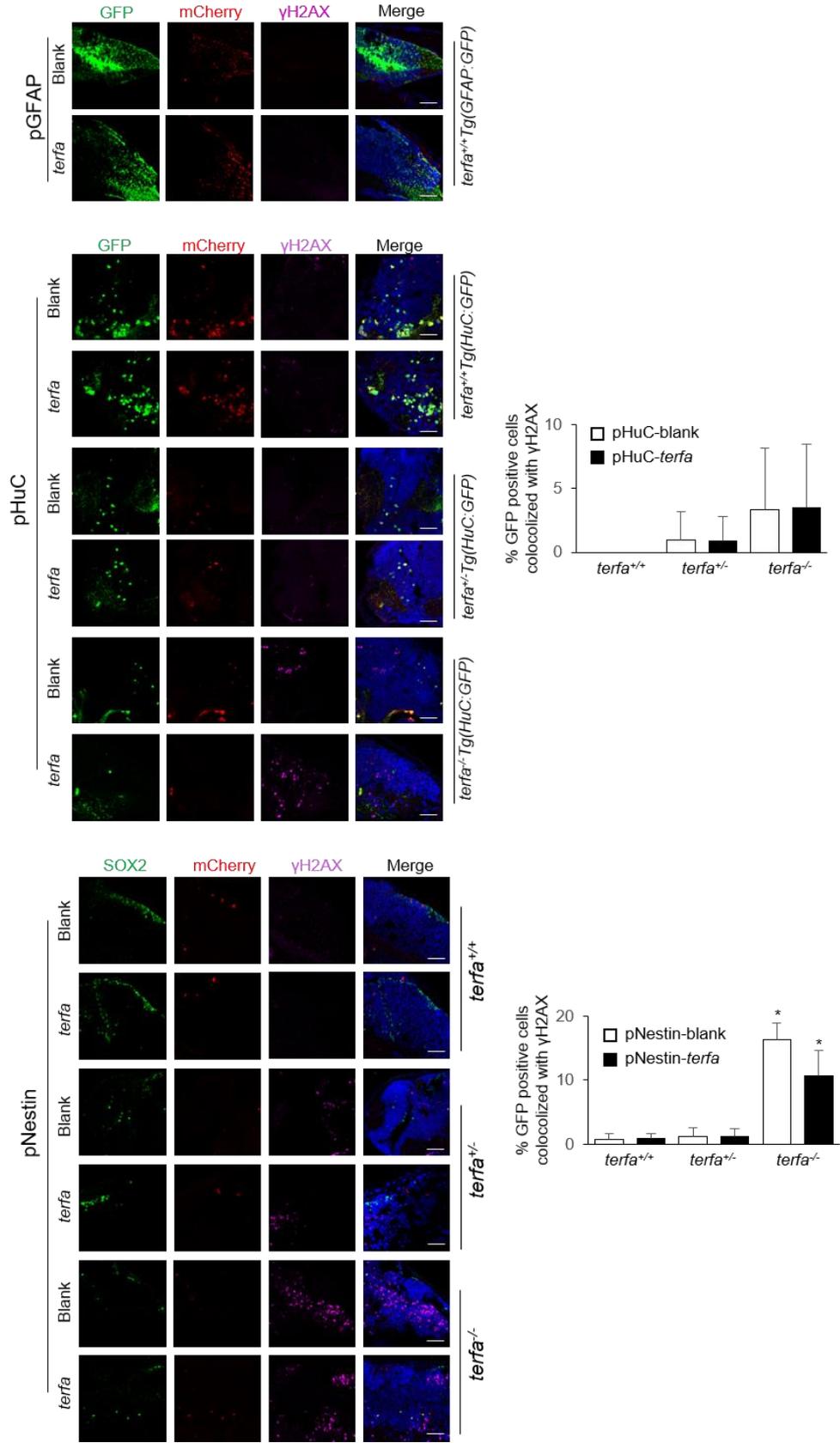
**b**



**c**



d



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