Research Paper

The differential spatiotemporal expression pattern of shelterin genes throughout lifespan

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ABSTRACT

Shelterin forms the core complex of telomere proteins and plays critical roles in protecting telomeres against unwanted activation of the DNA damage response and in maintaining telomere length homeostasis. Although shelterin expression is believed to be ubiquitous for stabilization of chromosomal ends. Evidences suggest that some shelterin subunits have tissue-specific functions. However, very little is known regarding how shelterin subunit gene expression is regulated during development and aging. Using two different animal models, the mouse and zebrafish, we reveal herein that shelterin subunits exhibit distinct spatial and temporal expression patterns that do not correlate with the proliferative status of the organ systems examined. Together, this work shows that the shelterin subunits exhibit distinct spatiotemporal expression patterns, suggesting important tissue-specific functions during development and aging.

INTRODUCTION

Telomeres are specialized chromatin structures, which cap chromosome ends and provide chromosome stability. The maintenance of telomeres requires accurate protections against DNA damage response (DDR) that would otherwise permanently stop cell division by checkpoint activation [ataxia telangiectasia mutated (ATM), and ATM- and Rad3-related (ATR) signaling] and lead to end-to-end chromosomal fusions by non-homologous end joining (NHEJ). Another threat to genome integrity stems from the inability of the conventional replication machinery to fully replicate the extremities of parental DNA, erosion compensated for by telomerase or recombination mechanisms [1, 2].

To achieve chromosome end protection, telomeres are composed of repetitive DNA sequences that can fold into a terminal loop (t-loop), nucleosomes, the non-coding telomeric repeat-containing RNA (TERRA), the protein complex shelterin, and an ill-defined network of nuclear factors [3]. Shelterin is essential for telomere protection and is composed of six subunits: three bind specifically to telomeric DNA (TRF1, TRF2, and POT1) and three establish protein–protein contacts: RAP1 with TRF2, TIN2 with TRF1 and TRF2, and

TPP1 with TIN2 and POT1. Each shelterin subunit appears to have a specific role in telomere protection, i.e., TRF2 blocks ATM signaling and NHEJ, while POT1 blocks ATR signaling [4].

Importantly, telomeres are dynamic structures during development, cancer and aging [5-7]. Indeed, the expression of telomerase is repressed in somatic tissues, leading to a progressive and cumulative telomere shortening with cell division ultimately leading to critically short telomeres triggering DDR and cellular senescence [8]. Therefore, telomeres have emerged as a key driver of aging.

In addition to their role in chromosome end protection, shelterin subunits are able to localize outside telomeric regions, where they can regulate the transcription of genes involved in metabolism, immunity and neurogenesis [9]. This delineates a signaling pathway by which telomeric changes (i.e. telomere shortening) control the ability of their associated factors to regulate transcription throughout the nucleus. This coupling between telomere protection, and tissue-specific transcriptional control might reflect the necessity of tissue homeostasis to rely on 'fine-tuned' coordination between telomeric dynamic (reflecting replicative history and the cumulative effects of various types of stress affecting telomere structure), cellular senescence and differentiation [9].

However, although many molecular and animal studies have manipulated the expression levels of shelterin subunits, only a few have examined the organ specificity of shelterin protein expression. We measured here shelterin gene expression levels in various tissues during development and early adulthood of mice and throughout the lifespan of zebrafish. This revealed distinct spatiotemporal regulation patterns of shelterin subunits during development and aging.

RESULTS

Shelterin genes are differentially expressed in mouse tissues

We measured the mRNA levels of mouse shelterin genes (*TERF1, TERF2, RAP1, TPP1, TINF2, POT1a,* and *POT1b*) in mouse brains, hearts, livers, and kidneys, commencing on embryonic day 10 (E10), at 2-day intervals up to postnatal day 1 (P1), and then on P7, P21, and P100 (Figure 1). The proliferating cell nuclear antigen (*PCNA*) gene served as a marker of proliferation in each organ system. In young mice, the relative expression levels of shelterin genes differed among the four tissues evaluated (Figure 1 and Table 1). *TPP1* was most prominently expressed in all tissues; whereas *TERF2* and *RAP1* were more highly expressed than was *TINF2* and *POT1a/b* in the brain but not the heart, liver, or kidney. This differential expression pattern appeared to be established during development. Indeed, we observed downregulation of *POT1a*/*b*, unchanged expression of *TINF2* and *TERF1*, and a significant increase in *TERF2, RAP1,* and *TPP1* expression through to adulthood (Figure 1, Table 2).

Table 1. Relative expression of shelterin genes.

Shelterin genes	Mouse E18				Zebrafish 9 months							
	Brain	Heart	Liver	Kidney	Brain	Gill	heart	Intestine	Liver	Muscle	Tail	Ovaries
TERF1	7	7	+/-	6	2	6	6	3	5	4	4	4
TERF2(m) *TERFA* (zf)	1	3	7	4	1	5	2	4	4	1	2	5
RAP1	2	2	4	1	4	4	3	2	3	6	5	3
TINF2	5	5	2	5	5	3	5	6	6	2	6	1
TPP1	4	1	1	2	3	1	1	5	2	3	3	6
POT1a (m)	6	4	6	7	na							
POT1b (m)	3	6	3	3								
POT1 (zf)	na				6	2	4	1	1	5	1	2

The relative order of the shelterin subunit expression was ranked from 1 to 7 (mouse) and from 1 to 6 (zebrafish) with 1 being the most expressed.

All shelterin components except *POT1a* were expressed at low levels in the heart, and the *POT1a* level decreased significantly during adulthood. The levels of *TPP1, RAP1*, and *TERF1* were higher in the liver than in the heart, and *POT1a*/*b* expression levels decreased during development. Kidney *TPP1* and *RAP1* levels were upregulated during adulthood; *POT1a*/*b* expression decreased, but no significant changes in the levels of *TERF1*, *TERF2*, or *TINF2* were observed (Figure 1). As expected, *PCNA* gene expression decreased significantly through to adulthood in all organs evaluated, indicating that the expression levels of the various shelterin genes were not associated with the proliferative status of the various tissues.

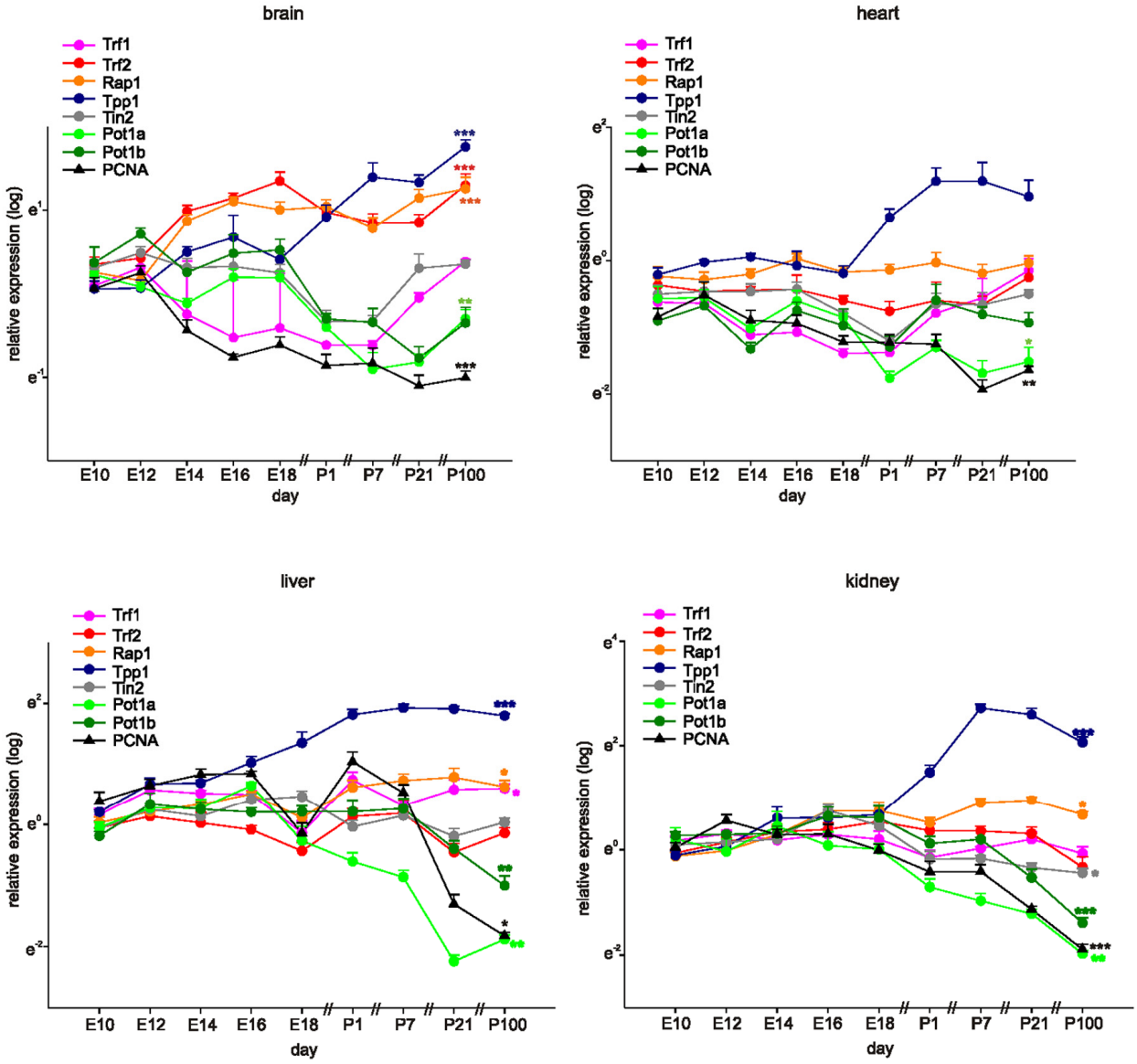

Figure 1. Shelterin components are differentially expressed during development and adulthood. Quantitative RT-PCRs for Shelterin components Trf1, Trf2, Rap1, Tpp1, Tin2, Pot1a, and Pot1b and PCNA as a marker for proliferation in mouse brains, hearts, livers, and kidneys at different time-points of development and in adulthood (*n*=4 each, the four samples for E10 were each pooled from 7 organs, at E12 and 14 the four samples were pooled from four organs each). Expression of each gene was normalized to the respective *Gapdh, actin, and Rplp0* expression. Next, the average of all organs and samples at E10.5 was calculated. Individual samples were then normalized against this average value. Significance was tested between E10 and P100 (adult). Data are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

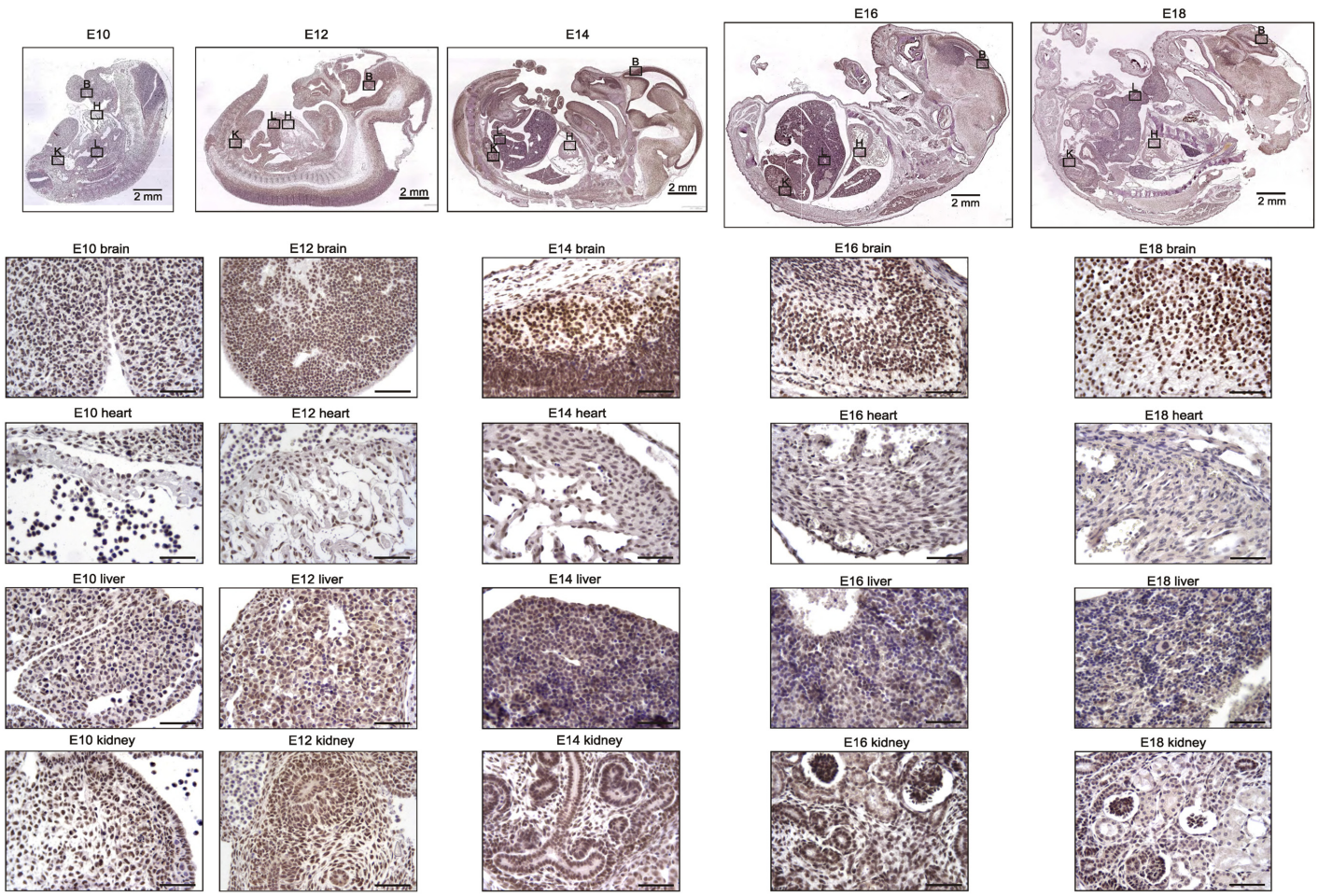

Figure 2. TRF2 is highly and ubiquitously expressed during embryonic development up to E16 and persists afterwards specifically in the brain. Representative photomicrographs of TRF2 immunostaining on sections of mouse embryos (3,3' diaminobenzidine (DAB) substrate, brown, hematoxylin counterstaining) at different stages before birth. B: brain, H: heart, L: liver, K: kidney. Unless otherwise indicated, scale bars represent 50µm.

TRF2 is highly expressed in the mouse neuronal system during both development and adulthood

To evaluate in detail differential TRF2 protein expression during development, we stained mouse tissues for TRF2 during development until young adulthood (i.e., from E10 to P100). TRF2 was highly expressed in all tissues until E16, at which time expression began to decrease in the heart, liver, and kidney but remained high in the brain (Figure 2). This finding is in contrast to what was reported by Cheng et al. [10], who found that TRF2 was not detected in the brain to E18. This may be attributable to technical problems, as the authors used a mouse-derived antibody to examine mouse tissues. In the absence of extensive blocking procedures, this may create false-negative results caused by an enhanced background. During later development and young adulthood, we found that TRF2 expression decreased in the heart, liver, and kidney but remained sTable 1n the brain; the protein was highly expressed in neurons (Figure 3, Supplementary Figure 1). In the heart, TRF2 continued to be expressed in some endothelial cells of the subepicardial vessels, but in the kidney, the expression thereof became restricted to glomerular podocytes and juxtaglomerular cells (Figure 3), in agreement with the recently described angiogenic properties of the protein and its expression regulation by the Wilms' tumor suppressor WT1 [11]. The persistent high level of TRF2 expression in the brain during both development and adulthood is in line with previous work showing that TRF2 expression specifically increases upon neural differentiation [12] [10, 13]. Such expression was accompanied by production of a brain-specific cytoplasmic form of

TRF2, termed TRF2-S, which lacks both the DNA-binding domain and the nuclear localization signal [14] [15]. However, we failed to detect marked cytoplasmic staining of TRF2 in neurons; the staining was predominantly nuclear (Figures 2, 3). Overall, the results suggest that the nuclear form of TRF2 plays a key role in brain development and function.

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). As in the mouse (Figure 1), the relative expression levels of shelterin genes varied among tissues. 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 down-regulation 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.

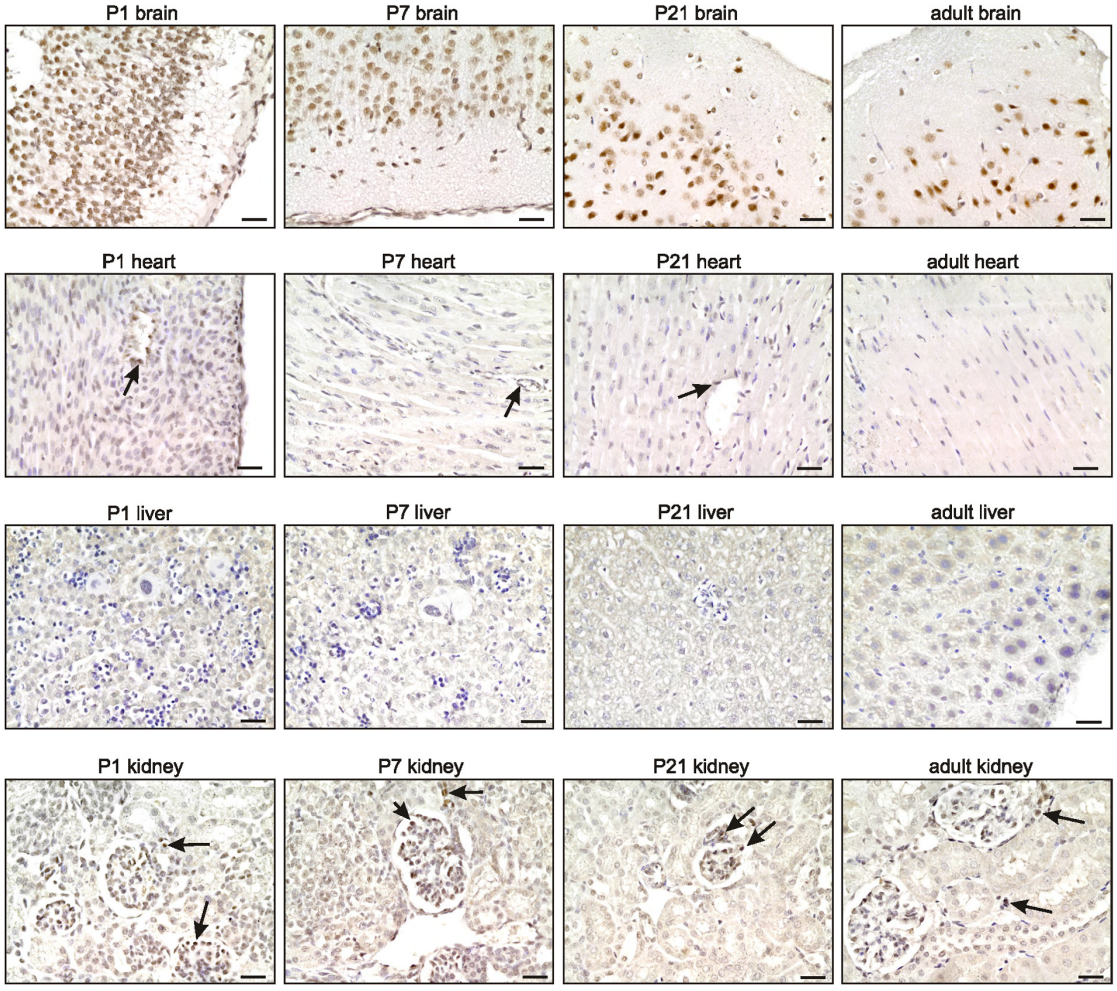

Figure 3. TRF2 expression remains high in the brain during adulthood. Representative photomicrographs of TRF2 immunostaining for the brain, heart, liver, and kidney (3,3' diaminobenzidine (DAB) substrate, brown, hematoxylin counterstaining) at different stages after birth. Note the persistent high expression of TRF2 in neurons of the brain (see also Figure S3), the specific expression in subepicardial endothelial cells of the heart, and glomerular podocytes and juxta-glomerular cells of the kidney (arrows). Scale bars indicate 50μm.

Table 2. Trend in shelterin gene expression during development, adulthood and aging.

Shelterin genes	Mouse development and young adulthood				Zebrafish adulthood and aging							
	Brain	Heart	Liver	Kidney	Brain	Gill	heart	Intestine	Liver	Muscle	Tail	Ovaries
TERF1	s	s	UP	s	DOWN	DOWN	s	DOWN	s	s	DOWN	DOWN
TERF2(m) *TERFA* (zf)	UP	s	s	s	DOWN	DOWN	s	DOWN	s	DOWN	DOWN	DOWN
RAP1	UP	s	UP	UP	DOWN	DOWN	s	DOWN	DOWN	DOWN	DOWN	DOWN
TINF2	s	s	s	s	DOWN	DOWN	DOWN	s	UP	DOWN	DOWN	DOWN
TPP1	UP	s	UP	UP	DOWN	DOWN	DOWN	s	DOWN	DOWN	DOWN	DOWN
POT1a (m)	DOWN	DOWN	DOWN	DOWN	na							
POT1b (m)	DOWN	s	DOWN	DOWN								
POT1 (zf)	na				DOWN	s	DOWN	DOWN	DOWN	s	DOWN	DOWN

UP : the general trend of expression is to increase.
DOWN : the general trend of expression is to decrease.
s : stable expression

We performed whole-mount *in situ* hybridization of zebrafish embryos for *TERFA* mRNA using a cDNA 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 [16], 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 [17] (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.

In zebrafish, *TERFA* specifically regulates the expression of neuronal genes

The above results suggest that TRFA has specific roles in zebrafish nervous system. Indeed, TRFA-compromised zebrafish show early-onset neuro-degenerative phenotypes [18]. Moreover, TRF2 downregulation in mammalian neural progenitor and tumour cells alters neuronal differentiation by mechanisms that are distinct from those regulating telomere protection [13, 19]. The ability of TRF2 to bind at or close to neuronal genes and to activate their transcription is an attractive mecahnism to explain the extratelomeric roles of TRF2 [9, 20, 21]. 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 [21]. 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 Phosphastase 2A (PP2A). We ruled out an off target of the MO against *TERFA* by showing a rescue of *PPP2R2C* 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

We unveil here a tissue-specific shelterin gene expression pattern that is largely conserved between mouse and zebrafish (Table 1). In particular, the relative expression level of *TPP1* mRNA was high in most mouse and fish tissues, whereas the *TERF2/TERFA* mRNA levels were specifically elevated in the brain. With the exception of a study on tissue-specific expression of human shelterin genes in response to physiological stress [22], the present study is, to the best of our knowledge, the first to show that shelterin gene expression levels change in a tissue-specific manner during development and aging. We propose that this spatiotemporal expression pattern of shelterin gene expression plays important roles during development, tissue homeostasis, and aging. The fact that TRF2/TRFA is highly expressed in the brain, a tissue of low proliferative activity, indicates that the tissue-specific roles played by TRF2 (and probably other shelterin components) may (at least in part) be independent of the functions in telomere protection. Indeed, we show in zebrafish embryos that TRFA is required to activate the transcription of several neuronal genes.

Our study had certain limitations. First, 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. However, we found that the TRF2 spatiotemporal expression pattern was identical, both, on the mRNA and the protein level, during mouse development, suggesting that any effects of post-transcriptional regulation may be limited. Overall, our results suggest that important tissue-specific shelterin subcomplexes exist. This is consistent with previous studies on the tissue-specific roles played by TRF2 [13, 14] and RAP1 [23, 24]. Consequently,

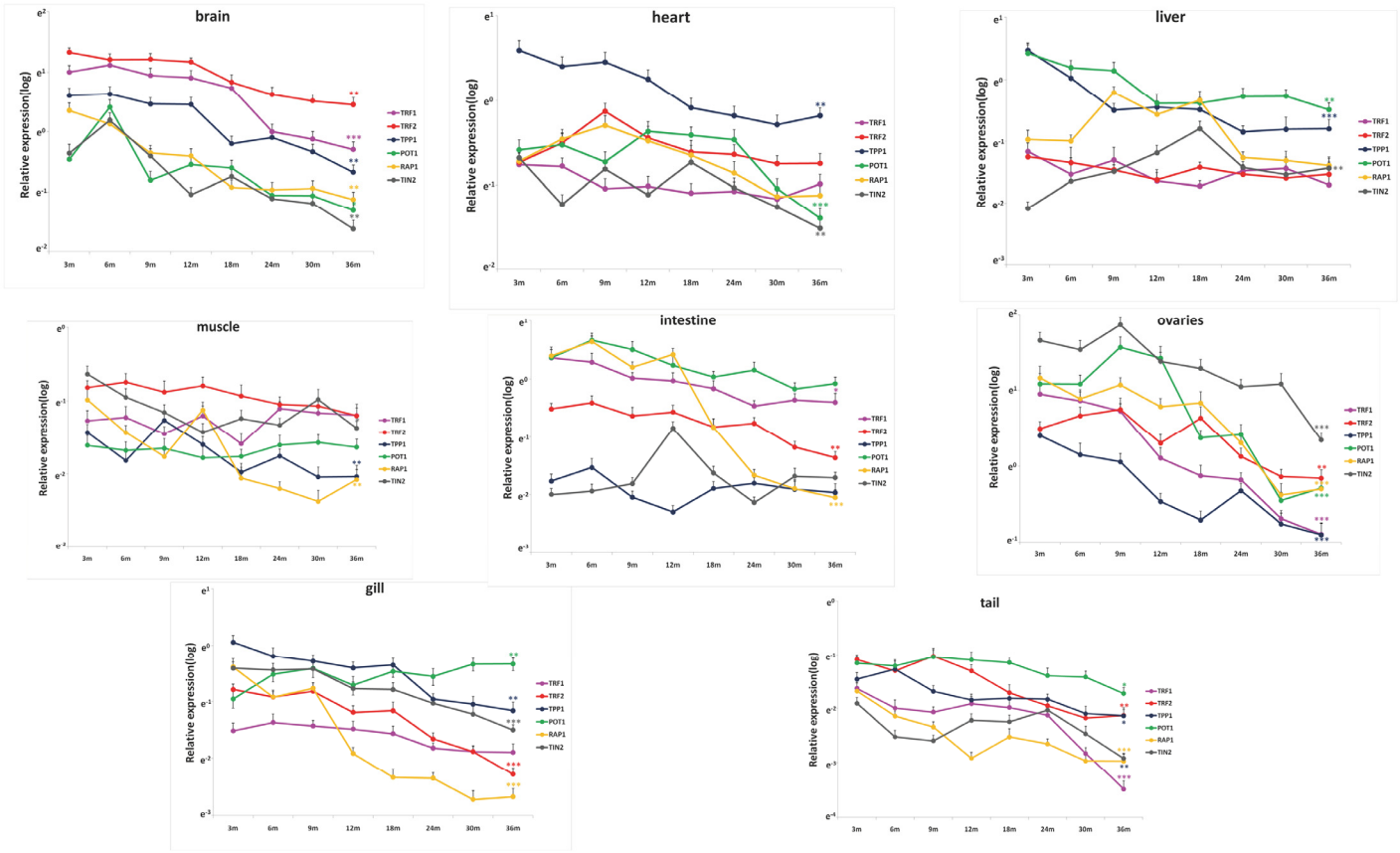

Figure 4. Shelterin genes are differentially expressed during zebrafish life span. Quantitative RT-qPCRs for Shelterin components TRF1, TRF2 (TERFA), RAP1, TPP1, TIN2 and POT1 in zebrafish's brain, heart, liver, intestine, muscle, gill, tail and ovary at different time-points of life span from 3 month to 36 month (n=6 each). Significance was tested between 3 month and 36 month. Data are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

the mechanisms by which shelterin and telomere structures affect cell fate may be more varied than previously thought; specific shelterin subcomplexes may be associated with different cell fates.

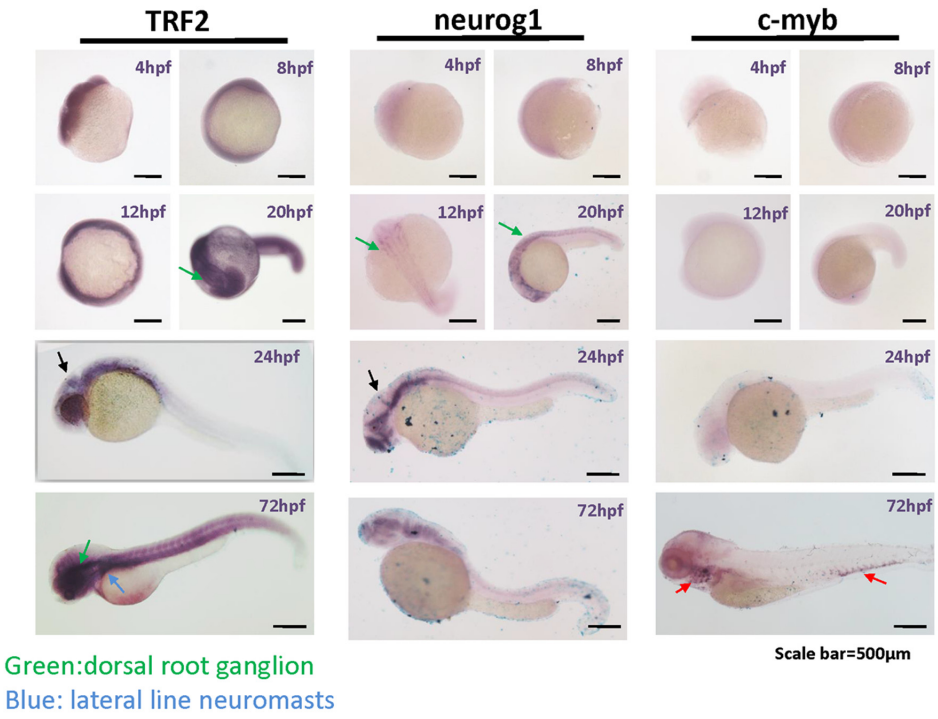

Figure 5. TERFA expression increase since neural development at embryonic stage and remains high in the brain during larval stage development. Representative photomicrographs of whole-mount in situ hybridization of *TERFA*, *Neurog1* and *c-MYB* mRNA. The RNA probe labelled with DIG was stained in dark blue. The green arrow indicates the dorsal root ganglion neuron and the blue arrow indicates the lateral line neuromasts. The black arrow in 24hpf indicates the midbrain boundary. The red arrow indicates the *c-MYB* signal marked hematopoietic tissue.

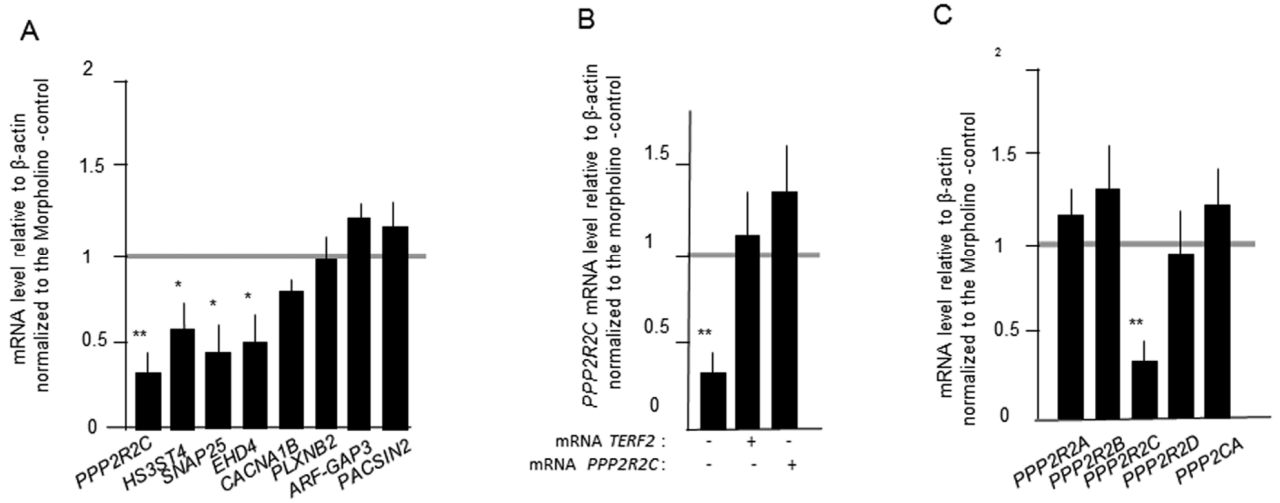

Figure 6. TERFA activates the expression of neuronal gene in zebrafish embryos. (**A**) Gene expression measured by RT-qPCRs in zebrafish embryos 24h after microinjection of MO targeting *TERFA* gene. (**B**) PPP2R2C expression upon co-injection of *TERFA* mRNA or *PPP2R2C* mRNA in zebrafish embryos after microinjection of MO targeting *TERFA* gene. (**C**) Expression of PP2A subunit genes in zebrafish embryos 24h after microinjection of MO targeting *TERFA* gene. Each qRT-PCR was repeated three times and the mRNA level of the PP2A genes was expressed relative to β-actin and normalized to the Morpholino -control. Data are mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

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 [6].

Our data form a solid foundation for future studies exploring the physiological role and regulation of the shelterin complex during development and aging. The work raises several questions. How is the spatio-temporal pattern of shelterin gene expression involved in tissue development, renewal, and function? How is shelterin gene expression regulated during development and over the subsequent lifespan? In this context, we have previously shown that the *TERF2* gene is a direct target of Wnt/beta-catenin and WT1 in both mouse and human cells [25] [11], suggesting that these signaling pathways play important roles in the spatiotemporal expression of shelterin during development and aging. If shelterin plays a critical role in telomere protection, how do the extratelomeric functions of shelterin subunits contribute to cell-type-specific functions? Interestingly, a global decrease in shelterin gene expression during aging was evident in most of the tissues evaluated. How is this decrease triggered during aging? Does the decrease actually cause aging, and, if so, can we stop aging by restoring normal levels of shelterin subunits? Future experiments addressing these questions will certainly shed new light on the increasingly complex and dynamic interaction between telomeres and lifespan.

MATERIALS AND METHODS

All animals were used in accordance with the guidelines of the French Coordination Committee on Cancer Research and local Home Office regulations.

More specific details on the experimental procedures used for morpholino design and microinjection, immunohistology and qRT-PCRs are given in the Supplemental Experimental procedures. Data are expressed as means ± SEMs. ANOVA together with the Bonferroni post-hoc or Mann-Whitney test was performed as indicated. A p value < 0.05 was considered to reflect statistical significance.

AUTHOR CONTRIBUTIONS

NW, KDW, EG, and JY designed the experiments. KDW, YLY, NW, WL, JJ, YC, and XFH performed the experiments. JY, EG, KDW, NW, YLY, and JFM analyzed the data. NW, KDW, EG, and JY wrote the paper. EG, NW and JY are co-senior authors because of their specific roles in coordinating the whole study (EG), the experiments with mice (NW) and the experiments with zebrafishes (JY).

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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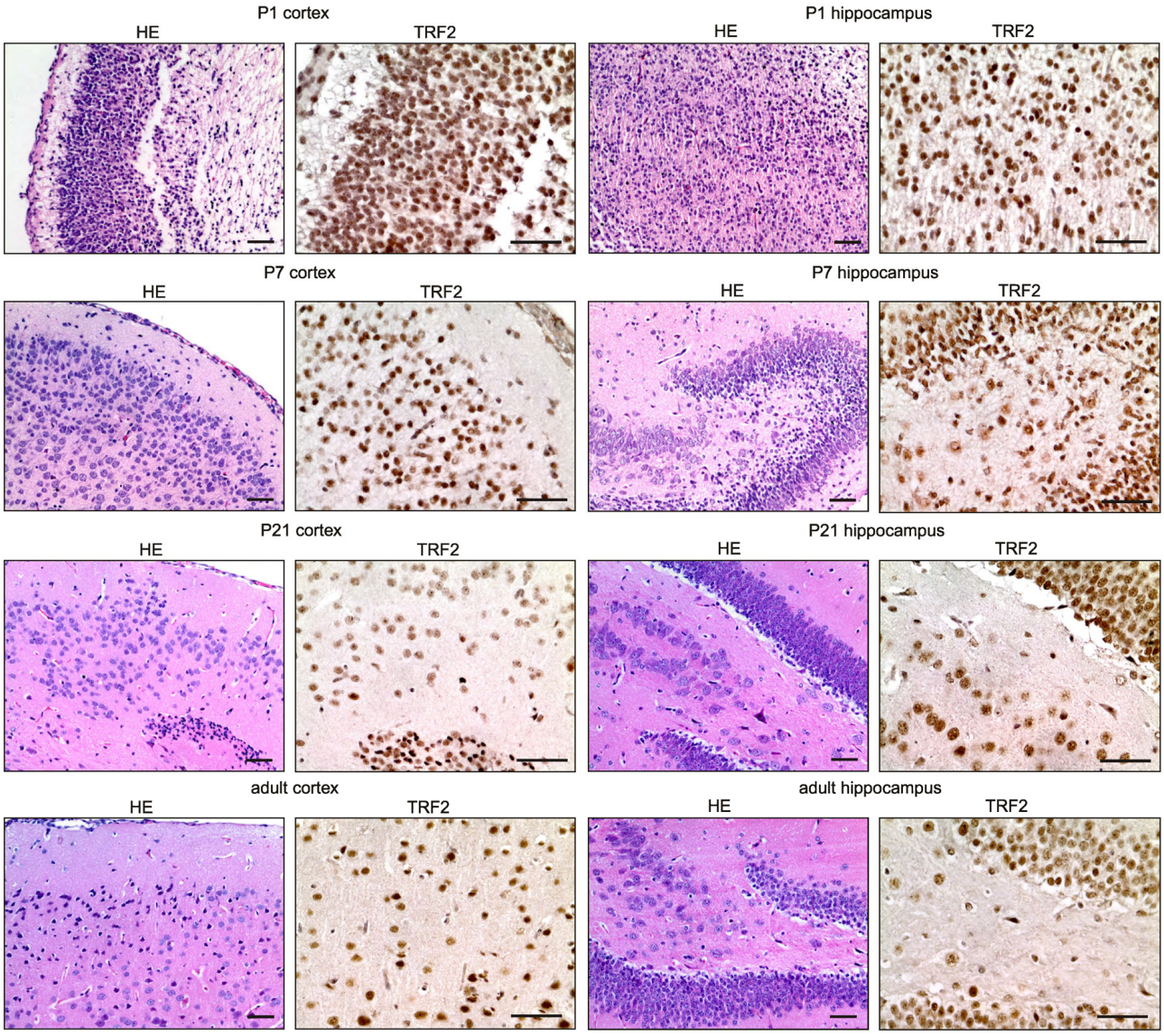

Figure S1 related to Figure 3. TERF2 continues to be highly expressed in neurons of the brain during adulthood representative photomicrographs of TERF2 immunostaining in the cortex and the hippocampal region of the brain (3,3' diaminobenzidine (DAB) substrate, brown, hematoxylin counterstaining) at different stages after birth. The left panels show hematoxylin-eosin (HE) stained sections for better orientation. Note the high expression of TRF2 in neurons independent from the brain section. Sale bars indicate 50µm.

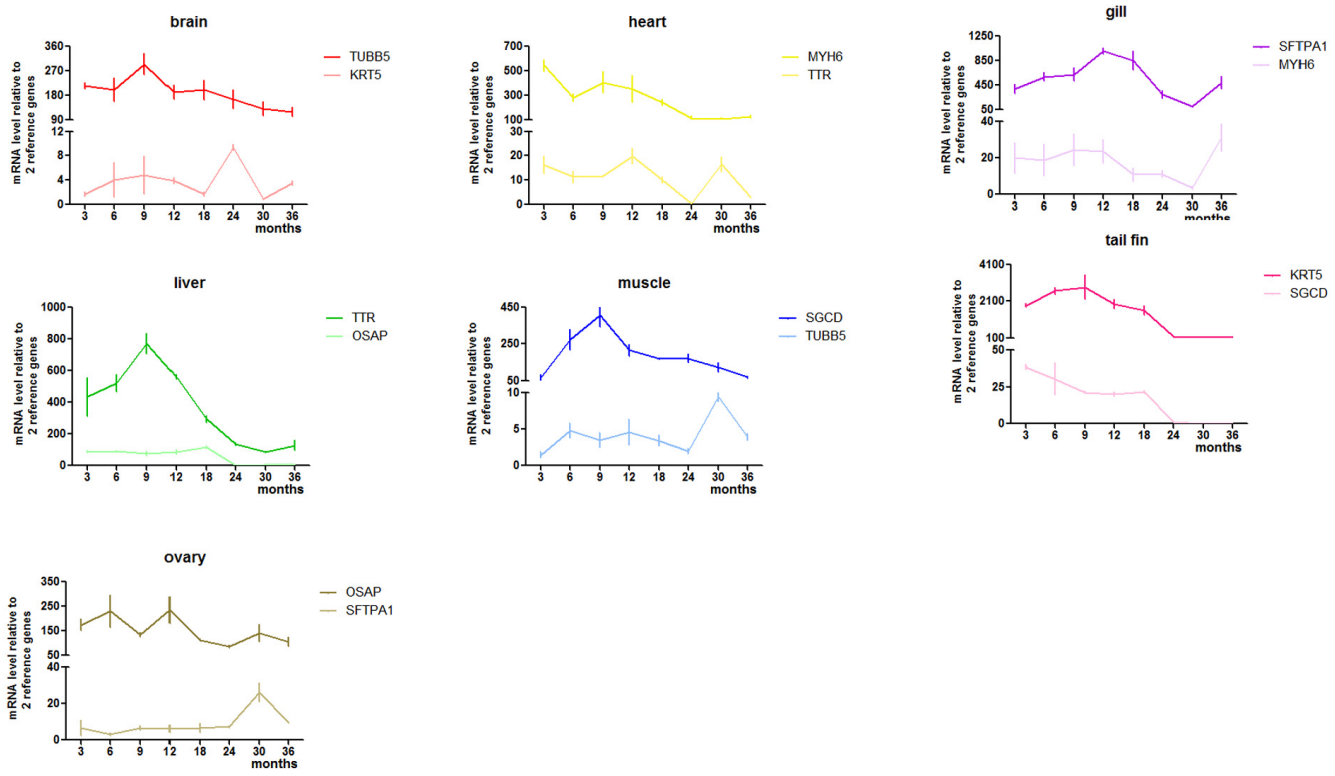

Figure S2 related to Figure 4. Expression of tissues specific markers in the dissected organs from zebrafishes at different ages. Quantitative RT-PCRs for different zebrafish organs was test by different tissues specific primers, including brain, heart, liver, intestine, muscle, gill, tail and ovary from 3 month to 36 month (N=6 each). Dta are presented at mean + SEM.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Mice, zebrafish, and tissue preparation

Timed pregnant mice (NMRI) were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Pregnant mice were killed using cervical dislocation, the cerebral wall at early development stages (embryonic day 10 (E10), 12, 14, and 16) or cerebral cortex at later developmental stages (E18, postnatal day 1 (P1), P7, P21) or in adults (P100), the heart, the kidney, and the liver were dissected, and tissues were used to prepare RNA. For immunohistochemistry, whole embryos were used up to E18, for later stages, organs were dissected. Timed zebrafish were purchased from Lab of Shanghai Institute of Haematology (Shanghai Jiaotong University affiliated Ruijin Hospital,Shanghai China). The maintenance, breeding and staging were performed as described previously [1]. The zebrafish were anaesthetized, and dissected on ice using ophthalmic forceps. The different tissues (brain, heart, liver, intestine, muscle, gill, tail and ovary) were separated and used to prepare RNA.

Real-time RT-PCR

Total RNA was isolated from organs using the Trizol reagent (Invitrogen). The RNA pellet was dissolved in diethyl pyrocarbonate-treated H2O. First-strand cDNA synthesis was performed with 0.5 µg of total RNA using oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen) for mouse tissues and Superscript I reverse transcriptase (Takara) for zebrafish tissues. One µl of the reaction product was taken for real time RT-PCR amplification (ABI Prism 7000, Applied Biosystems) using a commercial SYBR® Green kit (Eurogentec, Angers, France; and Tiangen, China). Primer sequences are available on request. Expression of each gene was normalized to the respective Gapdh, actin, and Rplp0 expression for mouse tissues and to the respective actin, and Rplp0 expression for zebrafish tissues.

Mouse tissue samples, histology and immunohistology Samples from at least three different animals per time-point were analyzed. Three µm paraffin sections were used for histological and immunohistological procedures. Haematoxylin-Eosin staining was routinely

performed on all tissue samples. For Trf2 immunohistology, after heat-mediated antigen retrieval and quenching of endogenous peroxidase activity, the antigen was detected after antibody application Trf2 (1:100, rabbit monoclonal, #13136, (Cell Signaling Technology) using the EnVisionTM Peroxidase/DAB Detection System from Dako (Trappes, France). Sections were counterstained with Hematoxylin (Sigma). Omission of the first antibody served as a negative control. Additionally, some slides were incubated with an IgG Isotype Control (1:100, rabbit monoclonal, clone SP137, Abcam) as a negative control. Slides were photographed using a slide scanner (Leica Microsystems, Nanterre, France) or an epifluorescence microscope (DMLB, Leica, Germany) connected to a digital camera (Spot RT Slider, Diagnostic Instruments, Scotland).

Cloning and constructs

The full-length cDNA of zebrafish TRF2(Terfa), neurog1 and c-myb was cloned into the pCS2+ vector. The EcoR I and Xho I enzymatic sites were incorporated into the end of cDNA sequence to facilitate the directional cloning. The following primers were used:
TRF2(Terfa): F:ATGAGCGACAAACCCTGCGAA; R: GACCATCTTGAGCTTGACCAT
Neurog1: F: CCCACCAATAAGGTTATCAA; R: GCAGACTGTCATTAAGGCAAA
C-myb: F: GGGTTGGACCATTGGAAGAA; R: TGTAAAGGCGAGGGTTGATG

Whole-mount in situ hybridization

Whole-mount in situ hybridizations with Digoxigenin- and fluorescein-labeled antisense mRNA probes were transcribed with T3 polymerase from EcoR I-linearized plasmid according to the manufacturer's instructions (Roche). As a control, sense mRNA probes were transcribed from Xho I-linearized plasmid using SP6 polymerase as previously described [2].

Primers used to determine Shelterin expression in zebrafish

TRF1
F- GCTGGAAGAACAGACAGATGTA; R- GAGGCATATTGCTGGTTGAAAG
TRF2
F- CTGCGCCTCATGCAGTTT; R- ATCACCAGCATCTCGCTGAT
TPP1
F- GGAGCACGTCAAGCCATATTA; R- CCACCACCAAACAGACTCAT
POT1
F- CTATCCCTGGTCCTTCTAGTT; R- CTGTAGACCAACCGAATGTG
RAP1
F- AGTTGTGCCGCCTTCTTTA; R- AAGATCTCCACTGGCTTTGAG
TIN2
F- CTGCTGTTCTGGAGGAATGT; R- CCCGAAAGAGGAAGGGAAAG

Primers used to confirm tissue specificity in zebrafish

TUBB5(Tubulin beta 5, brain)
F- GTCAGTGCGGAAACCAAATC; R- AGGCTCCAGATCCACTAGAA
MYH6(Myosin heavy chain 6, heart)
F- GGCACTGAAGACGCAGATAA; R- CTCACCACCATCCAGTTGAA
KRT5(Keratin 5, skin)
F- GACACATCAGTCGTTGTGGA; R- ATCTCAGCCTTGGTGTTACG
TTR(Transthyretin, liver)
F- CATCTCTGTTTGCCCTCTGT; R- ACCAGTCATGTCCACTTTCC
SFTPbb(Surfactant protein Bb, gill)
F- TTCAATGCTGGGACTGTGT; R- TGGCAACAAGCTCTCTACTG
SGCD(Dystrophin-associated glycoprotein, muscle)
F- CCGAGCCGTTCAAAGAGTTA; R- CAGACGTGGGAGCTTGATTT
MGARP(mitochondria-localized glutamic acid-rich protein, ovary)
F- TCCGGTGAGAACATCGTCTA; R- ATGGTTTGGGCTTCCACTC

Primers for housekeeping genes in zebrafish
RPLP0(36b4)

F- TGCTGCTGGCAAACAAAG; R- CACCTTGTCTCCAGGTTTGA
β-actin
F- GGGTATGGAATCTTGCGGTATC; R- CTTCATGGTGGAAGGAGCAA

Morpholino design and microinjection

Morpholino oligonucleotides (MOs) were obtained from Gene Tools (Philomath, OR, USA). The sequence of MOs against TERFA gene was 5'-TTCGCAGGGTTTGTCGCTCATTCTT-3' which was shown to effectively suppress the expression of TERFA in zebrafish. A standard control MO 5'-TCCACACAGTGGTTCAAATCCCACAT-3' was also used. The control MO provided by Gene Tools had no target and no significant biological activity. An MO solution was prepared with sterile water and contained

0.1% phenol red as a visualizing indicator. The MO was microinjected into embryos at the 1~2-cell stage with an IM-300 microinjector system (Narishige Scientific Instrument Laboratory, Tokyo, Japan). In preliminary tests, embryos injected with 2 ng of the control MO showed no significant differences in survival rates and morphology compared to WT embryos. Embryos injected with 2 ng of the TERFA MO showed no significant differences in survival rates compared with control MO but had a morphology difference.

Statistical analysis

Data are expressed as means±S.E.M. ANOVA with Bonferroni test as post-hoc test or Mann-Whitney tests were performed as indicated. A P-value of less than 0.05 was considered statistically significant.

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