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The role of ATF7 in the maintenance of stress-induced telomere shortening

(ストレスによるテロメア短縮の維持における ATF7 の役割)

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論　　文　　概　　要

○ 論文題目 The role of ATF7 in the maintenance of stress-induced telomere shortening

(ストレスによるテロメア短縮の維持における ATF7 の役割)

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Purpose: Telomeres, repetitive DNA located at the ends of chromosomes, shorten over the life course in response to the environmental stress. Epidemiological study indicated that stress exposure during intrauterine life is associated with shorter telomeres in young adulthood. The life histories of parents affect the telomere length of children. Nonetheless, empirical study for telomere length changing of offspring in response to *in uterus* stress and paternal stress has not been performed, and the mechanism for the maintenance of stress-induced telomere shortening remains unknown. ATF7 is a vertebrate member of the ATF2 subfamily of transcription factors, which belong to the ATF/CREB superfamily. ATF7 is phosphorylated by p38 in response to environmental stress such as psychological stress, nutritional stress, and pathogen infection. We recently found that tumor necrosis factor α (TNF-α), of which level in periphery is increased by various psychological stresses, induces phosphorylation of transcription factor ATF7 and a release of telomerase-ATF7 complex from telomere, leading to telomere shortening. In addition, ATF7 contributes to telomeric heterochromatin formation through recruiting Suv39H1 histone H3K9 trimethyltransferase. Furthermore, the stress-induced and dATF2-dependent epigenome change in *D. melanogaster* was inherited to the next generation either maternally or paternally. Thus, we would like to know whether paternal stress induces telomere shortening in mice offspring, and the role of ATF7 in the paternal-stress induced telomere shortening of offspring.

Methods: Pregnant mice, male wide-type and *Atf7*-deficient mice were used for TNF-α stimulation. TNF-α was intraperitoneally administered to pregnant mice or paternal adult mice daily. Telomere lengths of various tissues were measured by Q-PCR. Chromatin Immunoprecipitation assay was used to analysis the binding amounts of ATF7 and histone modification on telomere and subtelomere. TERRA (telomere repeat-containing RNA) RNA levels on several chromatins were measured by RT-PCR. TERRA RNA levels in spermatozoa were determined by Poly (A)-tailed PCR.

Results: *In utero* TNF-α treatment in mice induced telomere shortening in adulthood. Telomere shortening was observed in limited tissues such as bone marrow, spleen, and lung. In addition, telomere shortening was detected in the some range of age during adulthood. Under those conditions, telomere shortening was not observed in *Atf7*-deficient mice. Paternal TNF-α treatment also induced telomere shortening in

male mice offspring. However, under such condition, the telomere length of the paternal generation was not changed by TNF-α. The paternal TNF-α-induced telomere shortening of offspring was not observed in *Atf7*-deficient mice. TNF-α induced ATF7 phosphorylation, leading to a release of ATF7 from telomere and TERRA promoter region in paternal germ cells. The TNF-α-induced and ATF7-dependent decrease of H3K9me3 at telomere and TERRA RNA promoter region in paternal germ cells were observed. Oppositely, the increased level of H3K9me3 at both regions was observed in next generation MEFs. Furthermore, increased TERRA RNA levels in both paternal spermatozoa and next-generation MEFs were detected.

Conclusion: *In utero* TNF-α stress induced telomere shortening in young adult mice in an ATF7-dependent manner. This telomere shortening was occurred in the limited tissues and maintained for short period. Paternal TNF-α treatment also induced telomere shortening in mouse offspring in an ATF7-dependent manner. However TNF-α-induced and ATF7-dependent telomere shortening in mice offspring is independent on the telomere shortening of their fathers. ATF7 induced H3K9m3 and contributed to heterochromatin formation on both telomere region and TERRA promoter region, leading to repression of TERRA RNA expression. TNF-α induced TERRA RNA in sperm in an ATF7-dependent manner, which might be transmitted to the next generation and regulate the telomere length. Thus, ATF7 may play an important role in inheritance of telomere length, especially in response to environmental stress.

Content

Introduction

1. Telomere and Telomerase

1.1. Telomere and Telomere length

Telomeres were identified in the 1930s. It is typical heterochromatin region which located at the ends of chromosomes, composed of tandem repeats that are bound with shelterin multi-protein complex. The telomere structures are critical for chromosome integrity.

Telomeric DNA has typically heterochromatin structure composed of multiple short repeats that are often G/T rich. The mammalian telomeres are composed of double-stranded TTAGGG repeats and a single-stranded telomeric 3' tail end. The telomere lengths are variable among different species, for example telomere extends for 9-15 kb in humans but 100 kb in rodents. The telomere structure is characterized of T-loop and D-loop that is due to fold back and invade of telomere 3'-overhang to the double stranded region (Fig. 1-1A) [1].

Shelterin protein complex is known to cap with telomeric DNA. In mammals, there are six proteins: telomeric repeat-binding factor 1 (TRF1), TRF2, repressor and activator protein 1 (RAP1), TRF1-interacting nuclear protein 2 (TIN2), protection of telomeres 1 (POT1) and TPP1 (Fig.1-1A). In *S. cerevisiae*, Rap1, Cdc13, Stn1 and Ten1, chromosome end-binding proteins, are cap with telomere. In addition, Taz1 (homolog of TRF), Rap1, Pot1, Tpz1, Poz1 and Ccq1 (coiled-coil quantitatively-enriched 1) were reported to bind on the telomere forming the shelterin complex in yeast *S. pombe* [2]. Furthermore, some other proteins such as Ku70/80, yeast sir4, bind to telomere region. Ku70 and Ku80 are conserved from mammal to yeast, which are well known to form a heterodimer and contribute to DNA repair. The Ku heterodimer binds to double-stranded DNA (dsDNA) in a structure-specific and sequence-independent manner, and mediate a non-homologous end joining (NHEJ) to repair the DNA double-strand breaks. On telomere, the Ku heterodimer has a specific high-affinity interaction with TRF1 complex, acting in a unique way to prevent chromatin recombination. In addition, Ku complex could regulate the telomere length. Ku-mediated telomerase recruitment was reported in yeast and human [3-6].

Telomeres shorten in couple with DNA replication, due to end-replication problem. This progressive telomere attrition is a marker of organismal ageing, so called 'molecular clock' [1]. Several types of stress decrease telomere length: exposure to psychosocial stress is associated with telomere shortening; prenatal stress exposure causes shorter telomere length later in life; and oxidative stress shortens telomeres.

Epidemiological study indicated that people under high psychological stress have shorter telomere than people with low psychological stress. Psychological stress-induced telomere shortening is significantly associated with increase of oxidative stress and decease of telomerase activity in peripheral blood mononuclear cells [7]. In addition, Bucharest Early Intervention Project enrolled 136 children were carried out to judge whether the telomere length in childhood was affected by their early experiences. Telomere length of children was determined in foster care group and institutional care group. The time of institutional care was quantified to represent the strength of early adversity. Telomere shortening in middle childhood was significantly associated with time of institutional care [8]. In yeast cell model, stresses give rise to different outcomes on telomere length: longer telomere was observed after alcohol and acetic acid treatment, whereas stresses of caffeine and high temperatures induced telomere shortening. In addition, the telomere binding protein Rap1/Rif1 is identified to regulate the telomeric response to environmental signals [9].

Maternal stress is associated with shorter telomere length in offspring. A list of negative life events experienced *in uterus* including death or sudden severe illness of an immediate family member and loss of primary residence is provided to quantify the prenatal psychosocial stress. The Leukocyte telomere length (LTL) of young adult offspring was determined at about 28-years old using blood sample, which is a predictor of age-related disease onset. The LTL of young adult offspring exposed to prenatal stress showed a significant reduction, 178-bp, compare to control group [10]. Further, similar test is performed to determine the telomere length of newborn offspring. The 540-bp difference was observed in the high- vs low-stress group. Those finding provides that stress *in uterus* may exert a negative effect on telomere during early development that is already and more apparent at birth compare to the adult [11].

Inheritance of telomere length has been reported in human. The correlation between parents and offspring LTL is observed in some epidemiological studies,

although those results gave rise to some problems. One study examined in around 20,000 people and across four different populations. They found that a greater correlation between father and offspring compare to the mother-offspring correlation. However, this result is opposite to previous findings using relative small sample size, that the correlation between mother and offspring was greater [12, 13]. Although inconsistent inheritance of telomere length was observed, there might be some relationship between the telomere length of offspring and parents. Furthermore, a longer telomere length in offspring is significantly associated with age of their parents and telomere length of sperm. The longer telomere could be inherited to their grandchildren [14]. Thus, the telomere length is associated with life histories of their parents. However, there is no direct evidence to clarify the status of telomere in offspring when their parents suffering the stress.

We have focused on the maintenance of stress-induced telomere shortening. The tissue distribution of telomere shortening in offspring in response to stress *in uterus* was determined by empirical study. We have also examined the relationship of paternal stress-induced telomere shortening in offspring with telomere length of their fathers. Further, we have tried to clarify the mechanism of telomere shortening in offspring by paternal stress.

1.2. Telomerase

Telomerase is a unique DNA polymerase which is highly expressed in germ cells and some cancer cells to facilitate the solution of chromosome end-related problem. Telomerase contains telomerase reverse transcriptase (TERT) and a template-containing RNA component (TR). Encoding by TR, multiple tandem repeats of DNA are synthesized, to compensate for the telomere erosion with cell divisions. TERT is the core enzyme of telomerase containing about 1,000 amino acids and it is conserved among species such as human, mouse and yeast. Base on the domain analysis, the TERT protein has three major domains: the telomerase essential N-terminal (TEN) domain; the TERT RNA-binding domain (TRBD); and the reverse transcriptase domain (RT), which contains the fingers, palm and thumb subdomains and the active site for reverse transcription [2, 15].

Telomerase functions starting from telomerase recruitment. In human, shelterin component TPP1 plays a core role in telomerase recruitment. Through OB-

fold domain, TPP1 regulates the telomerase recruitment and mediates telomere elongation. By making point mutation of TPP1 OB-domain, a TPP1 glutamate (E) and leucine (L)-rich patch (TEL patch) which contains seven amino acids (E168, E169, E171, R180, L183, L212 and E215) was discovered to interact with telomerase. By separation-of-function mutations, the TEN-domain of hTERT was identified to interact with TPP1 both *in vivo* and *in vitro* with limited effect on the catalytic activity of telomerase. In addition, the telomerase recruitment defect was found in TIN2-R282H mutation, which unaffected the TPP1 binding sites on protein TIN2. Thus, TIN2-mediated telomerase recruitment might be independent of TPP1 [16-19] (Fig.1-1B).

In addition, the Ku70/80 heterodimer-mediated telomerase recruitment was shown in yeast *S. cerevisiae*. Ku70/80 heterodimer is a highly conserved DNA end-binding complex, which is well understood for non-homologous end-joining. In *S. cerevisiae*, Ku binds to a 74-nt hairpin in telomerase RNA via the DNA binding domain which is shown to interact with telomere. However, a co-factor Sir4 is needed to fascinate the telomerase recruitment, since the purified Ku complex cannot bind to telomere and telomerase concurrently [5, 6]. Although sequence of yeast TLC1 and human hTR has no similarity, Ku70/80 might bind to a 47-nt stem-loop region in 3' end of hTR both *in vitro* and *in vivo* [20]. Recently, our data demonstrated that Ku mediates TERT recruitment via ATF7, a transcription silencer. Immunoprecipitation assay showed that ATF7, Ku70/80 and TERT make a complex which binds to telomere. (Fig. 1-1C)

Telomerase activation and processivity occur after TERT recruitment by forming a protein complex though POT1, TPP1 and telomerase on telomeric ssDNA. The effect of POT1-TPP1 on telomerase activation and processivity was confirmed at individual steps of the telomerase reaction cycle. It is found that POT1-TPP1 decreases telomerase RNA template dissociation and improves the telomeric DNA translocation efficiency. Thus, the interaction among TPP1, POT1 and telomeric 3’ overhang is essential for telomerase processivity [21].

The mTR-deficient mice, lacking telomerase activity, was used to judge the biological meaning of telomere and telomerase. Telomerase deficient mice showed progressive telomere shortening and accumulated genetic instability in an age-

dependent manner. This may result in shorter lifespan, senescence, dysfunction of wound healing capacity and hematopoietic ablation. Further, in late-generation *Terc*$^{-/-}$ mice, the incidence of spontaneous tumors moderately increased, such as lymphomas, and teratocarcinomas. The appearance of these tumors might be due to accumulated genetic instability in telomerase deficient mice. Telomerase inhibitor BIBR1532 and TPP1-TEL patch mutations inhibit the growth of HeLa cells and induce cell apoptosis. Telomerase might an important target for anticancer therapy as 90% of human tumors are telomerase-positive [22, 23, 24]. Thus, telomerase and telomeres play important roles in aging and tumourigenesis, and it might be associated with several human diseases.

Telomere dysfunction is also associated with metabolic and mitochondrial compromise. Telomere dysfunction represses the PGC-1α and PGC-1β expression, since P53 was activated and bind to promoters of both genes when telomere shortening. This telomere-p53-PGC axis is critical for metabolic and mitochondrial biogenesis and function. *Tert*$^{-/-}$ mice shows defective mitochondrial biogenesis and function, impaired gluconeogenesis, cardiomyopathy, and increase of ROS resulted in worsening of organismal fitness and metabolic failure [25].

One of the famous telomerase associated human diseases is Dyskeratosis congenita (DC) that is a rare bone marrow failure syndrome. The DC disease is characterized as telomere syndrome containing a serial of abnormal somatic features and mucocutaneous features such as abnormal skin pigmentation, leucoplakia and nail dystrophy. In DC patient, mutations were identified in various telomerase components such as TERC, Dyskerin, NHP2, NOP10 and TCAB1which repressed the telomerase biogenesis, activation and processivity. Recently, mutation in shelterin components TIN2 and TPP1 and telomere replication proteins RTEL1 and CTC1 were identified as DC-causative mutation. DC patients with TIN2 mutations were characterized as short telomere and early onset of DC syndrome [19, 26, 27]. Collectively, telomere shortening and telomerase mutations are associated with various human diseases, such as cancers and dyskeratosis congenita, as well as changes in cellular metabolism.

1.3. Telomeric heterochromatin

Heterochromatin mainly contributes to gene silencing in the feature of a tightly packed form of DNA, which contains two types: constitutive and facultative

heterochromatin. Constitutive heterochromatin mainly located at centromeres, telomeres and repetitive sequences is characterized by DNA hypermethylation, hypoacetylation and hypermethylation of histone, most notably at histone H3, such as lysine 9 of histone H3 (H3K9). H3K9 trimethylation (H3K9me3) induced by Suv39H1/2, a histone methyltransferase (HMTase), is an important hallmarks of heterochromatin. H3K9me3 could recruit Heterochromatin Protein 1 (HP1) family proteins at pericentric chromatin to mediate heterochromatin formation.

Telomere is a bulk of constitutive heterochromatin displaying a serial of tightly packed nucleosomes that have a relative shorter repeat size. As a heterochromatin region, telomere tends to repress the nearby genes, so called telomere position effect.

In *S. cerevisiae,* Sir4 binds to Rap1, a telomere binding protein, to recruit Sir3 and Sir2 and induces the telomere heterochromatin formation. Sir2 is a NAD-dependent deacetylase which contributes to histones H3 and H4 deacetylation notably at lysine 16 of histone H4 (H4K16Ac), thereby creating a high affinity binding site for Sir3 and Sir4 [28, 29].

Vertebrate telomeres are enriched for the Suv39H1/2-dependent H3K9me3 marks, since decrease of H3K9me3 amounts on telomere was observed in Suv39H1/2-deficient mice (Suv39DN mice). H3K9me3 further creates a high affinity platform for HP1 family proteins promoting the imposition of the H4K20me3 marks. In addition, some other repressive histone marks, such as di-methyalted H3K79, histones H3 and H4 de-acetylated form are enriched on telomere region. Importantly, subtelomere region also contains multiply repressive chromatin marks, such as H3K9me3, HP1, H4K20me3, and H3 and H4 under-acetylated forms. However, it remains unclear how subtelomeric heterochromatin is established. There are two possibilities that subtelomeric heterochromatin is a consequence of spreading of heterochromatic element at telomere or it is established by recruiting some HMTases to subtelomere repetitive elements (Fig. 1-1D) [30, 31].

Furthermore, telomere length is regulated by H3K9me3 on telomere region. Suv39H1/2-deficient mouse primary cells have abnormally long telomeres relative to WT cells. Further, none of Suv39DN MEFs has mouse telomere dysfunction markers such as robertsonian-like fusions and higher frequencies of undetectable telomeres.

Thus, Suv39DN cells have abnormally long telomeres with normal capping function. Taken together, Suv39H1/2 and telomeric H3K9me3 levels may negatively regulate telomere length in mammalian [32].

1.4. TERRA RNA

For many years, telomeres have been considered to be transcriptionally silent, due to the heterochromatin structure. In 2007, telomeric repeat-containing RNA (TERRA) was first discovered in mammal, which is transcribed from several subtelomeric loci closed to chromosome end. TERRA molecules are heterogeneous in length of UUAGGG repeat sequences ranging from 100 bp to 9 kb. TERRA RNA is widely expressed in most mammalian tissues, including testicular germ cells. TERRA co-localizes with telomeres during mammalian meiosis and its expression progressively increases during spermatogenesis until the beginning of spermiogenesis. TERRA and TERT seems components of telomeres in mammalian germ cells (Fig. 1-2) [33, 34].

TERRA RNA is transcribed from the telomeric C-rich strand by DNA-dependent RNA polymerase II (RNAPII), containing various UUAGGG repeats. Approximately 7% human TERRA RNA has poly (A) tail, while most yeast TERRA molecular is polyadenylated. All the human TERRA RNA has a 7-methylguanosine (m^7G) on 5’ primer to increase the stability [35, 36].

TERRA RNA transcription is controlled by various mechanisms, including early embryo developmental stage, length of telomere, environment stress, stage of tumor and chromatin structure. Human TERRA RNA promoter is located at subtelomere region, which contains CpG dinucleotide-rich DNA islands, promoting transcription of TERRA molecules from various independent chromosome ends by RNAPII. This promoter is methylated by DNMT1 and DNMT3b since DNMT1 and DNMT3a/3b deficient cells display higher levels of TERRA RNA. Mouse TERRA RNA promoter region is different with the human, as TERRA RNA expression was reduced in mouse deficient for DNMT1 and DNMT3a/3b. Mouse TERRA RNA promoter region recently was identified at subtelomere region using a whole-genome RNA-sequencing approach. During the early mouse development, transcription of TERRA RNA starts in 2-cell stage and accumulates from the 4-cell and 8-cell embryo. Thus, the transcripts of TERRA RNA in early embryo are regulated in a strand-specific manner in time and space. It was reported that stress induces TERRA RNA

expression. TERRA RNA-FISH experiments were performed to quantify the amount of TERRA RNA. When immortalized MEFs were cultivated at 42 ℃ for 1 h, a kind of heat shock, the number of TERRA foci per nucleus highly increased compared to cells grown at 37 ℃. The number of TERRA RNA foci was recovered to normal levels, when transfer to normal conditions. However, the precise regulation mechanism of TERRA RNA remains elusive. [35, 37, 38, 39]

Furthermore, TERRA RNA expression is controlled by H3K9me3 level on telomere region. Cells with longer telomere have less TERRA RNA. This repression is mediated by increased H3K9me3 and HP1α density at telomeres, restricting human telomere position effect to telomere transcription [40]. On the other hand, TERRA RNA forms stable intermolecular or intramolecular G-quadruplexes with telomeric repeat DNA. TERRA RNA also interacts with several telomere-associated proteins, including TRF1, TRF2, subunits of the origin recognition complex (ORC), HP1 and H3K9me3. Depletion of TERRA RNA results in high level of telomere dysfunction-induced foci, abnormal telomeres structure in metaphase, and a loss of H3K9me3. It was shown that TERRA RNA facilitates telomere structural maintenance and heterochromatin formation via interaction with TRF2 and ORC [41]. Thus TERRA RNA contributes to a feedback loop to control the telomeric heterochromatin formation (Fig. 1-2).

TERRA RNA participates in telomere length regulation. In *S. cerevisiae*, a RNA exonuclease Rat1p degrades TERRA RNA and promotes telomere elongation. Furthermore, the natural TERRA RNA promoter was replaced by a doxycycline regulatable promoter to study the relationship between TERRA RNA and telomere length. Induction of TERRA RNA resulted in telomere shortening *in cis* and in a telomerase-independent manner. TERRA RNA interacts with Ku70/80 complex thus improving the activity of Exonuclease 1 (Exo1). Thus, TERRA RNA promotes telomere shortening through enhancement of Exo1-mediated resection of chromosome ends [42]. In mammal, TERRA RNAs is up-regulated in ICF (immunodeficiency, centromeric instability, facial anomalies) patient correlates with short telomeres [43]. TERRA RNA inhibits telomerase activity *in vitro* by binding to both the TR and TERT. TERRA RNA-induced increases of H3K9me3 on telomere region are negatively regulating the length of telomere. On the other hand, TERRA RNA tends to interact with telomerase components, and the TERRA-telomerase

cluster recruits telomerase to shortened telomeres in yeast cell. However the levels of TERRA declines at the late S phase, when telomerase preferentially elongates the shortest telomere. Although these finding raised some questions, TERRA RNA prefers to shorten telomere length [35, 36].

2. ATF7 and ATF2 subfamily protein

ATF7 is a vertebrate member of the ATF2 subfamily of transcription factors, which belong to the ATF/CREB superfamily, containing ATF2, ATF7 and CRE-BPa. These proteins are characterized by B-ZIP DNA-binding domains, which bind to the cyclic AMP response element (CRE: 5'-TGACGTCA-3') and a *trans*-activation domain consisting of a metal finger structure and phosphorylation sites for stress-activated protein kinases (SAPKs). [44-46] (Fig.1-3A)

ATF2 forms a homodimer and a heterodimer with c-Jun to activate a group of target genes. ATF2 phosphorylation sites (Thr^{69} and Thr^{71}) are activated by SAPKs such as p38 and Jun N-terminal protein kinase (JNK). In response to these various stresses, ATF2 was directly phosphorylated by p38 and JNK and enhance its *trans*-activating capacity. Such environmental stresses contain pathogen infection, psychological stress, heat stress, osmotic stress, and so on [47].

ATF7 represses target genes transcription through interaction with HMTases, such as ESET, G9a, Suv39H1/2, while ATF2 and CRE-BPa tend to activate transcription from CRE promoters via interaction with co-activator CBP. ATF7 is phosphorylated by p38, but not JNK, in responding to various stresses, including pathogen infection and psychological stress. Social isolation, a kind of psychological stress, can induce ATF7 phosphorylation by p38 in the brain, possibly by elevating the levels of inflammatory cytokines (e.g., TNF-α in the periphery). ATF7 phosphorylation causes a release of ATF7 and ESET from the gene encoding serotonin receptor 5b, leading to transcriptional activation that can be maintained for long periods. In absence of stress, ATF7 silences transcription of the target gene, by recruiting the ESET/SET-DB1 histone H3K9 trimethyltransferase to promote formation of a heterochromatin-like structure [48, 49]. Further, ATF7 also silences a group of innate immune genes via recruiting G9a in mouse macrophage. Pathogen infection induces ATF7 phosphorylation via Toll-like receptors-p38 pathway and a

release of ATF7-G9a, from the target genes leading to transcriptional activation and the long-term maintenance of the higher basal expression levels [50, 51].

In addition, Atf1, the fission yeast homolog of ATF2, and dATF2, *Drosophila melanogaster* homolog of ATF2, contribute to heterochromatin formation. In fission yeast, Atf1 and Pcr1, two ATF/CREB family proteins, were identified as critical factors for heterochromatin formation in a parallel mechanism to the RNAi pathway, since ATF1 and RNAi double mutants fail to assemble heterochromatin. In *Drosophila melanogaster*, the interaction between dATF2 and HP1 was found on heterochromatin and some specific loci in euchromatin. dATF2 was phosphorylated by some environmental stresses, such as heat shock or osmotic stress, leading to a release of dATF2 from heterochromatin. Thus, the stress-induced and dATF2-dependent heterochromatic disruption was observed and this epigenetic event was inherited to the next generation in a non-Mendelian fashion. When embryos were exposed to heat stress over multiple generations, the epigenetic event was inherited for multiple successive generations, although it gradually returned to the normal status. [52, 53]

On telomere region, ATF7-deficient mice showed telomere shortening. Immunoprecipitation assay showed that there is interaction among ATF7, Ku70/80 and telomerase TERT. In response to stress, the interaction of ATF7, Ku70/80 and TERT was disrupted, since ATF7 was phosphorylated via P38. This complex bound to telomere via Ku70/80. Chromatin Immunoprecipitation demonstrated that TERT binding amount on telomere was reduced in ATF7 knockdown cells, while telomeric ATF7 and TERT are decreased in Ku70 deficient cells. Stress stimulation induced a release of ATF7 and TERT from telomere. Furthermore, the interaction of ATF7 and Suv39H1was demonstrated in HeLa cells. Stress induces decrease of H3K9me3 level on telomere region by releasing Suv39H1 from telomere [54] (Fig. 1-3B).

Thus, we have focused on the role of ATF7 in the maintenance of stress-induced telomere shortening. We have examined the role of ATF7 in paternal stress and *in uterus* stress–induced telomere shortening in offspring.

3. Transgenerational epigenetic inheritance

Since 1970s, epigenetics has been established aimed to study mitotically or meiotically heritable changes in gene expression without any change on the DNA

sequence. In general, these epigenetic marks mainly contain DNA methylation, histone modification and RNA in sperm. Although those markers would be cleared and reestablished during germ cells development and early embryo development, there are a number of reports about transgenerational epigenetic inheritance in different model organisms [55].

3.1. DNA methylation

One of the best evidence for DNA methylation-mediated epigenetic inheritance is the study in the viable yellow agouti (A^{vy}) mouse model. The A^{vy} mouse was established by insertion of an intracisternal A particle (IAP) retrotransposon upstream of the transcription start site of the *Agouti* gene, in which the DNA methylation levels of IAP control *Agouti* gene expression. Since the agouti gene regulates the coat color of mouse, this mouse model is used to study nutritional and environmental alterations induced epigenetic inheritance. Pregnant female mouse fed with methyl donor supplementation such as folic acid, vitamin B12, or choline, altered the coat color of offspring toward the repressed state due to the hypermethylation of IAP-induced A^{vy} gene silencing [56].

Further, paternal nutrition stress induces the transgenerational epigenetic inheritance in offspring. The adult female offspring of paternal high-fat diet treatment showed impaired pancreatic β-cell function, such as impairment of insulin secretion and glucose tolerance. The expressions of 642 pancreatic islet genes in offspring were altered after paternal high-fat diet treatment. Among those, the expression *Il13ra2* gene was the highest increased, and methylation at some cytosine residue of *Il13ra2* was reduced in offspring after paternal high-fat diet. In addition, the offspring of paternal low-protein diet shows altered expression of a number of genes involved in lipid and cholesterol biosynthesis. The cytosine methylation of many DNA loci was changed in the livers of the offspring after paternal low-protein diet treatment. [57, 58].

3.2. Histone modification

Besides DNA methylation, histone modification was reported to mediate transgenerational inheritance. Although, most of histones are replaced by protamines, approximately15% histones were remained in human mature spermatozoa and few percent histones were remained in mouse. In *D. melanogaster*, the dATF2 contribute

to silence a white marker gene expression nearby the centromere in a heterochromatin-dependent manner. The flies exposed to heat shock stress and osmotic stress during early embryo development showed red eyes because the white gene silencing was most effectively disrupted. This stress-induced and dATF2-dependent heterochromatic disruption was inherited to the next generation in a non-Mendelian distribution. When embryos were exposed to heat stress over several generations, the epigenetic event was inherited for multiple successive generations, although it gradually returned to the normal status [52]. In fission yeast, deficient for histone demethylase *Epe1*, H3K9methylation was found to be a heritable epigenetic mark since Clr4-induced-H3K9me3 on target gene and the silent chromatin status were remained after mitotic and meiosis cell divisions, when Clr4 was released from its target gene [59].

3.3. RNA in sperm

It had been accepted that there is no rRNA in the highly condensed sperm nucleus, since most of their cytoplasm is extruded during the spermatogenesis. However, the presence of c-MYC mRNA in mature ejaculated human sperm was reaffirmed by RT-PCR analysis and *in situ* hybridization in 1993 [60, 61]. Then, the sperm RNA transcripts in healthy fertile men and mice were clarified using microarray and RNA sequencing. Moreover, paternal messenger RNAs were transmitted to the oocyte which is critical for embryo development. Thus, it is believed that not only the haploid DNA but also numbers of RNA transcriptions can be passed from father to his children. The RNA in spermatozoa appears to be a complex mixture of short RNA, ranging from 13 to 248 nt, which are small RNA and some break down products from rRNA, tRNA and mRNA. Bioinformatic analysis of the small RNA in sperm shows the dominant component is repeat-associated small RNAs, around 65%. Besides, microRNA and Piwi-interacting piRNAs also exists in sperm [62-66].

Interestingly, RNA transcriptions in sperm may mediate transgenerational epigenetic inheritance. *Kit* mutant mouse is characterized by the white tail tip and white feet. However, the white spots were found in the homozygous WT offspring when matting with two heterozygotes parents. This paramutation phenotype was in non-mendelian distribution. The accumulation of RNA in spermatozoa was observed and microinjection exogenous *Kit*-specific microRNAs into fertilized eggs could induce a heritable white tail phenotype [67]. In addition, unpredictable maternal

separation combined with unpredictable maternal stress (MSUS) was used to test the behavioral responses of offspring affected by traumatic stress across several generations. The offspring of MSUS mice exhibited a serial of abnormal behaviors such as reduced avoidance, fear, and altered response to aversive conditions. In sperm of father mouse with MSUS, small non-coding RNA expression pattern was altered. Injection of sperm RNAs from MSUS mice into fertilized zygotes could reproduce the abnormal behavioral and metabolic alterations in the offspring [68].

We have checked the histone modification in both father and son in response to paternal stress. We have also analyzed the TERRA RNA in both father and son after paternal stress. Further, we have identified the TERRA RNA expression in mature sperm, which may contribute to the inheritance of telomere length in response to stress.

Chapter 1. *In utero* TNF-α treatment induces telomere shortening of young adult mice in an ATF7-dependent manner

Epidemiological study indicated that stress exposure during intrauterine life is associated with shorter telomeres in young adulthood. Furthermore, it was suggested that telomere length in early life is correlated with lifespan. However, empirical study for those phenomena has not been performed, and the mechanism for stress-induced telomere shortening remains unknown. We recently reported that tumor necrosis factor α (TNF-α), of which level in periphery is increased by various psychological stresses, induces phosphorylation of transcription factor ATF7 and a release of telomerase-ATF7 complex from telomere, resulting to telomere shortening. Here, we report *in utero* TNF-α treatment in mice induces telomere shortening in adulthood. Telomere shortening was observed in limited tissues such as bone marrow, spleen, and lung. In addition, telomere shortening was detected in the some range of age during adulthood. Under those conditions, telomere shortening was not observed in *Atf7*-deficient mice. Thus, the conditions under which *in utero* TNF-α treatment induces telomere shortening in adulthood have been described.

1. Method

1.1. *Mice*

Congenic wild-type (WT) and *Atf7*$^{-/-}$ in the C57BL/6 genetic background, which were described previously [49], were used. Experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of the RIKEN Institute.

1.2. TNF-α administration

TNF-α (10 or 20 μg/kg weight) was intraperitoneally administered to pregnant mice daily during E2.5 ~ E18.5. DNAs were prepared from blood cells or various tissues of 1-6-weesk-old male mice, and used for Q-PCR to measure telomere length.

1.3. Preparation of DNA from various tissues

Various tissues were isolated from mice offspring at different time. In case of blood sample, red blood cell lysis buffer (0.155 M NH4Cl, 0.01 M KHCO3 and 0.1 mM EDTA, pH 7.4) was used to remove red blood cells. DNAs were prepared from various mice tissues by DNeasy Blood & Tissue Kit (Qigen).

1.4. Measurement of telomere length by Q-PCR

Using a real-time quantitative PCR method previously described [69, 70], average telomere length was measured in total genomic DNA prepared from various tissues. In this assay, average telomere length ratio is determined by quantitating telomeric DNA using a specifically designed primer sequence, and then dividing that value by the level of a single-copy gene measured in the same sample (36B4). The primer sequences used are shown in Table 1. Real-time PCR, using 1.5 ng DNA as a template, was performed using an ABI 7500 real-time PCR instrument and SYBR Green PCR Master Mix (Applied Biosystems), as previously described. The PCR conditions for telomeres were as follows: 95 ℃ for 10 min, and 25 cycles of 95 ℃ for 15 s and 54 ℃ for 2 min. The PCR conditions for 36B4 were as follows: 95 ℃ for 10 min, and 25 cycles of 95 ℃ for 15 s, 52 ℃ for 20 s, and 72 ℃ for 32 s. For each PCR reaction, a standard curve was made using serial dilutions of known amounts of DNA. The telomere signal (T) was normalized to the signal from the single-copy gene 36B4 (S) to generate a T/S ratio, indicative of relative telomere length. Equal amounts of DNA were used for each reaction, and several replicates of each reaction were run.

1.5. ChIP-Slot blot hybridization

ChIP assays were carried out essentially as described [54]. Splenocytes were crosslinked in 0.5% formaldehyde for 8 min at room temperature, and then glycine was added to a final concentration of 0.125 M to quench the crosslinking reaction. The chromatin was solubilized and extracted with lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% SDS, and protease inhibitor cocktail). The DNA was sheared into ~500 bp fragments by sonication, and then diluted 10-fold by dilution buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% Deoxycholate). Immunoprecipitation was carried out overnight at 4 ℃ with anti-ATF7 (2F10), anti-H3 (ab1791, Abcam) and anti-H3K9me3 (ab8898, Abcam) antibodies. Normal anti-mouse IgG antibodies were used as controls. Immunocomplexes were recovered using Dynabeads Protein G beads (10009D, Life

Technologies), and then incubated at 65℃ in 130 μl of IP elution buffer (1% SDS, 0.1 M $NaHCO_3$, 250 mM NaCl, 200 mg/ml proteinase K, and 10 mM DTT) to release proteins. The DNA was further purified using a QIAquick PCR Purification Kit (Qiagen) and eluted in 100 μl of elution buffer. Eluted DNA samples were blotted on Biodyne B membrane. The membrane was hybridized overnight using hybridization buffer containing 7% NaDodecyl sulfate and 1% BSA, 0.5 M phosphate buffer (pH7.2), and 1 mM EDTA at 42 ℃ with ^{32}P-labeled oligonucleotides telomeric probes: G24 probe, $[TTAGGG]_4$; C24 probe, $[CCCTAA]_4$. The signals were visualized and quantitated by using Image Analyzer 2500 (Fuji).

1.6. Statistics

Results are presented as means ± standard deviation (SD). Differences between groups were investigated for statistical significance using Student's t-test.

2. Result

2.1. In utero TNF-α treatment induces telomere shortening in blood cells of infant and young adult mice

We have first tested whether TNF-α injection into pregnant mice affects the telomere length of young adult. It was reported that injection of TNF-α into mice induces expression of multiple genes. Based on such papers, we examined the expression levels of some genes, and confirmed that expression of *Cxcl10*, *Cxcl9*, *NF-κB p65*, and *Cd14* were significantly induced in liver when 20 μg/kg of TNF-α was administrated into pregnant mice [54, 71]. Pregnant mice were intraperitoneally administrated everyday with 10 or 20 μg/kg of TNF-α or saline during E2.5~E18.5, and DNA was prepared from blood cells of new born mice at 1-week after birth. Telomere length was examined using Q-PCR method. Results indicated that telomere shortening was observed in blood cells of 1-week-old mice which were treated with 10 or 20 μg/kg of TNF-α during E2.5~E18.5 (Fig. 2-1A). We then also examined telomere length of blood cells at later stages. When we examined telomere length of blood cells of 3-week-old mice which were treated with 20 μg/kg of TNF-α during E2.5~E18.5, similar telomere shortening was observed (Fig. 2-1B). However, such telomere shortening was not observed in blood cells of 6-week-old mice. Thus, *in utero* TNF-α treatment induced telomere shortening in blood cells of 1- and 3-week-old mice, but not in 6-week-old mice.

2.2. In utero TNF-α treatment induces telomere shortening in some adult tissues

In human being, maternal psychosocial stress is associated with shorter telomere length in offspring, however the samples to measure telomere length is limited, mainly blood cells [10, 11]. Using mice, we next examined telomere length of various tissues of 1-week-old mice which had been exposed to *in utero* TNF-α treatment. When 10 or 20 μg/kg of TNF-α was administrated daily into pregnant mice during E2.5~E18.5, telomere shortening was observed in bone marrow of 1-week-old mice treated with 20 μg/kg of TNF-α , but not with 10 μg/kg of TNF-α. In spleen and lung, telomere shortening was detected with both 10 and 20 μg/kg of TNF-α treatment *in utero* (Fig. 2-2A). However, telomere shortening was not observed in thymus, brain, heart, liver, and kidney of 1-week-old mice. Thus, at 1 week after birth, only bone marrow, spleen and lung exhibited the telomere shortening induced by *in utero* TNF-α treatment.

We then examined the telomere shortening induced by *in utero* TNF-α treatment at 3-week after birth. After injection of 20 μg/kg of TNF-α into pregnant mice, such telomere shortening in bone marrow, spleen, and lung was not observed in 3-week-old mice (Fig. 2-2B). Thus, *in utero* TNF-α treatment induces telomere shortening of certain types of cells, including blood cells, bone marrow, spleen, and lung of infant mice, 1-week-old mice. However, such telomere shortening was canceled in most of tissues of young adult 3-week-old mice, and retained only in blood cells.

2.3. In utero TNF-α treatment does not induce telomere shortening in Atf7 deficient mice

We recently demonstrated that TNF-α injection into pregnant mice induced telomere shortening in mouse embryonic fibroblasts (MEFs) in an ATF7-dependent manner [54]. Since TNF-α induces ATF7 phosphorylation via p38, which causes a release of ATF7 and TERT from telomere in HeLa cells, similar mechanism may occur in MEFs. To examine whether the *in utero* TNF-α-induced telomere shortening in blood cells of young adult mice is also depend on ATF7, we mated *Atf7*-deficient ($Atf7^{-/-}$) male and female mice, and the generated pregnant mice were intraperitoneally administrated everyday with 20 μg/kg of TNF-α during E2.5~E18.5. Telomere shortening was observed in blood cells of WT but not $Atf7^{-/-}$3-week-old mice (Fig. 2-3A). These

results indicated that the *in utero* TNF-α-induced telomere shortening in bone marrow, spleen, and lung of 1-week-old mice is also depend on ATF7.

We also tested whether *in utero* TNF-α-induced telomere shortening in blood cells of young adult mice is also depended on ATF7. Telomere shortening was not observed in any tissues examined of 1-week-old *Atf7*$^{-/-}$ mice (Fig. 2-3B). Thus, the *in utero* TNF-α -induced telomere shortening in infant and young adult mice is mediated by ATF7.

2.4. In utero TNF-α treatment induces a release of ATF7 in adult splenocytes

We recently demonstrated that TNF-α induces ATF7 phosphorylation via p38, which causes a release of ATF7 and Suv39H1 histone H3K9 trimethyltransferase from telomere in HeLa cells. Therefore, we examined whether TNF-α treatment during E2.5~E18.5 also induce a release of ATF7 from telomere in splenocytes of 1-week-old mice. Splenocytes were prepared from 1-week-old mice which had been treated 20 μg/kg of TNF-α or saline during E2.5~E18.5, and the chromatins were cross-linked, fragmented, and immunoprecipitated using anti-ATF7 monoclonal antibody. Immunoprecipitated DNA was hybridized with telomere G or C probe. Results indicated that *in utero* TNF-α treatment reduced the amount of ATF7 on telomere of splenocytes from 1-week-old mice (Fig. 2-4A). These results suggest that once ATF7 is released from telomere by TNF-α treatment, it is not returned back.

We also examined whether TNF-α treatment during E2.5~E18.5 also induce a decrease in histone H3K9 trimethylation (H3K9me3) in splenocytes of 3-week-old mice. Results of similar ChIP/Slot blot hybridization experiments using anti-H3K9me3 indicate that *in utero* TNF-α treatment reduced the level of H3K9me3 on telomere of splenocytes from 3-week-old mice (Fig. 2-4B).

3. Discussion

Epidemiological study indicated that stress exposure in intrauterine life is associated with shorter telomere length in young adulthood. In the present empirical study, injection of TNF-α into pregnant mice, which is induced in the periphery by various psychological study, causes telomere shortening in young adult mice. Thus, the results of epidemiological study has now supported empirically. Furthermore, this telomere shortening occurred in an ATF7-dependent manner, indicating that TNF-α induces ATF7 phosphorylation via p38, which leads to a release of ATF7 and TERT from

telomere, resulting to telomere shortening. Telomere shortening occurred in such way was retained until young adult mice. At 3 weeks after birth, *in utero* TNF-α treatment-induced telomere shortening was observed only in blood cells, indicating that telomere shortening was retained about one month. TNF receptor, p38, and ATF7, which are involved in TNF-α-induced and ATF7-dependent telomere shortening, are expressed not only in blood cells but also other tissues, indicating that this specificity is not due to the expression specificity of these factors. Although the mechanism underlying this tissue specificity remain unknown at preset, it might be interesting that the lifespan of blood cells is around one month after differentiating from hematopoietic stem cells.

Even in the blood cells, *in utero* TNF-α treatment-induced telomere shortening detected at 3 weeks after birth was lost at 6 weeks after birth. We previously demonstrated that TNF-α shorten or lengthen telomere by different mechanisms. TNF-α induces ATF7 phosphorylation via p38, which causes a release of ATF7 and TERT, leading to telomere shortening. On the other hand, TNF-α also induces the nuclear entry of NF-κB p65 which brings TERT from cytosol into nuclei, leading to telomere lengthening. Furthermore, the TNF-α-induced ATF7 phosphorylation also causes a release of Suv39H1 histone H3K9 trimethyltransferase [54, 72]. In fact, we observed that *in utero* TNF-α treatment reduced the level of H3K9me3 on telomere of splenocytes from 3-week-old mice. In light of the observation that Suv39H1/2 mutations induce telomere lengthening [32], it is possible that the TNF-α-induced decrease in H3K9me3 on telomeres may cause telomere lengthening in a similar manner. Thus, TNF-α may shorten or lengthen telomere depending on the balance between different mechanisms. Amount of NF-κB p65, Suv39H1, and other factors that regulate the level of histone H3K9me3 might be changed during development, and such change could cause a loss of *in utero* TNF-α-induced telomere shortening detected at 6 weeks after birth.

Although epidemiological study indicated that stress exposure in intrauterine life is associated with shorter telomere length in young adulthood, only limited type of cell sample such as blood cells was used in such epidemiological study. Results of present study suggest that such telomere shortening might be detected only in limited types of tissue and cells. Thereore, such telomere shortening may not necessarily link to the short lifespan, altough that telomere length is widely belived to be correlated

with lifespan. Rather, such telomere shortening in the limited types of tissus may chage metabolism and increase the level of reactive oxygen species as reported [25], which may be toxic for other tisues as a diffusible molecule and cause certen type of disease.

In summary, we found that *in utero* TNF-α treatment induced telomere shortening in certain tissues of infant and young adult mice in an ATF7-dependent manner. This finding may be useful to understand the mechanism of stress-induced telomere-related diseases of human being.

Chapter 2. Paternal TNF-α treatment programs telomere shortening in mouse offspring

The life histories of parents affect the telomere length of children. Nonetheless, telomere length inheritance in response to paternal stress has not been confirmed. Transcription factor ATF7 regulates telomere length and telomeric heterochromatin structure. Stress-induced and dATF2-dependent epigenome change in *D. melanogaster* was inherited to the next generation either maternally or paternally. Thus we attempt to examine whether paternal TNF-α treatment induces telomere shortening in offspring, and the role of ATF7 in telomere length inheritance in response to stress. Here, we have found that paternal TNF-α treatment induces telomere shortening in male mice offspring. However under such condition the telomere length of their fathers was not changed. The telomere shortening of offspring was not observed in *Atf7*-deficient mice under the same condition. Paternal TNF-α treatment induced ATF7 phosphorylation, leading to a release of ATF7 from telomere and TERRA RNA promoter region in paternal germ cells. Thus, TNF-α-induced and ATF7-dependent decreases of H3K9me3 in germ cells at telomere and TERRA RNA promoter region were observed. Oppositely, increases of H3K9me3 at both regions were observed in next generation MEFs. Furthermore, increases of TERRA RNA levels in both paternal spermatozoa and son were observed. Thus, TNF-α-induced and ATF7-dependent increase of TERRA RNA in sperm could be inherited to children and regulate the telomere length, and ATF7 might play an important role on inheritance of telomere length in response to stress.

1. Methods

1.1. *Mice*

Congenic wild-type (WT) and *Atf7*$^{+/-}$ in the C57BL/6 genetic background, which were described previously [49], were used. Experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of the RIKEN Institute.

1.2. TNF-α administration

TNF-α (5, 10 or 20 μg/kg weight) was intraperitoneally administered to adult mice (8 weeks) daily for 4-8 weeks. The offspring was prepared by matting with WT female

mice (9 weeks). Blood or spleen of offspring was collected from 3 and 8-weesk-old male mice. MEFs were prepared from embryo at E14.5. Genotype and gender were confirmed by PCR.

1.3. Preparation of DNA from various tissues

Various tissues were isolated from mice offspring at different time. In case of blood sample and spleen, red blood cell lysis buffer (0.155 M NH4Cl, 0.01 M KHCO3 and 0.1 mM EDTA, pH 7.4) was used to remove red blood cells. DNAs were prepared from various mouse tissues by DNeasy Blood & Tissue Kit (Qigen).

1.4. Measurement of telomere length by Q-PCR

Same as above.

1.5. ChIP-Slot blot hybridization

Same as above.

1.6. qRT-PCR

RNA was purified from various tissues and cells by Trizol reagent (Invitrogen). TERRA RNA from various chromatins were examined by qRT-PCR using SYBR green realtime PCR Master Mix (TOYOBO). Primers used were described in Table 1.

1.7. TERRA expression in Spermatozoa

Mature spermatozoa was isolated from cauda epididymis by squeezing the cauda epididymis in HTF medium (Millipore), purified by density gradient separation using Sperm Separation Media (SAGE), followed by washing in somatic cell lysis buffer (0.1% SDS, 0.5% Triton X-100). Sperm RNA was purified by Trizol reagent (Invitrogen) with DNase I treatment. TERRA RNAs in spermatozoa were measured by ploy (A)-tailed qRT-PCR. Poly (A) tailing and first-strand cDNA synthesis was performed using miRNA cDNA Synthesis Kit (Invitrogen), and cDNA level was measured by SYBR green qPCR Mix (TOYOBO). Primers used were described in Table 1.

1.6. Statistics

Results are presented as means ± standard deviation (SD). Differences between groups were investigated for statistical significance using Student’s t-test.

2. Result

2.1. Paternal TNF-α treatment induces telomere shortening in mice offspring

To analyze whether paternal stress induces telomere shortening in offspring, we analyzed the telomere length of peripheral blood mononuclear cell (PBMC) in offspring, after paternal saline or TNF-α (10 μg/kg weight) treatment for 4, 6 and 8 weeks. Telomere length was examined using Q-PCR method. Results indicated that telomere length of offspring was about 19.0% shorter than saline-treated WT offspring, when paternal TNF-α (10 μg/kg weight) treatment for 6 weeks was given (Fig.3-1A). However, when TNF-α was paternally treated for 4 or 8 weeks daily, telomere shortening in offspring was not observed (Fig. 3-1A). Further, when 10 or 20 μg/kg weight TNF-α was administrated to mice for 6 weeks, telomere length reduction was observed, about 19% and 16.6%, respectively (Fig. 3-1B), while 5 μg/kg TNF-α treatment did not give any significant telomere shortening in offspring. Thus, in the following experiments, we used the condition of 10 μg/kg weight TNF-α treatment for 6 weeks.

To understand the role of ATF7 in paternal TNF-α-induced-telomere shortening; we measured the telomere length of offspring at 3-weeks old of WT and *Atf7*$^{+/-}$ mice. In the pups of *Atf7*$^{+/-}$ mice, the telomere length was not significantly different between paternal saline and paternal TNF-α treatment group, while 13.6% reduction was observed in the WT offspring of after TNF-α treatment (Fig. 3-1C). By using the male and female separately, we found telomere length of male pups was significantly reduced after paternal TNF-α treatment, while female pups did not (Fig. 3-1D). Furthermore, we determined the telomere length of PBMC at 8-week old, to analyze the telomere length in the young adult mice. 15.6% reduction in the offspring of WT mice was observed after paternal TNF-α treatment, but not in the pups of *Atf7*$^{+/-}$ mice (Fig. 3-1E). This suggested that paternal stress-induced telomere shortening in offspring occurs in an ATF7-dependent manner.

2.2. Paternal TNF-α-induced ATF7-dependent telomere shortening in offspring is not associated with the telomere length of their fathers

We measured the telomere length in the F_0 generation. When TNF-α was injected for 6 weeks with 10 μg/kg weight, the telomere shortening was not observed in somatic cells (i.e., blood and spleen) or germ cells (testicular germ cell and spermatozoa) (Fig. 3-2A, B). Surprisingly, this is opposite from the telomere length of their children,

although TNF-α treatment for 8 weeks could induce telomere shortening in mature spermatozoa and it is in dose dependent manner (Fig. 3-2C). Thus, we speculate that paternal TNF-α-induced ATF7-dependent telomere shortening in offspring is not directly linked to the telomere length of their fathers.

2.3. Paternal TNF-α treatment induces a release of ATF7 from telomere.

We recently demonstrated that TNF-α induces ATF7 phosphorylation via p38, leading to the release of ATF7 from telomeres in HeLa cells [54]. Western blot was performed in TNF-α–treated paternal testicular germ cells to detect the phosphorylation level of ATF7. Results showed that one hour after TNF-α addition, ATF7 phosphorylation level increased compared with saline-treated mouse. TNF-α treatment for 6 weeks also increased the phosphorylation level of ATF7 (Fig. 3-3A). Using testicular germ cells, chromatin immunoprecipitation was performed to check the ATF7 amount on telomere. The chromatins were cross-linked, fragmented, and immunoprecipitated using anti-ATF7 monoclonal antibody. Immunoprecipitated DNA was hybridized with telomere G or C probe, Telomeric ATF7 amounts were decrease after TNF-α treatment for 6 weeks in testicular germ cell (Fig. 3-3B).

2.4. Paternal TNF-α-induced ATF7-denpendent decrease of H3K9me3 was not observed in offspring.

It was reported that ATF7 and orthologs of ATF7, *drosophila* ATF2 (dATF2) and yeast *S. pombe* Atf1, contribute to heterochromatin formation via recruiting HMTases such as G9a, ESET and Suv39H1 [49, 54]. Transgenerational epigenetic inheritance of heterochromatin has been found in various organisms. Thus, we determined the H3K9me3 and H3K9me2 level on telomere region by ChIP slot-blot hybridization in both paternal germ cells and MEFs from their children. Telomeric H3K9me2 and H3K9me3 levels in WT paternal germ cells were significantly decreased by TNF-α-treatment, whereas such decrease was not observed in *Atf7*$^{+/-}$ germ cells (Fig. 3-4A). Oppositely, H3K9me3 level on telomere region significantly increased after paternal TNF-α-stimulation in the offspring of WT mice. Paternal TNF-α did not change telomeric H3K9me3 level in the offspring of *Atf7*$^{+/-}$ mice (Fig. 3-4B). These results suggest that TNF-α-induced decrease of H3K9me3 in father is not directly inherited to the next generation and there could be a feedback regulation system to control the heterochromatin formation during early embryo development of the next generation.

2.5. Paternal TNF-α-induced ATF7-denpendent increase of TERRA RNA in both fathers and son.

TERRA RNA is a telomere transcription which contributes to regulate the telomere length and telomeric heterochromatin formation [35, 36, 41]. We first performed the ATF7 ChIP to check the binding of ATF7 on TERRA promoter region. Although all of TERRA RNA promoters were not completely analyzed, the TERRA RNA promoter on chromosome 8 was reported [38]. We found one CRE-like site (TGAAGTCA), which ATF7 may recognize, in TERRA-8q promoter region. Primers to detect non-CRE and CRE-like sites were used to determine the binding of ATF7 on TERRA-8q promoter. Result showed that ATF7 bound to both CRE and non-CRE sites. TNF-α induced a release of ATF7 from the both sites on TERRA-8q promoter in germ cells. (Fig. 3-3C) Together with previous findings, ATF7 may regulate telomeric heterochromatin formation via regulating TERRA RNA expression.

TERRA RNA on chromosome 8q, 11q and 5q were measured by qRT-PCR analysis in both father and children. In F_0 testicular germ cells, TNF-α induced TERRA RNA (8q, 11q, 5q) expression in WT mice, but not in *ATF7*$^{+/-}$ mice (Fig. 3-5A). Similarly, in both F_1 male MEFs and spleen, paternal TNF-α treatment increased TERRA RNA (8q, 11q, 5q) expression in the offspring of WT mice, compared to paternal saline-treated pups. On the other hand, in the male MEFs and spleen cells from *ATF7*$^{+/-}$ offspring, TERRA RNA expression was not significantly changed after paternal TNF-α treatment (Fig. 3-5B). While the expression level of TERRA RNA did not change in response to paternal TNF-α treatment in F_1 WT female MEFs (Fig. 3-5C).

To further understand how ATF7 regulates the TERRA RNA expression, H3K9me3 level on TERRA-8q promoter was measured by ChIP analysis. In F_0 testicular germ cells of WT mice, H3K9me3 level was decreased by TNF-α treatment, but not in the *ATF7*$^{+/-}$ germ cells (Fig. 3-5D). On the other hand, paternal TNF-α induced increase of H3K9me3 on TERRA-8q promoter in the offspring of WT mice, but not in the pups of *ATF7*$^{+/-}$ mice (Fig. 3-5E). These data suggests that ATF7 regulates the TERRA RNA expression through regulating the H3K9me3 level on telomere and TERRA RNA promoter region. Paternal TNF-α treatment induces a release of ATF7 from telomere and TERRA RNA promoter region may lead to increase of TERRA RNA expression, which can be inherited to the next generation.

2.6. Paternal TNF-α-induced ATF7-denpendent increase of TERRA RNA in paternal spermatozoa

The significant amounts of RNA in mature spermatozoa led to speculate its possible role of embryonic development. It was discovered that RNA-mediated inheritance of epigenetic traits in mouse led us to conclusion that sperm RNAs may act as transgenerational epigenetic inheritance [67, 68]. Here we determined the TERRA RNA amount in mature sperm. We first purified the sperm from cauda epididymis followed by density gradient separation and somatic cell lysis buffer washing. To confirm most contaminants were removed, RT-PCR was performed as previous reported [66] to check the markers of blood cells, germ cells, and reproductive system tissue fragments (Cd45, c-Kit, and E-cadherin), and the results showed that most of the contaminants were eliminated (Fig.3-6A). Using the primer for the 5’ end of TERRA RNA on subtelomere region, no PCR product was observed (Fig. 3-6A). As sperm RNA appeared as a complex mixture of break down products, we used the ploy (A)-tailed RT-PCR to amplify the 3’ end (UUAGGG repeat sequence). By polyadenlyated and reverse-transcribed all the miRNAs using poly (A) polymerase and a specially designed universal RT primer, we measured the TERRA RNA level in sperm using a universal primer and telomere primer. Gel electrophoresis of the PCR product showed a clear signal band (Fig. 3-6B). Spr-13, mouse spermatozoa specified noncoding RNA, and let-7a, lower expression in sperm, were used as controls [65]. Further, quantitative Q-PCR data showed that TNF-α treatment increased the TERRA RNA level in WT mouse sperm, while there was no significant difference between saline and TNF-α treatment in *Atf7*$^{+/-}$ mice (Figure 3-6C). This suggested that sperm could carry stress-induced higher amount of TERRA RNA to zygote and regulate the telomere homeostasis in the next generation.

Thus, paternal TNF-α treatment induced telomere shortening in offspring in an ATF7-dependent manner. ATF7 regulates TERRA RNA expression through histone modification on its promoter region and telomere region. TNF-α-induced release of ATF7 from telomere and TERRA RNA promoter may lead to increase of TERRA RNA expression. TNF-α-induced and ATF7-denpendent increase of TERRA RNA seems to be retained in spermatozoa, which could be passed to zygote and mediate the telomere shortening and enhance of H3K9me3 on telomere and subtelomere region in the next generation.

3. Discussion

Epidemiological study showed that telomere length of offspring is associated with life histories of their parents [14]. Here, we found that paternal TNF-α induces telomere shortening in male offspring without the change of telomere length of their fathers in an ATF7-dependent manner. Our previous data showed that ATF7 recruits TERT to telomere and that TNF-α induces a release of ATF7 and TERT from telomere, leading to telomere shortening in MEFs and HeLa cells [54]. Epidemiological study also indicated that psychological stress-induced telomere shortening is significantly associated with an increase of oxidative stress and a decrease of telomerase activity in peripheral blood mononuclear cells [7]. This is consistent with our resluts that TNF-α treatment for 8 weeks induceed telomere shortening in sperm. However, TNF-α treatement for 6 weeks did not change the telomere length in our system, which could be due to the relatively shorter period and the less strength of stress. It aso might bo correlated with that the turnover time of blood cells and time course of spermatogenesis are about one month in mouse [73]. ATF7 is highly expressed in spermatogonia and spermatocyts. Thus, treatment for 6 weeks may not be sufficient to induce telomere shortening in sperm cells, while it might be sufficient to induce some epigenetic modification and RNA expression in spermatogonia and spermatocyts which are maintained in mature sperm. On the other hand, 8-weeks-treatment did not induce telomere shortening in mice offspring. This may be correlated with the TERRA RNA level in sperm. The stress-induced telomere shortening in offspring was not observed in female mice, which might be due to no change of TERRA RNA in female MEFs. Thus, the stress-induced and ATF7-dependent telomere shortening in offspring was not associated with the telomere length of their fathers.

TNF-α induced ATF7 phosphorylation in testicular germ cell, which is consistent with the previous reports using MEFs, HeLa cells, macrophage, and mouse brain. We have found that ATF7 binds to telomere and TERRA RNA promoter region. After TNF-α treatment, ATF7 was released from its target genes. ATF7 recruits histone HMTases, such as ESET, G9a, Suv39H1/2 to contribute to heterochromatin formation and silent the gene expression [49, 51, 54]. In the present study, we found that the H3K9me3 levels on both telomere and TERRA RNA promoter region were decreased after TNF-α treatment in an ATF7-dependent manner. It is well known that TERRA RNA expression is negative regulated by H3K9me3 level on telomere region,

and that heat shock stress induces TERRA RNA expression in MEFs [35, 40]. These reports may be consistent with our result that TNF-α induced TERRA RNA expression in an-ATF7-dependent manner in sperm cells, which and might be caused by both decrease of H3K9me3 level on telomere and TERRA promoter region.

TERRA RNA interacts with several telomere-associated proteins, including TRF1 and TRF2, subunits of ORC, HP1, and H3K9me3. Thus, TERRA facilitates telomere structural maintenance and heterochromatin formation via interaction with TRF2 and ORC [41]. We have found the paternal TNF-α treatment induces the increase of H3K9me3 level in F_1 MEF, which may suggest that there might be a TERRA RNA-mediated feedback control system in early embryo development to counterbalance the stress-induced heterochromatin disruption.

Induction of TERRA RNA transcription leads to telomere shortening in yeast and mammalian [42, 43]. TERRA RNA inhibits telomerase activity *in vitro* by binding to both the TR and TERT. TERRA RNA level declines at the late S phase when telomerase preferentially elongates the shortest telomere [35, 36]. Thus, TERRA RNA may negatively regulate telomere length. These observations suggest that the increase of TERRA may induce the telomere shortening in offspring. However, some reports showed that TERRA RNA could mediate telomerase recruitment to shortened telomeres. Thus, the mechanism of telomere length regulation by TERRA RNA is not completely clarified. We have found the TERRA RNA was up-regulated in male MEFs but not in female cells, which might be consistent with the results that paternal TNF-α treatment induced telomere shortening in male offspring but not in female. This may also support the notion that that TERRA RNA induces telomere shortening in response to stress.

In conclusion, paternal stress induces telomere shortening in male offspring via TERRA RNA which is repressed by ATF7. The stress-induced and ATF7-dependent induction of TERRA RNA in sperm may be transmitted to the next generation and control the telomere length and telomeric heterochromatin formation during early embryo development.

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Figure Legend

Fig. 1-1. Telomere structure and Telomerase recruitment

(A) Telomere structure and Telomere binding protein in mammalian. (B) Telomerase recruitment by TPP1. (C) ATF7 mediated telomerase recruitment via Ku70/80 heterodimer, and stress induced a release of TERT from telomere. (D) Telomeric and subtelomeric heterochromatin structure.

Fig. 1-2. TEERA RNA

TERRA RNA is transcript from subtelomere to telomere by RNAPII. TERRA contributes to heterochromatin formation.

Fig. 1-3. ATF2 family proteins

(A) Domain structures of ATF2 family proteins. (B) ATF7 silent gene expression and contribute to telomeric heterochromatin. Stress induces a release of ATF7 from telomere.

Fig. 2-1. *In utero* TNF-α treatment induces telomere shortening in adult blood cells.

(A) WT pregnant mice were intraperitoneally injected with saline or TNF-α (10 or 20 μg/kg weight) daily from E2.5 to E18.5. Blood cells were prepared from 1-week-old mice, and used for telomere length measurement by Q-PCR. (B) WT pregnant mice were intraperitoneally injected with saline or TNF-α (20 μg/kg weight) daily from E2.5 to E18.5. Blood cells were prepared from 3- or 6-week-old mice, and used for telomere length measurement by Q-PCR. Relative telomere length, expressed as T/S ratio (see Methods), is shown ± SD (n = 6-8). *, $p < 0.05$; N.S., no significant difference.

Fig. 2-2. *In utero* TNF-α treatment induces telomere shortening in some adult tissues.

(A and B) WT pregnant mice were intraperitoneally injected with saline or TNF-α (10 or 20 μg/kg weight) daily from E2.5 to E18.5. DNAs were prepared from indicate tissues of at 1-week-old (A) or 3-week-old (B) mice, and used for telomere length measurement by Q-PCR. Relative telomere length, expressed as T/S ratio, is shown ± SD (n = 6-8). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; N.S., no significant difference.

Fig. 2-3. *In utero* TNF-α treatment does not induce telomere shortening in Atf7-deficient mice.

(A and B) WT or *Atf7* deficient (*Atf7*$^{-/-}$) pregnant mice were intraperitoneally injected with saline or TNF-α (20 μg/kg weight) daily from E2.5 to E18.5. Since *Atf7*$^{-/-}$ pregnant mice were generated by mating with *Atf7*$^{-/-}$ male mice, all mice born were *Atf7*$^{-/-}$ mice. DNAs were prepared from indicate tissues of mice at 3-week-old (A) or 1-week-old (B), and used for telomere length measurement by Q-PCR. Relative telomere length, expressed as T/S ratio, is shown ± SD (n = 3-4). ***, $p < 0.001$; N.S., no significant difference.

Fig. 2-4. *In utero* TNF-α treatment induces a release of ATF7 and a decrease in histone H3K9me3 in adult splenocytes.

(A and B) WT pregnant mice were intraperitoneally injected with TNF-α (20 μg/kg weight) daily from E2.5 to E18.5. Splenocytes were prepared from 1-week-old mice (n=3), and used for chromatin immunoprecipitation with anti-ATF7 or IgG (A). Splenocytes were prepared from 3-week-old mice (n=4), and used for chromatin immunoprecipitation with anti-histone H3K9me3 or IgG. Average signals relative to input ± SD are shown, and typical data from slot-blot hybridization are shown below. (B). Recovered DNAs were subjected to slot-blot hybridization with a ^{32}P-labeled telomere probe. Average signals relative to H3 ± SD are shown, and typical data from slot-blot hybridization are shown below. *, $p < 0.05$; **, $p < 0.01$;***, $p < 0.001$; N.S., not significant.

Fig. 3-1. Paternal TNF-α treatment induces telomere shortening in mouse offspring via ATF7.

Telomere length of offspring was analyzed by Q-PCR in blood cells. (A and B) WT male mice were daily administered with saline or various doses TNF-α for indicated weeks. Blood was collected from their offspring (n=5-14) which were prepared by matting with naïve WT female mice. (C, D and E) WT and *Atf7*$^{+/-}$ mice were daily treated with saline or TNF-α (10 μg/kg weight) for 6 weeks. Telomere length was analysis by using all the pups (n=10-12) at 3-week old (c). Telomere length of 3-week-old mice was calculated by using male (n=6) or female pups (n=4-6), respectively (d). Telomere length of blood cells was determined in male mice at 8-week old (n=4-5, e). Relative telomere length, expressed as the T/S ratio, is shown ± SD. *, $p < 0.05$; **, $p < 0.01$; NS. not significant.

Fig. 3-2. Paternal TNF-α-induced and ATF7-dependent telomere shortening in offspring is not associated with the telomere length of their father.

Telomere length of somatic cells (A) and germ cells (B) was determined by Q-PCR in F_0 mice. WT and *Atf7*$^{+/-}$ mice were daily administered with saline or TNF-α (10 μg/kg weight) for 6 weeks. (C) Telomere length of spermatozoa in F_0 mice, which were treated by saline or TNF-α (5, 10 and 20 μg/kg weight) for 8 weeks. Relative telomere length, expressed as the T/S ratio, is shown ± SD (n=3). *, $p < 0.05$; NS. not significant.

Fig. 3-3. Paternal TNF-α treatment induces release of ATF7 from telomere and TERRA RNA promoter in germ cells.

(A) TNF-α induces phosphorylation of ATF7 in testicular germ cells. Testicular germ cells were prepared from WT mice after saline or TNF-α (10 μg/kg weight) single treatment for indicted time. Testicular germ cells were also prepared from mice daily administrated with TNF-α (10 μg/kg weight) for 6weeks. Western blotting was performed using whole cell lysis. (B) TNF-α induces a release of ATF7 from telomere in testicular germ cells. Chromatin immunoprecipitation was performed using anti-ATF7 and IgG antibodies in testicular germ cells. Mice were treated as Fig. 3-2A. Recovered DNAs were subjected to slot-blot hybridization with ^{32}P-labeled telomere probes. Average value relative to input ± SD is shown (n =3), and typical data from slot-blot hybridization are shown below. *, $p < 0.05$; **, $p < 0.01$. (C) TNF-α induces a release of ATF7 from TERRA RNA promoter in testicular germ cell. Chip was performed as above. TERRA-8q promoter sequence was used as report. Primers of CRE region and non-CRE region, as indicted, were used for Q-PCR. Average value relative to input ± SD is shown (n = 3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 3-4. Paternal TNF-α-induced and ATF7-denpendent decrease of H3K9me3 was not observed in offspring.

(A) TNF-α induces decrease of H3K9me3 and H3K9me2 on telomere in F_0 germ cells. ChIP was performed by anti-H3, H3K9me3 and H3K9me2 antibodies in WT or *Atf7*$^{+/-}$ testicular germ cells (n=4). Mice were treated as Fig. 3-2A (B) Paternal TNF-α treatment induced increase of H3K9me3 on telomere in F_1 male MEFs. MEFs were prepared at day E14.5 (see Methods). ChIP was performed by anti-H3 and H3K9me3 antibodies in WT or *Atf7*$^{+/-}$ F_1 male MEFs (n=3). Paternal mice were treated as Fig. 3-2A. Recovered DNAs were subjected to slot-blot hybridization with ^{32}P-labeled

telomere probes. Average value relative to H3 ± SD is shown, and typical data from slot-blot hybridization are shown below. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS. not significant.

Fig. 3-5. Paternal TNF-α-induced and ATF7-denpendent increase of TERRA RNA in both fathers and son.

(A) TERRA RNA expression was determined by qRT-PCR in testicular germ cells from father mice. (B) MEFs and spleen cells were isolated from F_1 male mice to measure the TERRA RNA level. (C). MEFs isolated from F_1 female mice embryo were used to analysis the TERRA RNA level. Average value relative to U6 snRNA ± SD is shown (n=3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS. not significant. (D) TNF-α induces decrease of H3K9me3 on TERRA RNA promoter region in F_0 germ cells in an ATF7-dependent manner. (E) Paternal TNF-α treatment induced increase of H3K9me3 on TERRA RNA in F_1 male MEFs in an ATF7-dependent manner. H3K9me3 Chip assay was performed as Fig. 3-4. Recovered DNAs were amplified by qPCR. Primers of non-CRE region were used to determine the TERRA RNA promoter level. Average value relative to H3 ± SD is shown (n = 3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS. not significant.

Fig. 3-6. Paternal TNF-α-induced ATF7-denpendent increase of TERRA RNA in paternal spermatozoa.

(A) 5’ TERRA RNA was not observed in spermatozoa. RT-PCR was using to amplified 5’ TERRA RNA, 18S rRNA and some possible contaminant markers such as E-cadherin, Cd45 and c-Kit. The PCR products were electrophoresed on 3% agarose gel and stained with ethidium bromide. (B and C) 3’ TERRA RNA expression level in spermatozoa. Poly (A) tailed PCR was used to analysis the level of TERRA RNA in sperm (C). PCR products were electrophoresed on 3% agarose gel and stained with ethidium bromide (B). Spr-13 and let-7a are controls. Average value relative to 18S rRNA ± SD is shown (n = 3). *, $p < 0.05$; NS. not significant.

Figure

Figure 1-1.

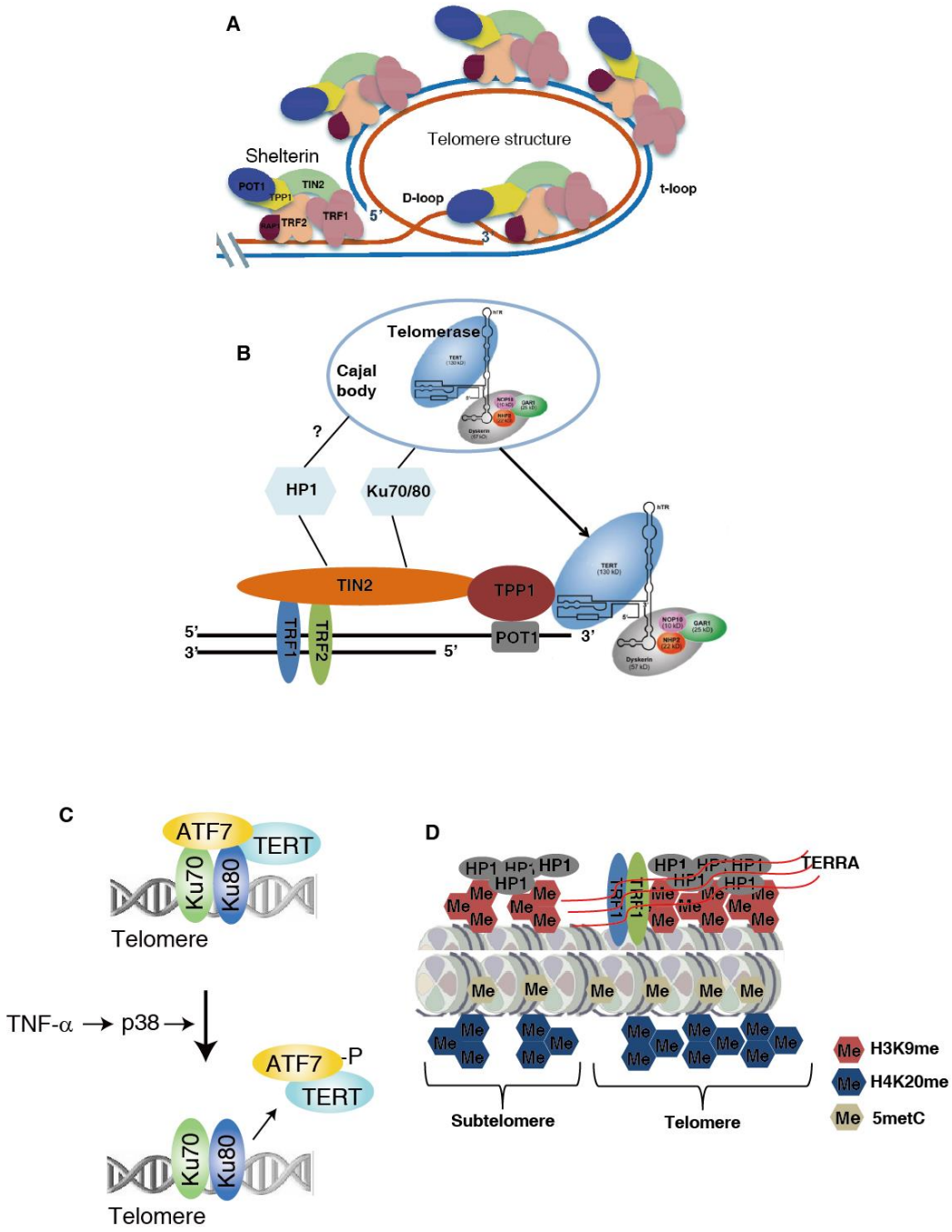


Figure 1-2.

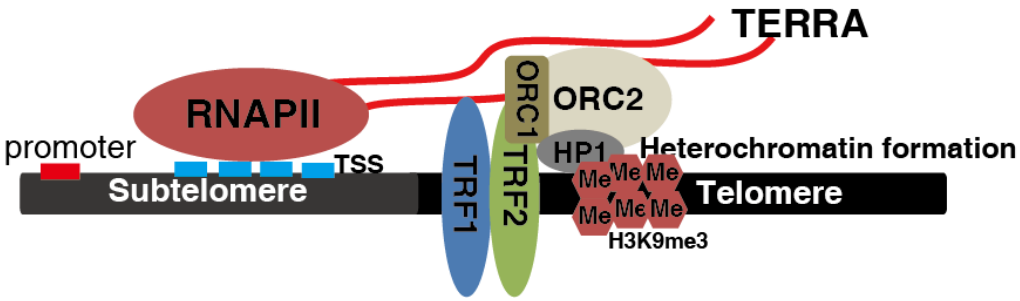

TERRA
RNAPII
ORC1
ORC2
HP1
Heterochromatin formation
promoter
TSS
Subtelomere
TRF1
TRF2
Telomere
Me
H3K9me3

Figure 1-3.

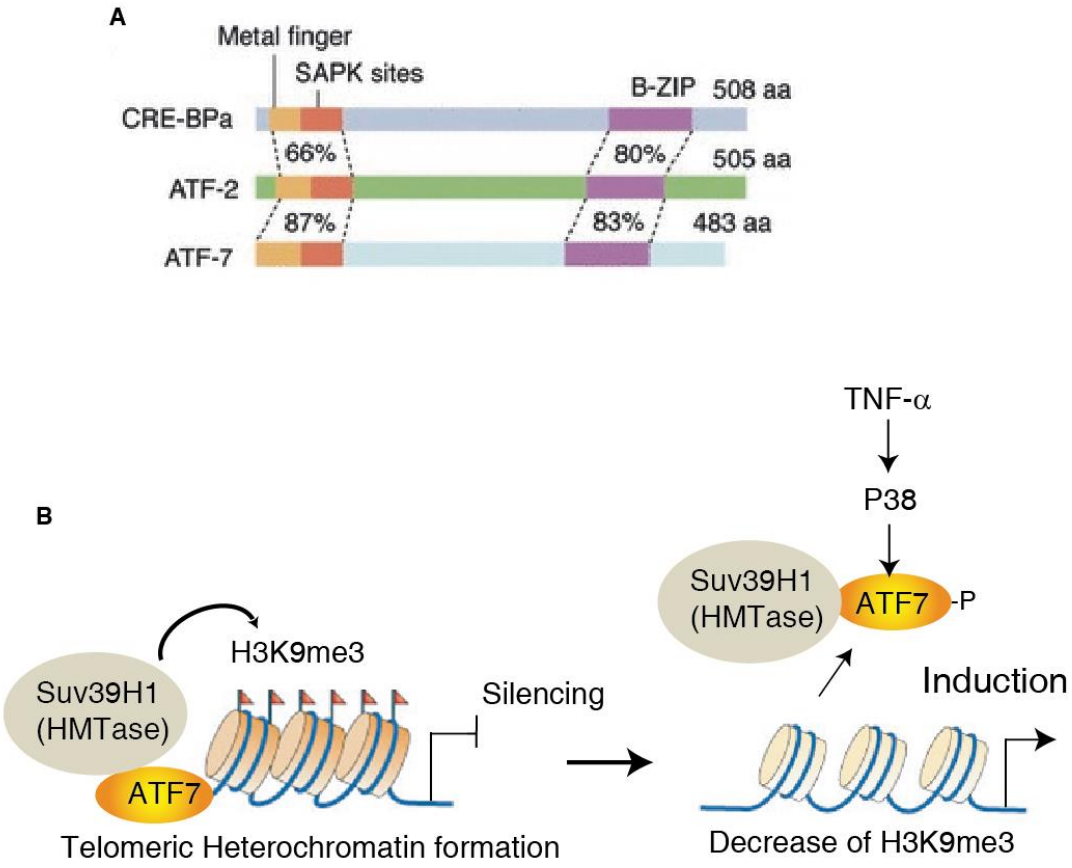

A
Metal finger
SAPK sites
B-ZIP
CRE-BPa
508 aa
66%
80%
505 aa
ATF-2
87%
83%
483 aa
ATF-7
B
TNF-α
P38
Suv39H1
(HMTase)
ATF7
-P
H3K9me3
Suv39H1
(HMTase)
Silencing
Induction
ATF7
Telomeric Heterochromatin formation
Decrease of H3K9me3

Figure 2-1.

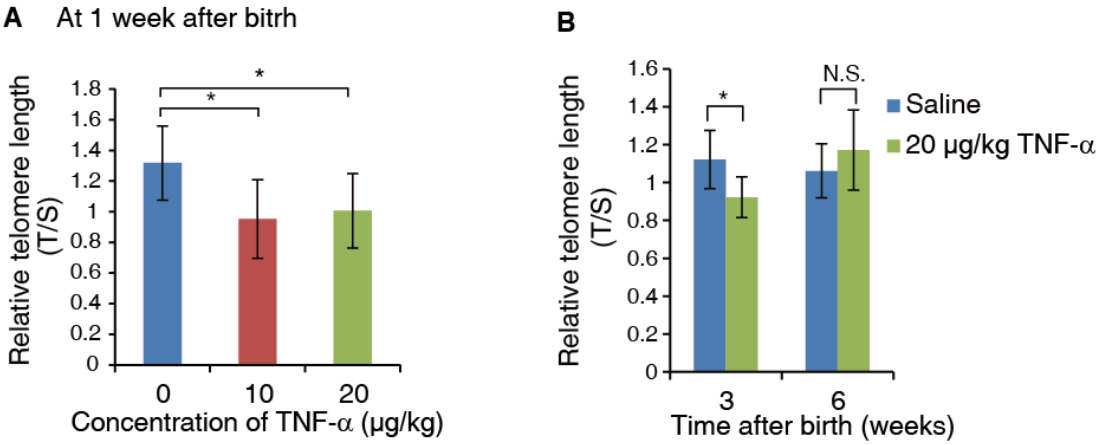
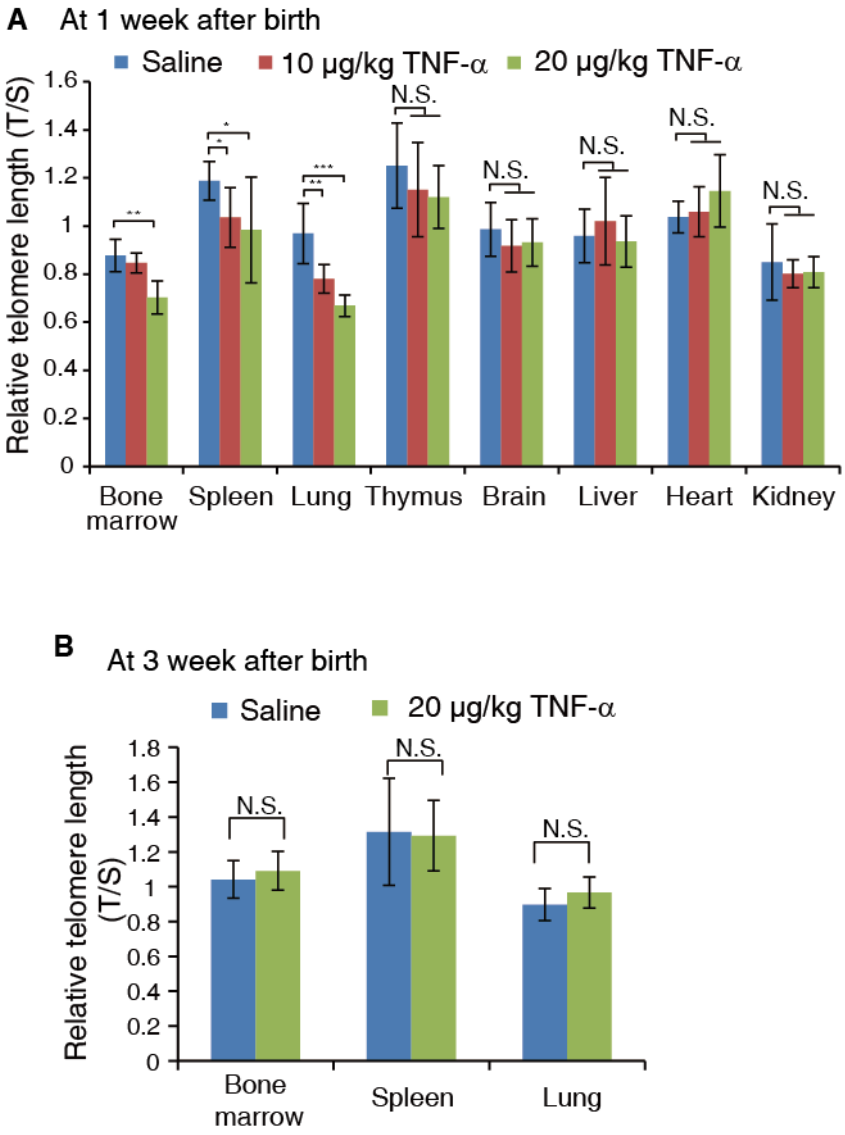

A
At 1 week after bitrh
Relative telomere length (T/S)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
0
10
20
Concentration of TNF-α (μg/kg)
B
Relative telomere length (T/S)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
N.S.
Saline
20 μg/kg TNF-α
3
6
Time after birth (weeks)

Figure 2-2.


A
At 1 week after birth
Saline
10 μg/kg TNF-α
20 μg/kg TNF-α
Relative telomere length (T/S)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
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**
*
***
N.S.
N.S.
N.S.
N.S.
N.S.
Bone marrow
Spleen
Lung
Thymus
Brain
Liver
Heart
Kidney
B
At 3 week after birth
Saline
20 μg/kg TNF-α
Relative telomere length (T/S)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
N.S.
N.S.
Bone marrow
Spleen
Lung

Figure 2-3.

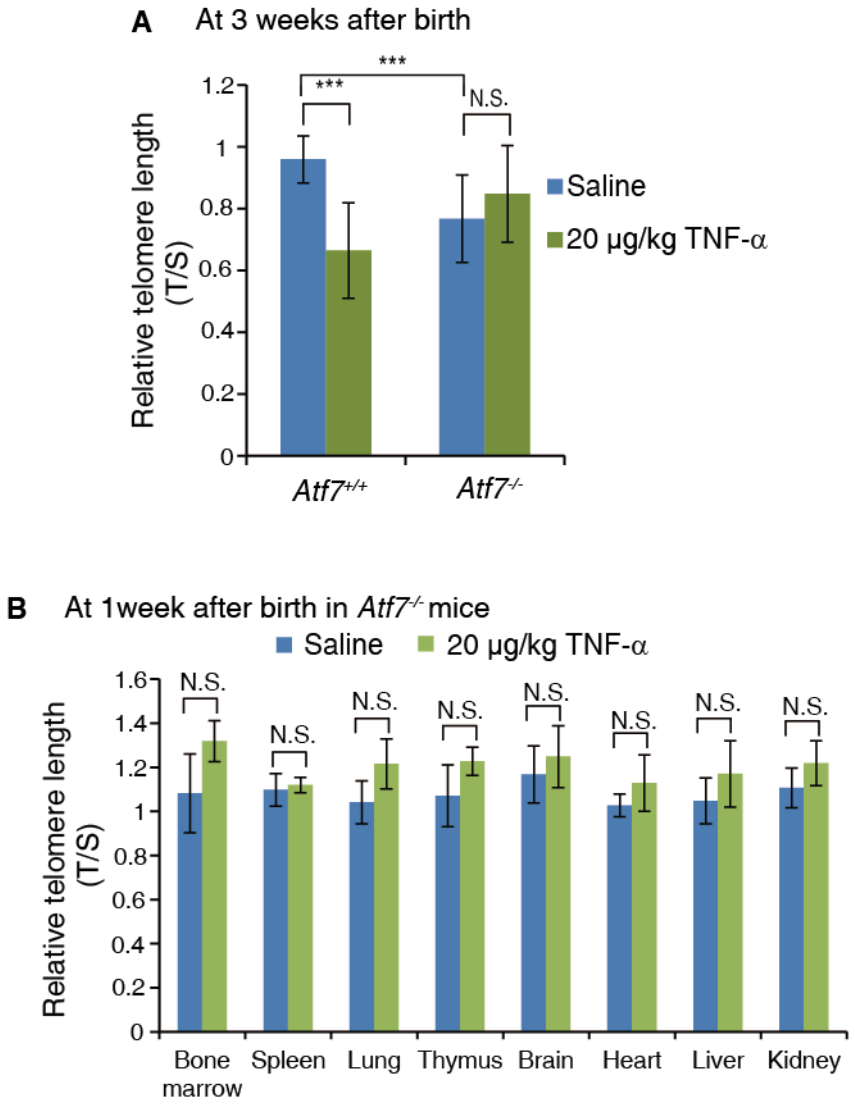
A
At 3 weeks after birth
***
***
N.S.
Relative telomere length (T/S)
1.2
1
0.8
0.6
0.4
0.2
0
Saline
20 μg/kg TNF-α
Atf7+/+
Atf7-/-
B
At 1week after birth in Atf7-/- mice
Saline
20 μg/kg TNF-α
Relative telomere length (T/S)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
Bone marrow
Spleen
Lung
Thymus
Brain
Heart
Liver
Kidney

Figure 2-4.

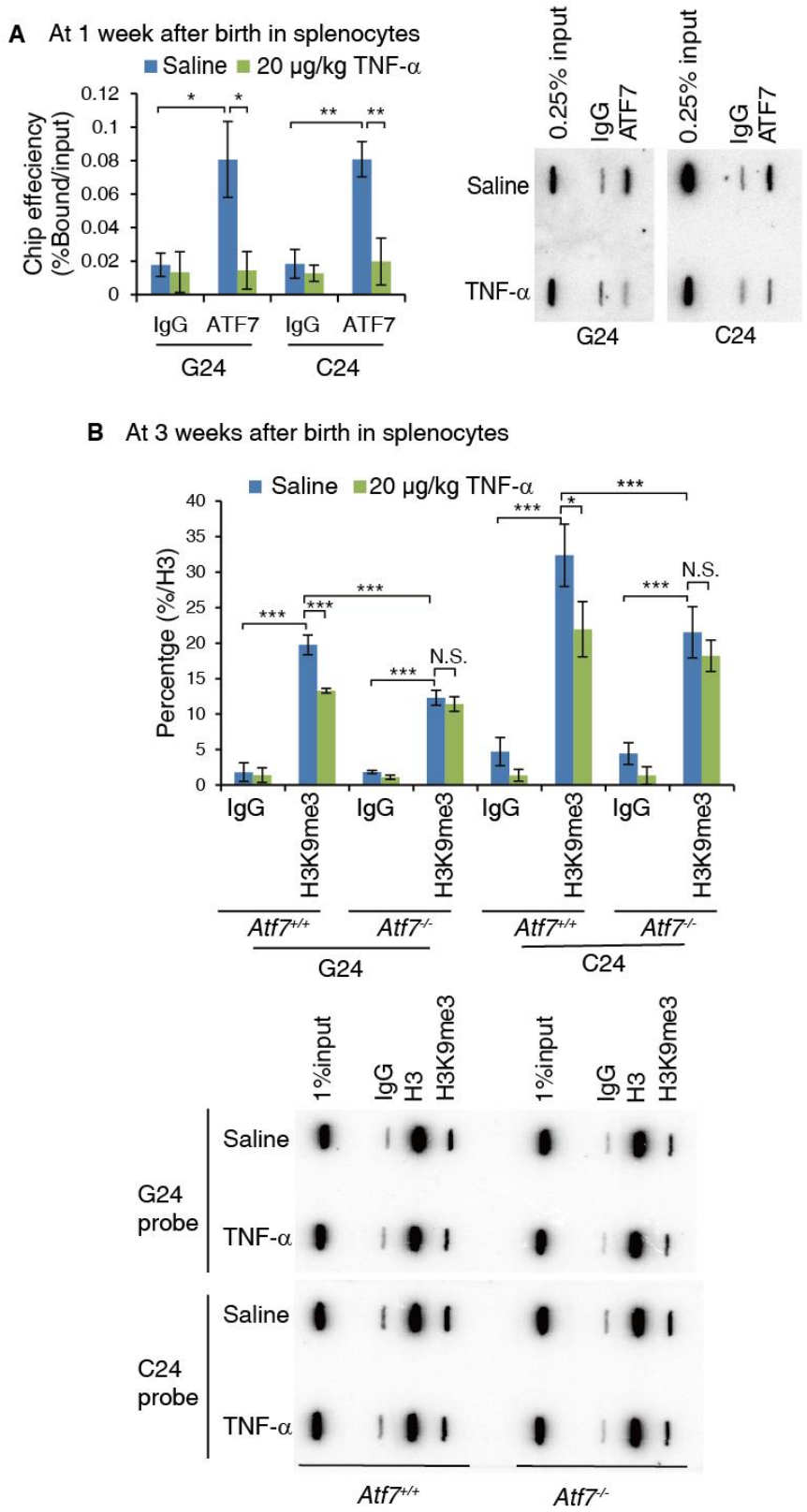
A At 1 week after birth in splenocytes
Saline
20 μg/kg TNF-α
Chip effeciency (%Bound/input)
0.12
0.1
0.08
0.06
0.04
0.02
0
IgG ATF7 IgG ATF7
G24 C24
0.25% input
IgG
ATF7
Saline
TNF-α
G24 C24
B At 3 weeks after birth in splenocytes
Saline
20 μg/kg TNF-α
Percentge (%/H3)
40
35
30
25
20
15
10
5
0
N.S.
IgG H3K9me3 IgG H3K9me3 IgG H3K9me3 IgG H3K9me3
Atf7+/+ Atf7-/- Atf7+/+ Atf7-/-
G24 C24
1%input
IgG
H3
H3K9me3
G24 probe
C24 probe
Saline
TNF-α
Atf7+/+ Atf7-/-

Figure 3-1.

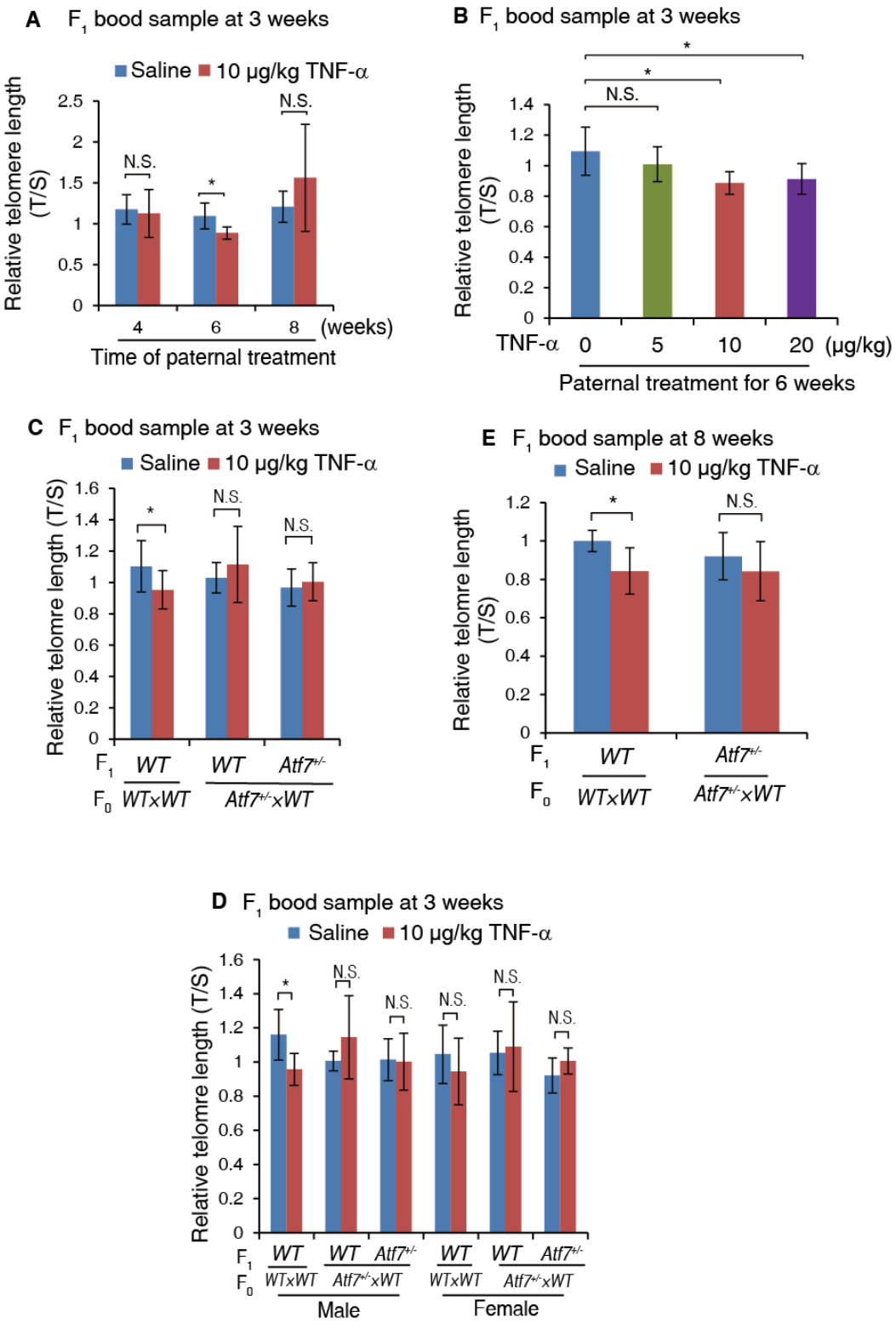
A F1 bood sample at 3 weeks
Saline 10 μg/kg TNF-α
Relative telomere length (T/S)
N.S.
*
N.S.
4 6 8 (weeks)
Time of paternal treatment
B F1 bood sample at 3 weeks
Relative telomere length (T/S)
N.S.
*
*
TNF-α 0 5 10 20 (μg/kg)
Paternal treatment for 6 weeks
C F1 bood sample at 3 weeks
Saline 10 μg/kg TNF-α
Relative telomre length (T/S)
F1 WT WT Atf7+/-
F0 WTxWT Atf7+/-xWT
E F1 bood sample at 8 weeks
Saline 10 μg/kg TNF-α
Relative telomre length (T/S)
F1 WT Atf7+/-
F0 WTxWT Atf7+/-xWT
D F1 bood sample at 3 weeks
Saline 10 μg/kg TNF-α
Relative telomre length (T/S)
F1 WT WT Atf7+/- WT WT Atf7+/-
F0 WTxWT Atf7+/-xWT WTxWT Atf7+/-xWT
Male
Female

Figure 3-2.

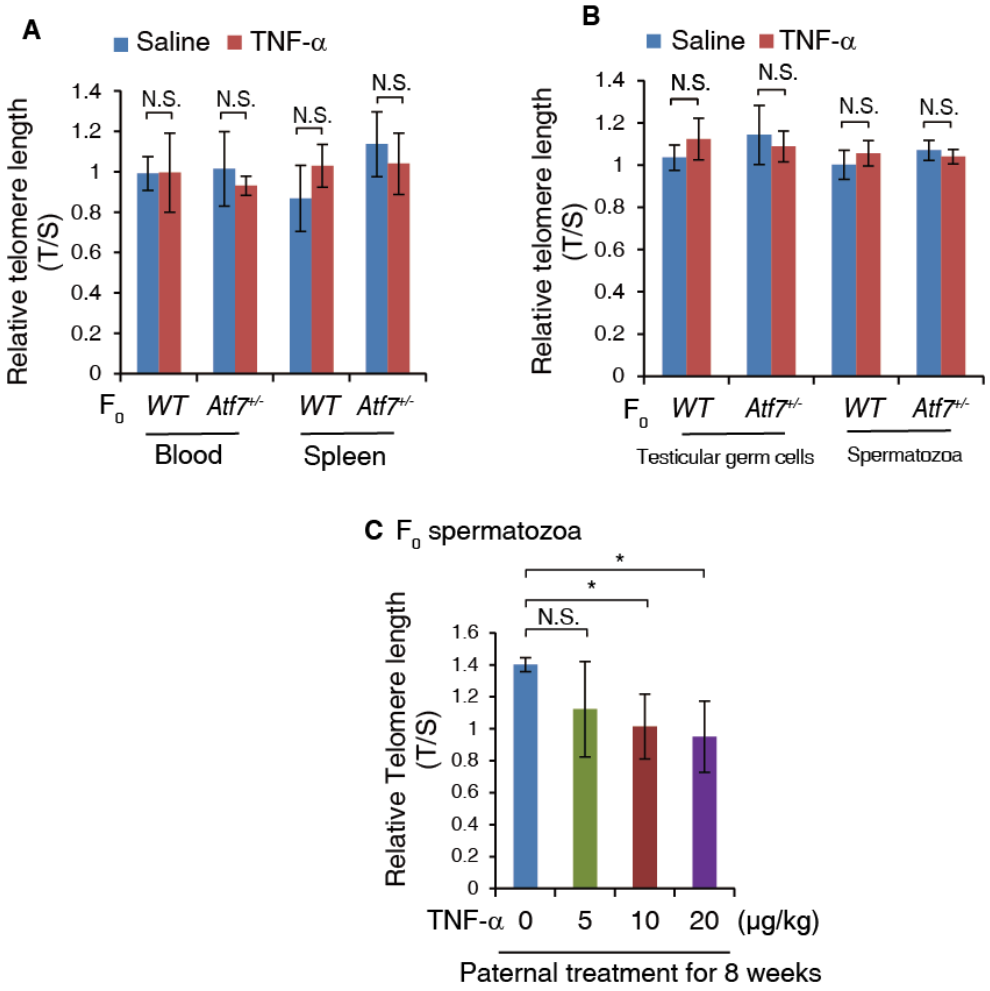
A
Saline
TNF-α
Relative telomere length (T/S)
N.S.
F0 WT Atf7+/- WT Atf7+/-
Blood
Spleen
B
Saline
TNF-α
Relative telomere length (T/S)
N.S.
F0 WT Atf7+/- WT Atf7+/-
Testicular germ cells
Spermatozoa
C F0 spermatozoa
Relative Telomere length (T/S)
*
*
N.S.
TNF-α 0 5 10 20 (μg/kg)
Paternal treatment for 8 weeks

Figure 3-3.

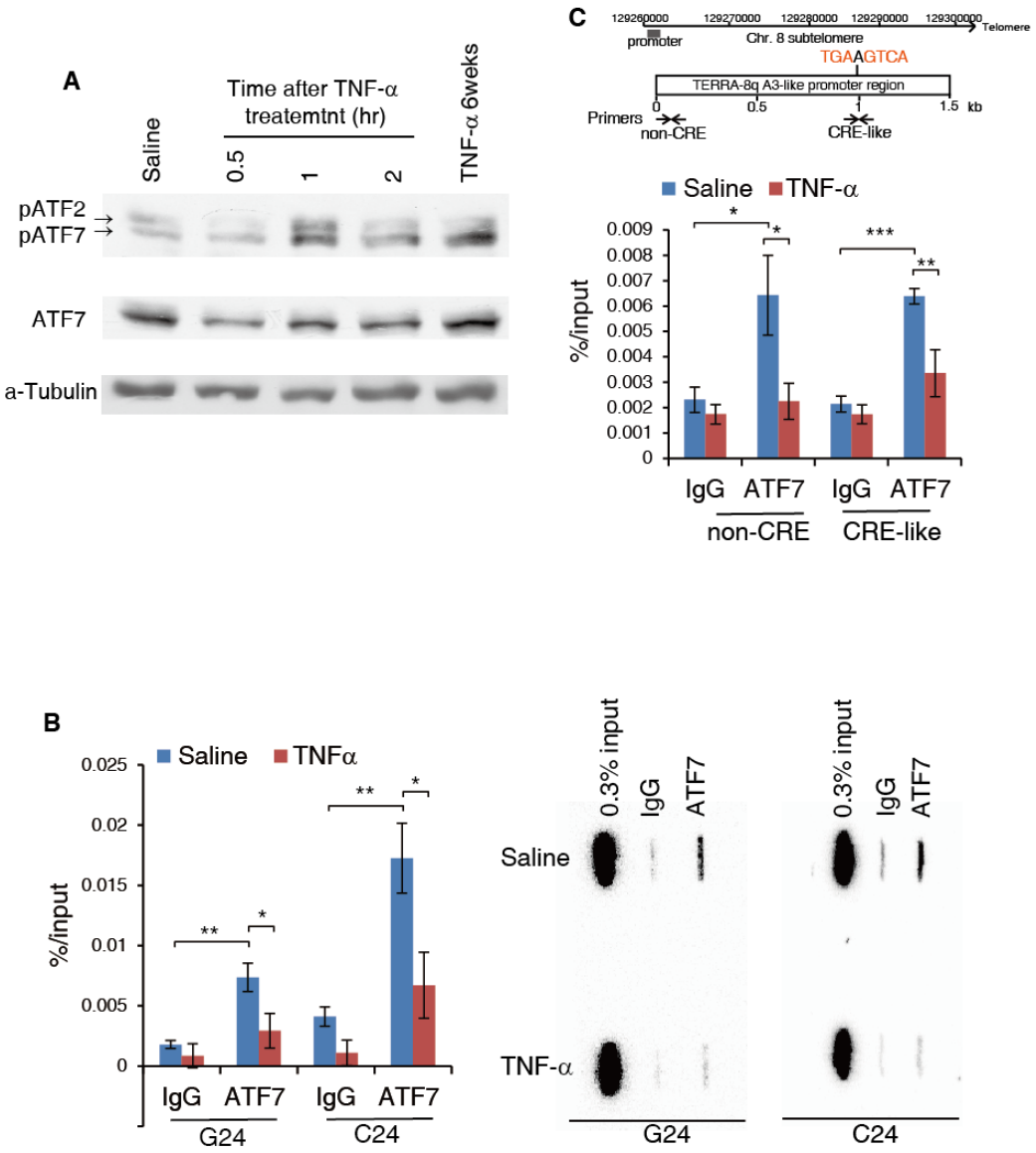
A
Time after TNF-α treatemtnt (hr)
Saline
0.5
1
2
TNF-α 6weks
pATF2
pATF7
ATF7
a-Tubulin
C
129260000
129270000
129280000
129290000
129300000
Telomere
promoter
Chr. 8 subtelomere
TGAAGTCA
TERRA-8q A3-like promoter region
0
0.5
1
1.5 kb
Primers
non-CRE
CRE-like
Saline
TNF-α
%/input
IgG
ATF7
non-CRE
CRE-like
B
Saline
TNFα
%/input
IgG
ATF7
G24
C24
0.3% input
Saline
TNF-α

Figure 3-4.

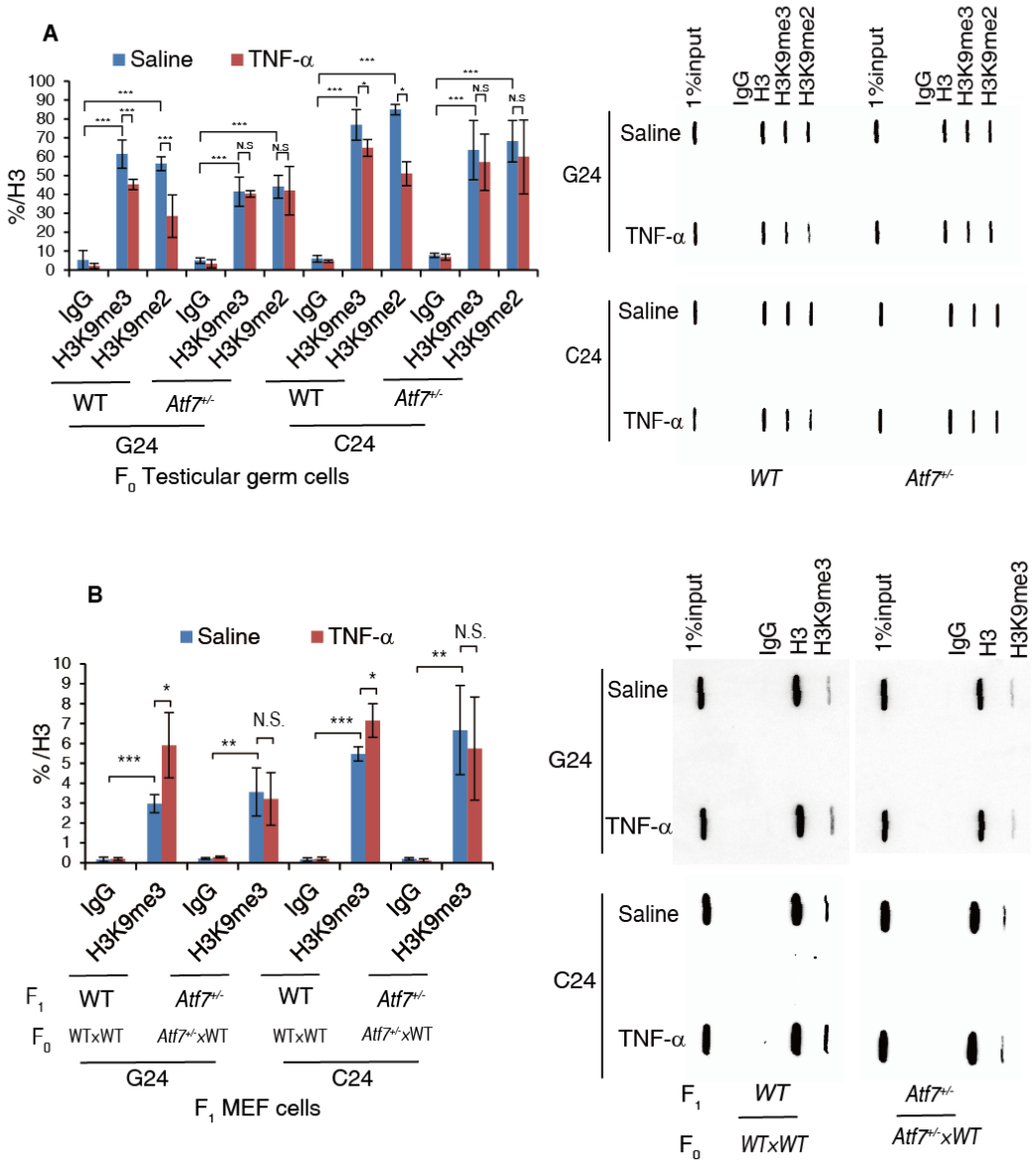
A
Saline
TNF-α
%/H3
IgG
H3K9me3
H3K9me2
WT
Atf7+/-
G24
C24
F0 Testicular germ cells
1%input
IgG
H3
H3K9me3
H3K9me2
Saline
TNF-α
G24
C24
WT
Atf7+/-
B
Saline
TNF-α
% /H3
N.S.
IgG
H3K9me3
F1 WT Atf7+/- WT Atf7+/-
F0 WTxWT Atf7+/-xWT WTxWT Atf7+/-xWT
G24
C24
F1 MEF cells
1%input
IgG
H3
H3K9me3
Saline
TNF-α
G24
C24
F1 WT Atf7+/-
F0 WTxWT Atf7+/-xWT

Figure 3-5.

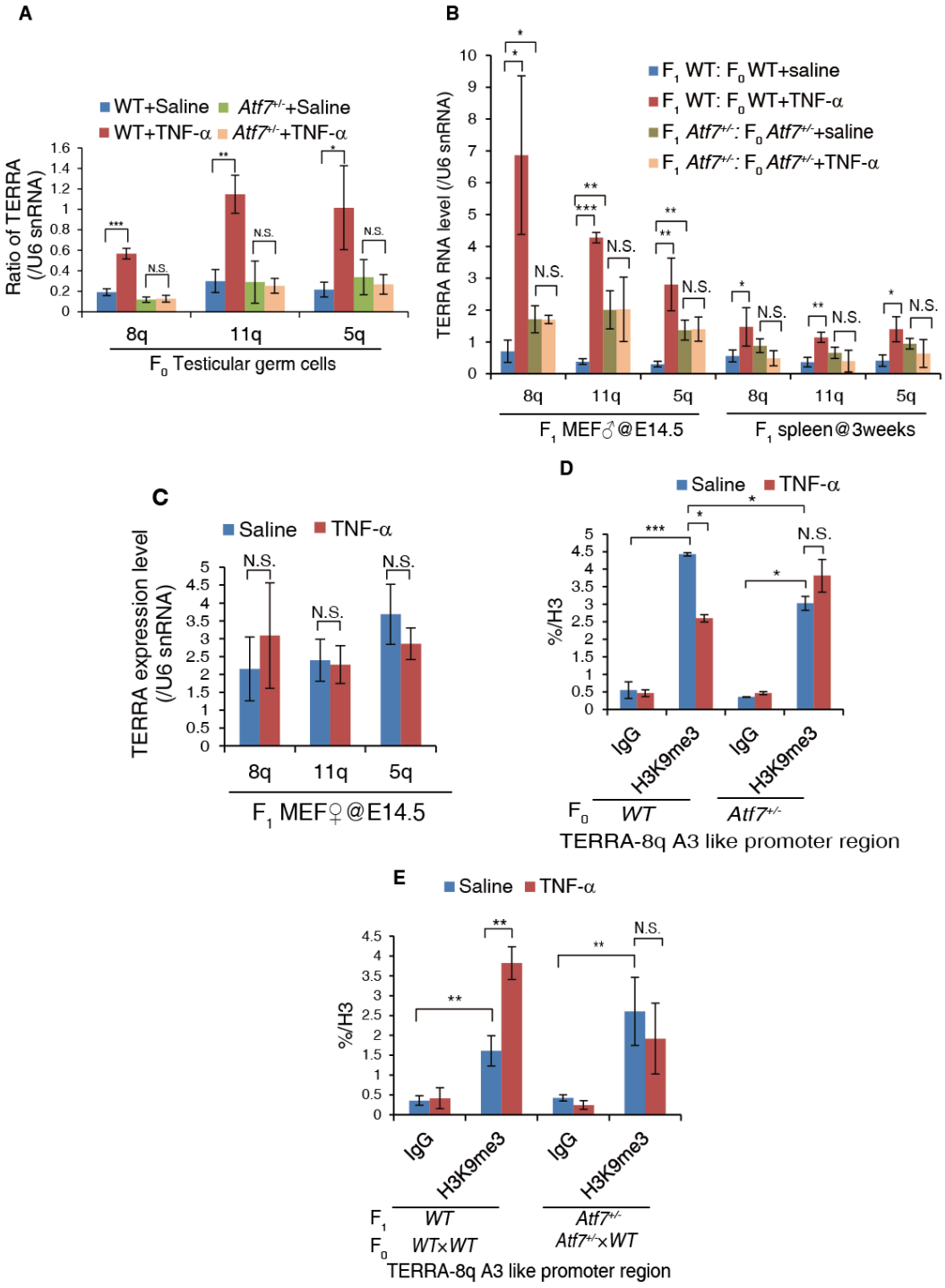
A
WT+Saline
Atf7+/-+Saline
WT+TNF-α
Atf7+/-+TNF-α
Ratio of TERRA (/U6 snRNA)
8q
11q
5q
F0 Testicular germ cells
B
F1 WT: F0 WT+saline
F1 WT: F0 WT+TNF-α
F1 Atf7+/-: F0 Atf7+/-+saline
F1 Atf7+/-: F0 Atf7+/-+TNF-α
TERRA RNA level (/U6 snRNA)
F1 MEF♂@E14.5
F1 spleen@3weeks
C
Saline
TNF-α
TERRA expression level (/U6 snRNA)
F1 MEF♀@E14.5
D
%/H3
IgG
H3K9me3
F0 WT
Atf7+/-
TERRA-8q A3 like promoter region
E
F1 WT
Atf7+/-
F0 WT×WT
Atf7+/-×WT
TERRA-8q A3 like promoter region

Figure 3-6.

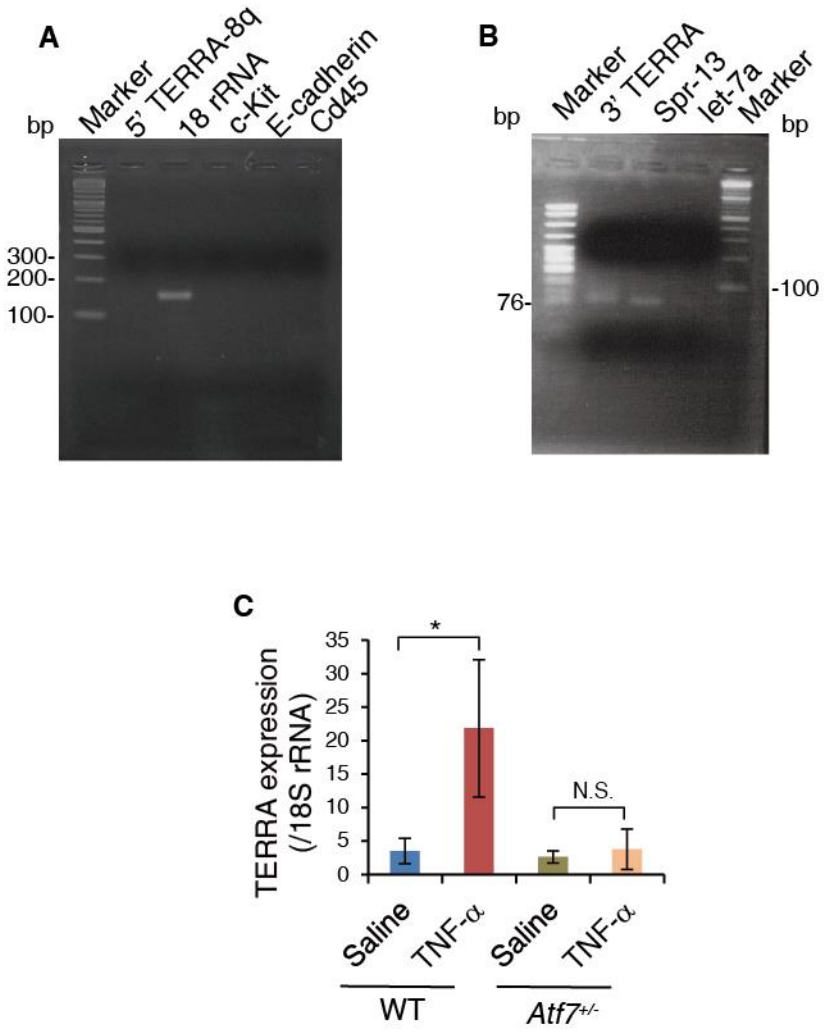
A
bp
Marker
5' TERRA-8q
18 rRNA
c-Kit
E-cadherin
Cd45
300-
200-
100-
B
bp
Marker
3' TERRA
Spr-13
let-7a
Marker
bp
76-
-100
C
TERRA expression
(/18S rRNA)
35
30
25
20
15
10
5
0
*
N.S.
Saline
TNF-α
Saline
TNF-α
WT
Atf7+/-

Table 1. Sequences of oligonucleotides and primers used.

qPCR primer		
Gene or region	Forward	Reverse
Telomere	GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT	TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA
Mouse 36B4	ACTGGTCTAGGACCCGAGAAG	TCAATGGTGCCTCTGGAGATT
8q-CRE-like	GGGACAGATGGATAACTCCTC	CCCTCACTCAGTAGCCTTCTT
8q-non-CRE	TGGCATCACTTCACAACAG	TTCCCACATCCTCAGTTTG
Probe		
Telomere	G24: $[TTAGGG]_4$	C24: $[CCCTAA]_4$
RT-PCR primer		
Gene or region	Forward	Reverse
TERRA-8q	TCCCACTGTCAATAACAGAC	CAAGCACAGGCTAGAAGTG
TERRA-11q	AGCAGATGGGTCCCTGGTAAA	TTGTCCGCCCTCACCTAGCTT
TERRA-5q	ATTAACAAGCACAAGAGGGTAGCA	CAACCATACCTGAAATGCCTAGATC
U6 snRNA	GGAATCTAGAACATATACTAAAATTGGAAC	GGAACTCGAGTTTGCGTGTCATCCTTGCGC
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Spr-13	CAGGGTGGGGCGGGGCG	Universal primer
let-7a	TGAGGTAGTAGGTTGTATAG	Universal primer
3’-TERRA	$[TTAGGG]_4$	Universal primer
Cd45	CGCACCACTGAATCCACACCCC	CCGGGAGCAGGCGTGAGTGT
c-Kit	GCAGAGGGATTCCCGGAGCC	GGCGGAACTCTTGCCCACATCG
E-cadherin	CTCACCTCTGGGCTGGACCGA	GGCGATCCGGGCATTGACCT

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参　考　論　文

1. Liu B, Maekawa T, Ishii S, *In utero* TNF-α treatment induces telomere shortening of young adult mice in an ATF7-dependent manner. *FEBS Open Bio.* (2016) DOI: 10.1002/2211-5463.12006.