Supplementary Information for

Telomere shortening by transgenerational transmission of TNF-α-induced TERRA via ATF7

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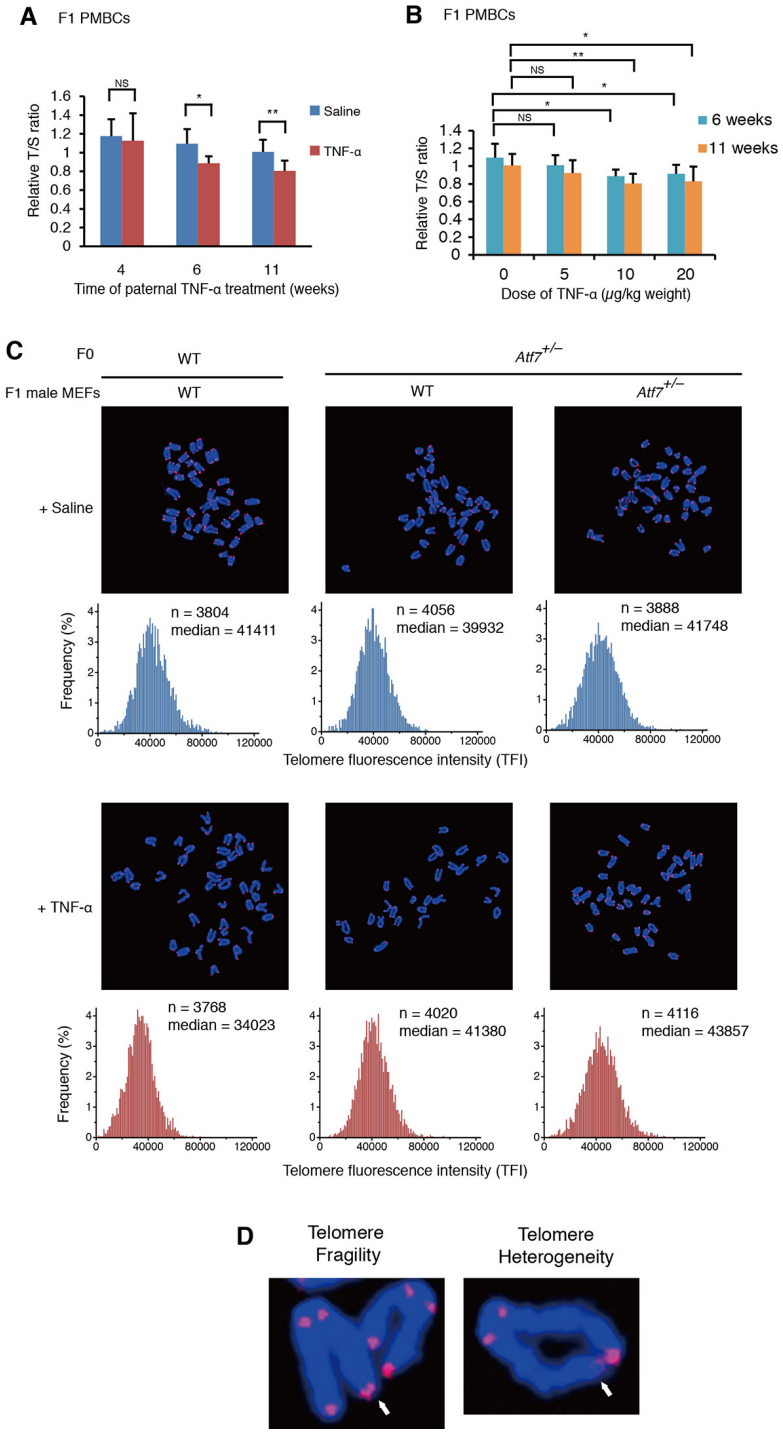
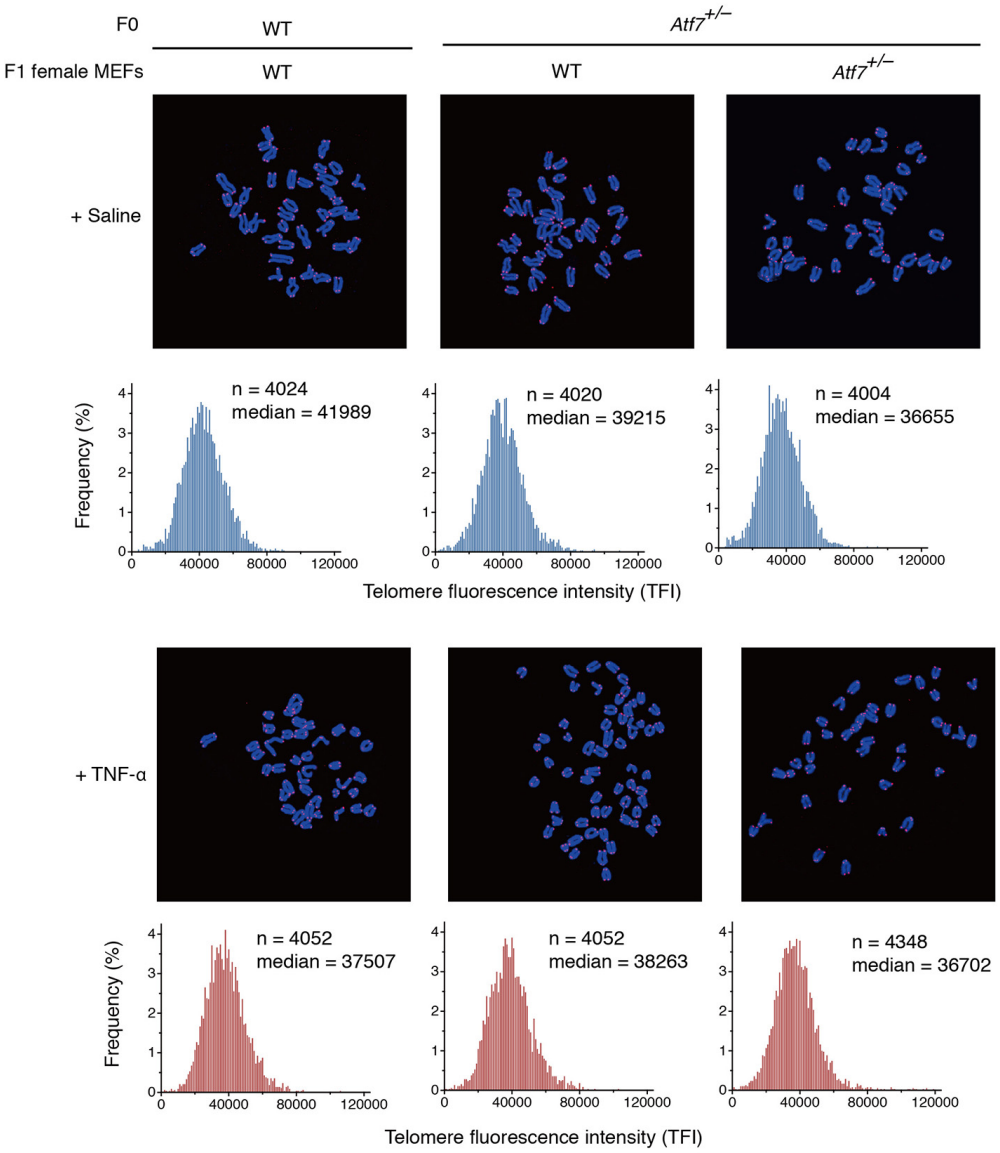
Figure S1. Liu, B. *et. al.*



Supplementary Figure S1. Paternal TNF-α treatment induced telomere shortening in the offspring. **(A)** Wide-type (WT) 8-week-old male mice (F0) (n = 3) were daily administered with TNF-α (10 μg/kg weight) or saline for 4, 6 or 11 weeks, and then mated with WT female mice (n = 3). Peripheral blood mononuclear cells (PMBCs) (n = 8, 14; 7, 5; 8, 8 for each group from three independent pregnant mice) were prepared from the 3-week-old pups (F1) (mixture of male and female), and telomere length was determined by Q-PCR. *, $p < 0.05$; **, $p < 0.01$; NS, not significant. **(B)**

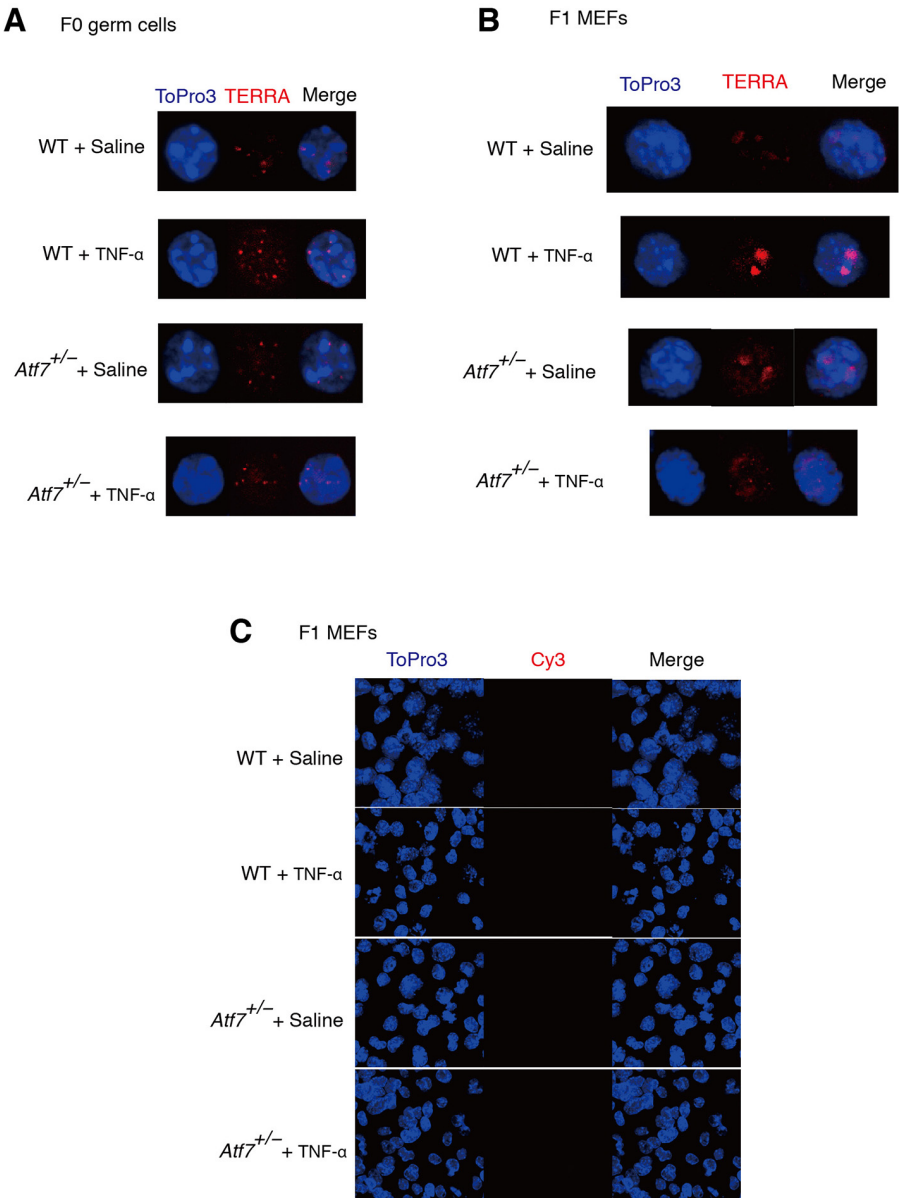
WT 8-week-old male mice (F0) (n = 3) were daily administered with TNF-α (5, 10 or 20 μg/kg weight) or saline for 6 and 11 weeks, and then mated with WT female mice (n = 3). PMBCs (n = 7, 8; 8, 8; 5, 8; 6, 8 for each group from three independent pregnant mice) were prepared from the 3-week-old pups (F1) (mixture of male and female), and telomere length was determined by Q-PCR. *, $p < 0.05$; **, $p < 0.01$; NS, not significant. **(C)** Analysis of paternal TNF-α-induced telomere shortening in F1 male MEFs by Q-FISH. Raw Data of Q-FISH of Figure 1B. Experiments were performed as described in Figure 1B. Telomere FISH images are shown. Blue, chromosomes; red, telomeres. Histograms express the telomere fluorescence intensity (TFI) and frequency of all individual telomere spots are shown. The number of individual telomere signals and the median of TFI are shown. **(D)** Paternal TNF-α exposure increases the frequency of the telomere fragility and telomere length heterogeneity in MEFs of male offspring. Experiments were done as described in Figure 1D. Telomere FISH images of MEFs are shown. White arrows indicate the telomere fragility and telomere length heterogeneity.

Supplementary Figure 2. Liu, B. *et. al.*



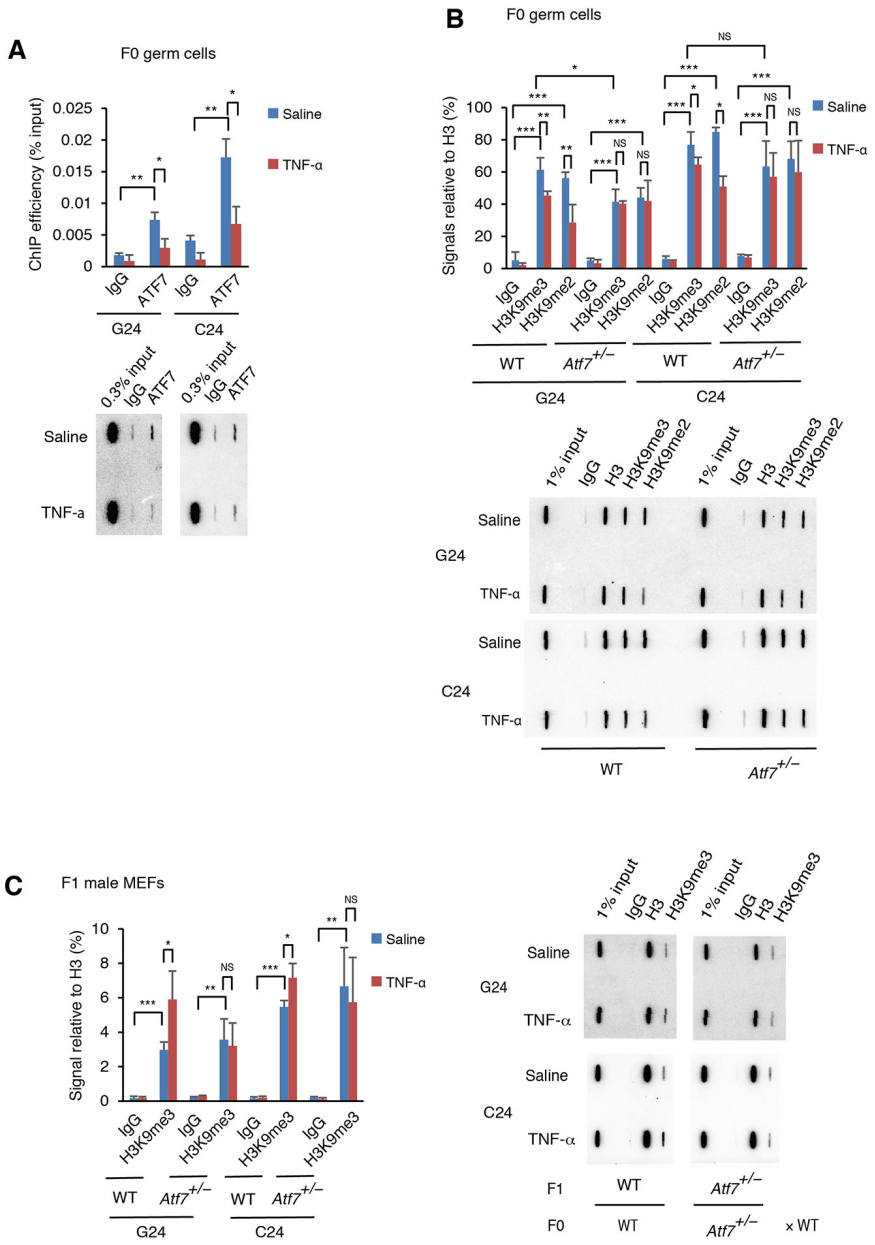
Supplementary Figure S2. Paternal TNF-α treatment does not induce telomere shortening in the F1 female MEFs. Raw Data of Q-FISH of Figure 2A. Experiments were performed as described in Figure 2A. Telomere FISH images are shown. Blue, chromosomes; red, telomeres. Histograms express the telomere fluorescence intensity (TFI) and frequency of all individual telomere spots are shown. The number of individual telomere signals and the median of TFI are shown.

Figure S3. Liu, B. *et. al.*



Supplementary Figure S3. TNF-α increases TERRA level in an ATF7-dependent manner in F0 testicular germ cells and F1 male MEFs. **(A)** TERRA expression in F0 testicular germ cells was determined by RNA FISH as described in Figure 3A. Expanded image of single cell is shown. **(B)** TERRA expression in F1 male MEFs was determined by RNA FISH as described in Figure 3D. Expanded image of single cell is shown.**c,** Negative control experiments for RNA FISH. RNA FISH of TERRA in MEFs from male F1 embryos without Tel-Cy3 probe. Typical data is shown.

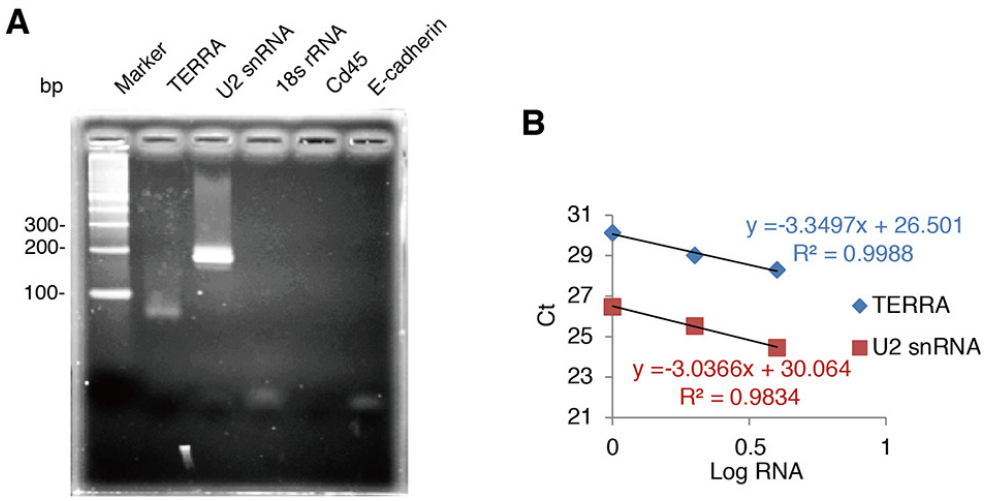
Figure S4. Liu, B. *et. al.*



Supplementary Figure S4. TNF-α exposure decreases H3K9me3 level on telomere in F0 testicular germ cells, but increases in F1 MEFs. **(A)** TNF-α treatment induces a release of ATF7 from telomere in F0 testicular germ cells. WT male mice (n = 3) were administered with TNF-α or saline as described in Figure 1A. Testicular germ cells were prepared, and used for ChIP with anti–ATF7 antibody. Recovered DNAs were subjected to slot-blot hybridization with ^{32}P-labeled telomere probes (G24 and C24). Average value relative to input ± SD is shown, and typical data for slot-blot hybridization are shown. *, $p < 0.05$; **, $p < 0.01$. **(B)** TNF-α exposure decreases H3K9me3 level on telomere in F0 testicular germ cells in an ATF7-dependent manner. WT and Atf7$^{+/-}$ male mice (n = 3) were administered with TNF-α or saline, and mated with WT female as described in Figure 1A. Testicular germ cells

were prepared, and used for ChIP with anti-H3K9me3, anti-H3K9me2, and anti-H3 antibodies. Recovered DNAs were subjected to slot-blot hybridization with ^{32}P-labeled telomere probes (G24 and C24). Average value relative to input ± SD is shown, and typical data for slot-blot hybridization are shown. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant. **(C)** Paternal TNF-α treatment increases H3K9me3 level on telomere in WT but not Atf7$^{+/-}$ male MEFs of offspring. MEFs were prepared as described in Figure 1A, and used for ChIP with anti-H3K9me3 and anti-H3 antibodies. Recovered DNAs were subjected to slot-blot hybridization with ^{32}P-labeled telomere probes (G24 and C24). Average value relative to input ± SD is shown, and typical data for slot-blot hybridization are shown. *, $p < 0.05$; **, $p < 0.01$; ***. $p < 0.001$; NS, not significant.

Figure S5. Liu, B. *et. al.*

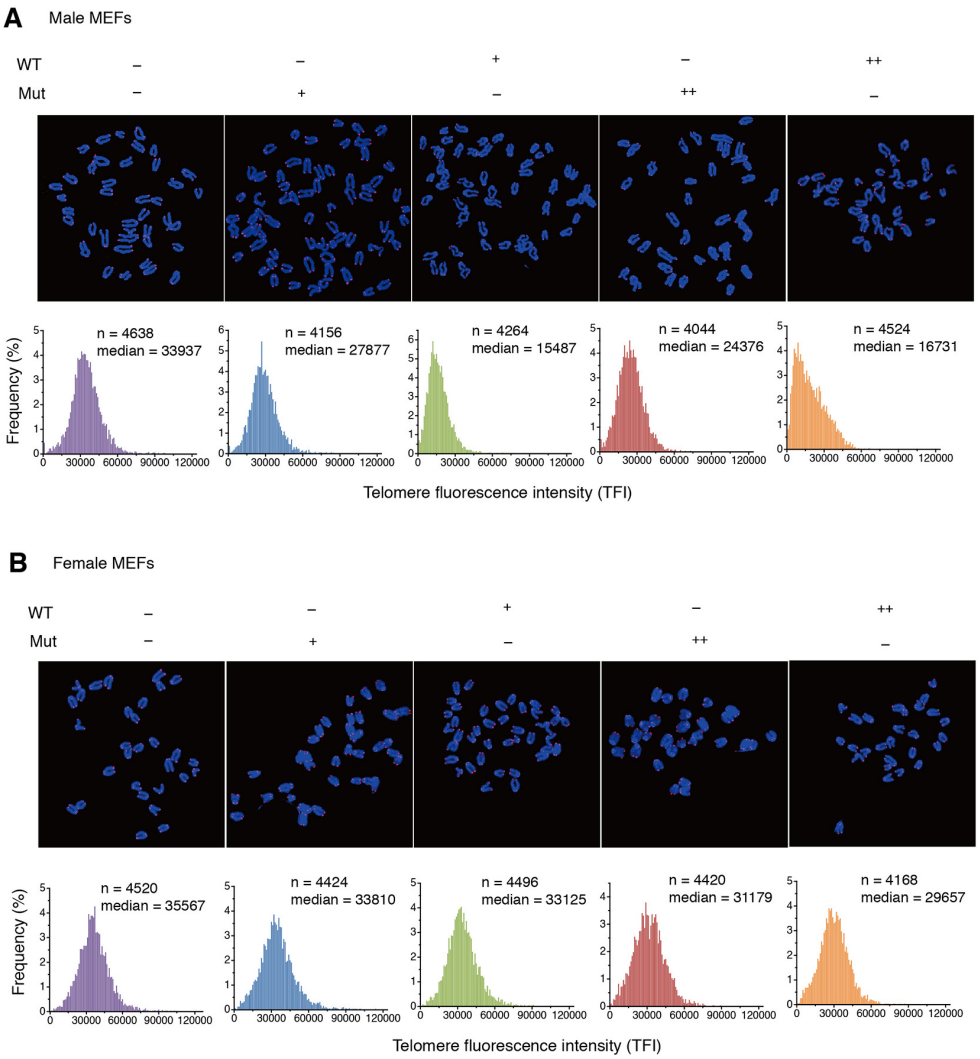


Supplementary Figure S5. PCR product of Poly(A)-tailed qRT-PCR of TERRA. **(A)** poly(A)-tailed qRT-PCR was used to amplify TERRA, U2 snRNA, 18S rRNA, Cd45 mRNA, and E-cadherin mRNA. The PCR products were electrophoresed on 3% agarose gel and stained with ethidium bromide. Since it was reported that Cd45 mRNA, and E-cadherin mRNA was not detected in spermatozoa (69) and 18S rRNA was located in cytoplasm but not sperm head, these mRNAs were used as a negative control. Note that the small-size bands in the lanes of 18S rRNA and E-cadherin are primers. **(B)** Control data for Figure 5A. Standard curves used to calculate the relative DNA concentration (log DNA) from the Ct of real-time PCR products by serial dilution of known amounts of RNA prepared from WT mature sperm. Ct (threshold cycle) is the interpolated intersection of the amplification curve (fluorescence intensity vs. cycle number) and the detection threshold. Blue squares, TERRA; red squares, U2 snRNA control. Regression equations and the R^2 of Ct versus log DNA are shown inside the graphs.

Reference

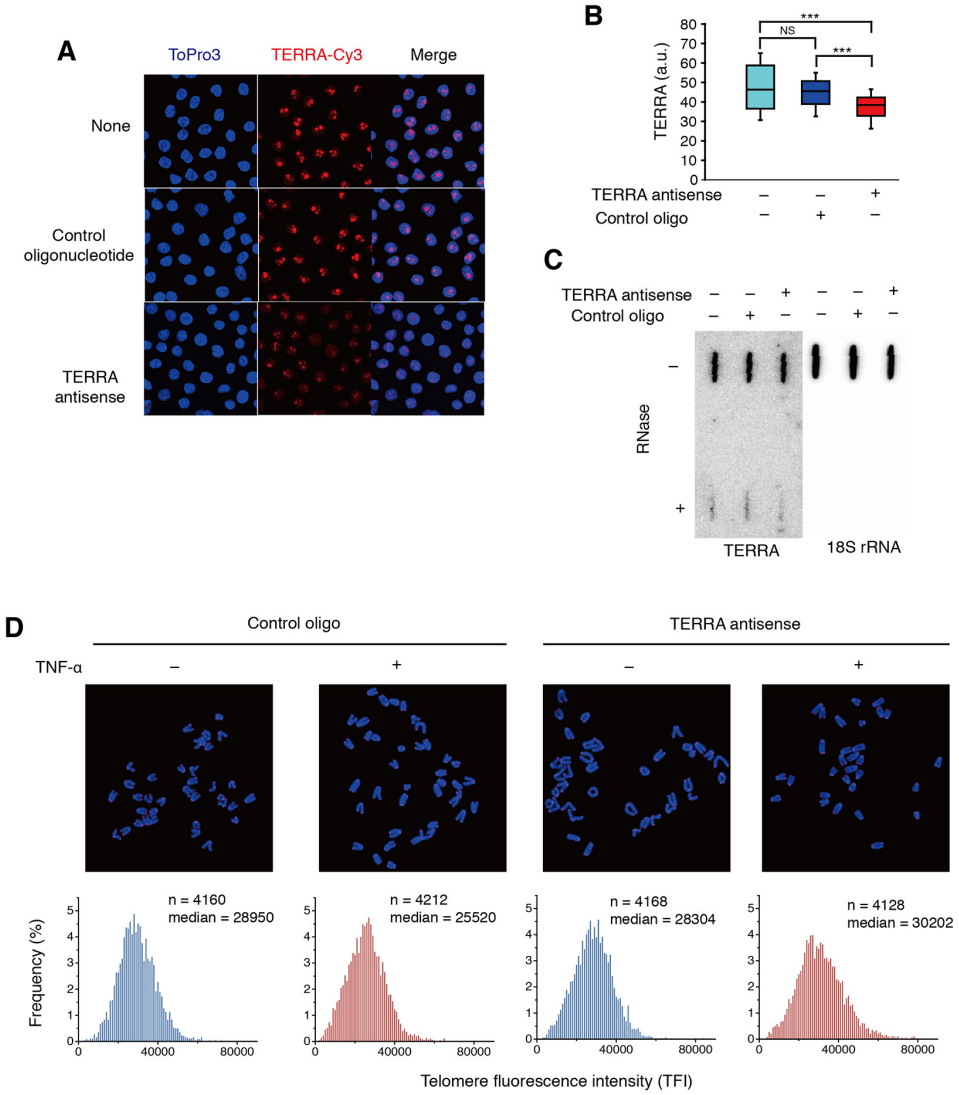
69. Fang, P., Zeng, P., Wang, Z, Liu, M., Xu, W., Dai, J., Zhao, X., Zhang, D., Liang, D., Chen, X., Shi, S., Zhang, M., Wang, L., Qiao, Z. and Shi, H. (2014) Estimated diversity of messenger RNAs in each murine spermatozoa and their potential function during early zygotic development. *Biol. Reprod.*, **90,** 94.

Figure S6. Liu, B. *et. al.*



Supplementary Figure S6. Injection of TERRA into zygote induces telomere shortening in male but not female MEFs. Raw data of Figures 6B and 6D. Experiments were done as described in Figures 6B and 6D. Telomere FISH images are shown. Blue, chromosomes; red, telomeres. Histograms express the telomere fluorescence intensity (TFI) and frequency of all individual telomere spots are shown. The number of individual telomere signals and the median of TFI are shown.

Figure S7. Liu, B. *et. al.*



Supplementary Figure S7. TERRA antisense oligonucleotide masks TERRA. **(A, B)** HeLa S3 cells were non-treated or transfected with TERRA antisense or control oligonucleotides by RNAi MAX. After 48 h, RNA-FISH was performed as above to detect free TERRA. Typical data from RNA-FISH is shown (A). Box plots representing the fluorescence intensity (a.u.) of individual TERRA foci from nuclei (n = 583, 514, 475) are shown (B). ***, $p < 0.001$; NS, not significant. **(C)** TERRA antisense oligonucleotide does not change the TERRA level. Total RNAs from HeLa S3 cells were used to slot-blot hybridization with ^{32}P-labelled C24 probe or 18S rRNA probe. Total RNA treated with RNase A was used as negative control. **(D)** Raw data of Q-FISH of Figure 7B. Experiments were done as described in Figure 7B. Telomere FISH images and histograms express TFI are shown as described in Supplementary Figure S1C.

Supplementary Table S1. Effect of TNF-α injection in F0 testicular germ cells F1 male MEFs.

	F0 testicular germ cells	F1 male MEFs
Telomere length	No change*	Shortened
TERRA	Increased*	Increased**
H3K9me3 on TERRA gene promoter	Decreased	Increased***
H3K9me3 on telomere	Decreased	Increased***

* The absence of telomere shortening induced by TERRA in F0 testicular germ cells despite TERRA induction might be due to a relatively high level of telomerase in testicular germ cells[47].

** Transmitted TERRA to offspring might enhance the transcription of the telomere region to which TERRA and ATRX localize, as TERRA increases transcription by antagonizing ATRX[27].

*** Transmitted TERRA to offspring might increase the H3K9me3 level, as TERRA increases heterochromatin formation by interacting with TRF2[36].

Supplementary Table S2. Sequences of primers, oligonucleotides, and template DNA used.

qPCR primer		
Gene or region	**Forward**	**Reverse**
Telomere	GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTC	TCCCGACTATCCCTATCCCTATCCCTATCCCTAT
Mouse 36B4	ACTGGTCTAGGACCCGAGAAG	TCAATGGTGCCTCTGGAGATT
8q-CRE-like	GGGACAGATGGATAACTCCTC	CCCTCACTCAGTAGCCTTCTT
8q-non-CRE	TGGCATCACTTCACAACAG	TTCCCACATCCTCAGTTTG
RT-PCR primer		
TERRA-8q	TCCCACTGTCAATAACAGAC	CAAGCACAGGCTAGAAGTG
TERRA-11q	AGCAGATGGGTCCCTGGTAAA	TTGTCCGCCCTCACCTAGCTT
TERRA-5q	ATTAACAAGCACAAGAGGGTAGCA	CAACCATACCTGAAATGCCTAGATC
U6 snRNA	GGAATCTAGAACATATACTAAAATTGGAAC	GGAACTCGAGTTTGCGTGTCATCCTTGCGC
U2 snRNA	CTCGGCCTTTTGGCTAAGAT	CGTTCCTGGAGGTACTGCAA
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Cd45	CGCACCACTGAATCCACACCCC	CCGGGAGCAGGCGTGAGTGT
E-cadherin	CTCACCTCTGGGCTGGACCGA	GGCGATCCGGGCATTGACCT
Probe		
Telomere	G24: $(TTAGGG)_4$	C24: $(CCCTAA)_4$
18S rRNA	CGGAACTACGACGGTATCTG	
U2 SnRNA	TACCAGGTCGATGCGTGGAGTGGACGGAGC	
Cy3-TelC	$(CCCTAA)_3$	
LNA antisense oligo		
TERRA	A*A*C*C*C*T*A*A*C*C*C*T*A*A*C*C	
Negative control	A*C*G*T*C*T*A*T*A*C*G*C*C*C*A	
Template DNA for in vitro transcription		
Telomere WT	$(TTAGGG)_{50}$	
Telomere mutant	$(ATACCG)_{17}ATAC(ATACCG)_{25}CG(ATACCG)_7$	