SUPPLEMENTARY APPENDIX

Therapeutic effect of androgen therapy in a mouse model of aplastic anemia produced by short telomeres

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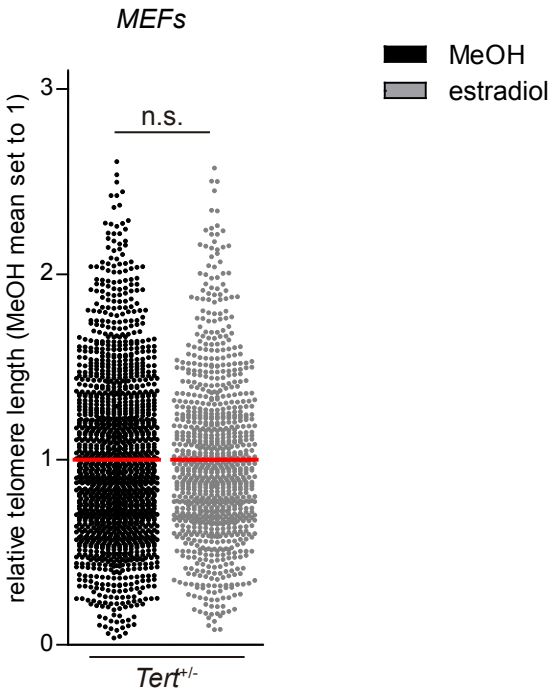
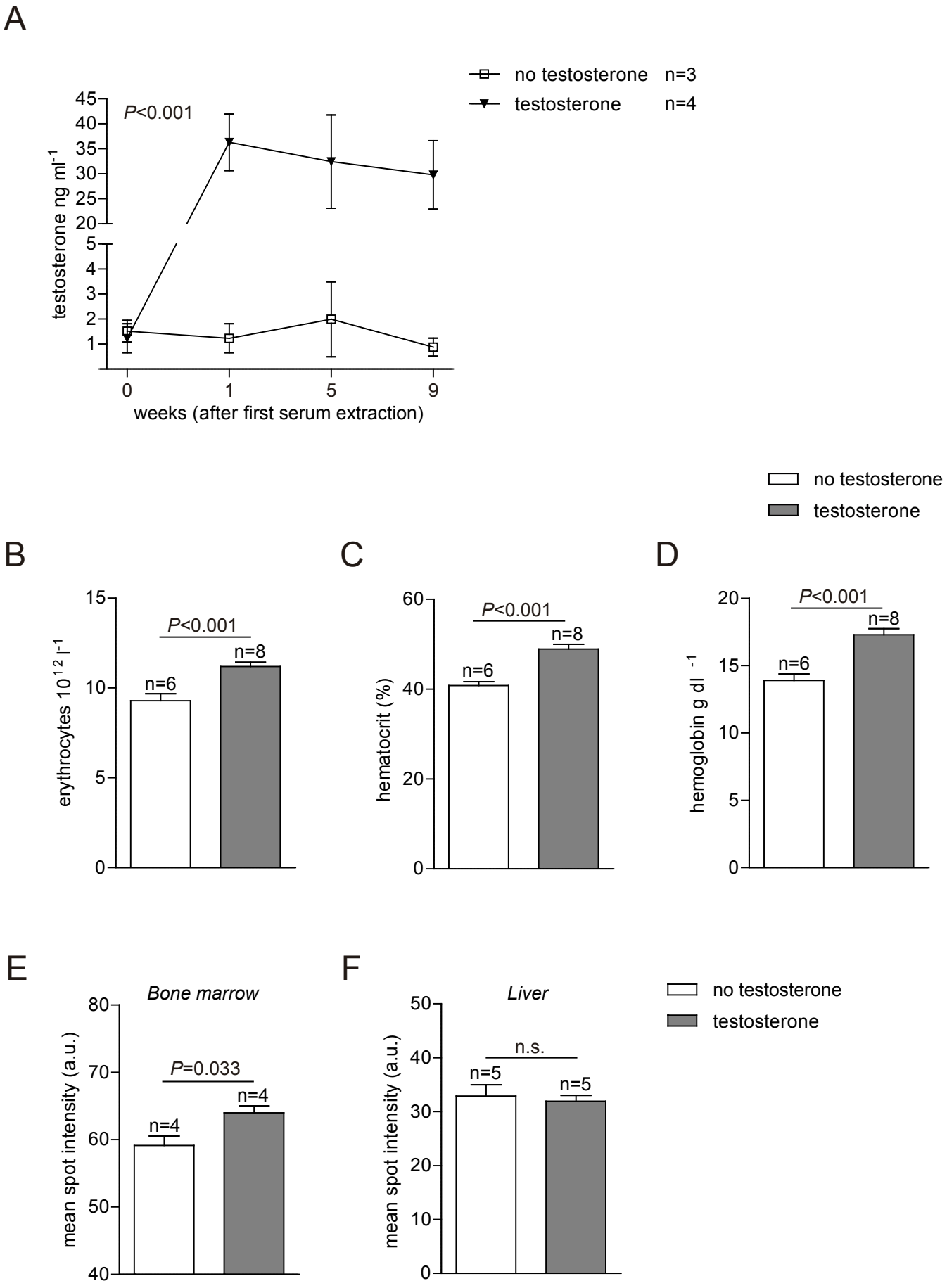
Supplementary Figure 1



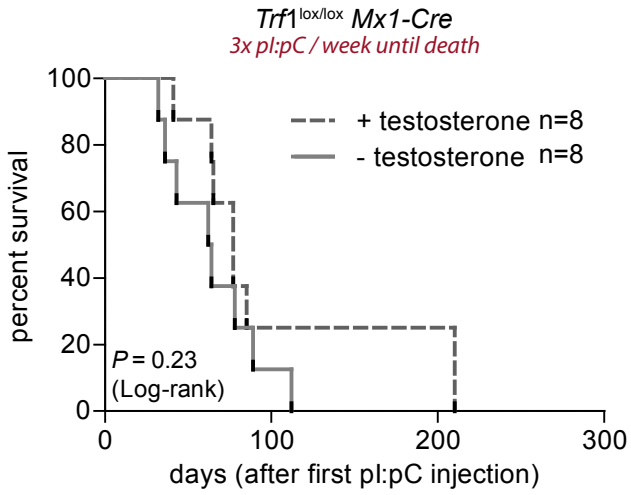
Supplementary Figure 1 | Q-FISH analysis in *Tert*$^{+/-}$ MEFs. Relative TL (arbitrary units of fluorescence) of *Tert*$^{+/-}$ MEFs incubated with 1 µM estradiol for 4 passages. n = 899 telomeres for estradiol and n = 1576 telomeres for methanol treatment. Two-sided Student's *t*-test was used for statistical analysis. n.s. = not significant.

Supplementary Figure 2

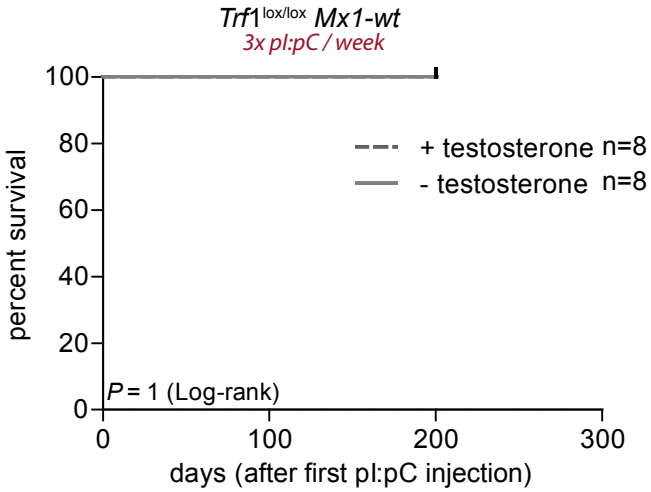


Supplementary Figure 2 | Blood analysis of pI:pC treated mice after testosterone administration. (A) Time course of serum testosterone concentration in mice implanted with a slow release testosterone pellet and mice without. Serum for ELISA analysis was extracted at indicated time points. (B) Erythrocyte count, (C) hematocrit and (D) hemoglobin levels in mice with or without testosterone treatment. (E) TL determined in Q-FISH analysis on bone marrow cross sections from mice with or without testosterone therapy represented as mean telomere spot intensity (arbitrary units of fluorescence, a.u.). (F) Q-FISH TL analysis represented as mean telomere spot intensity in liver tissue section from the same mice as in (E). n = number of mice. Graphs show mean values, error bars indicate s.e.m. Two-way ANOVA test was used for statistical analysis in figure A, two-sided Student's *t*-test was used for figures B-F. *P*-values are indicated.

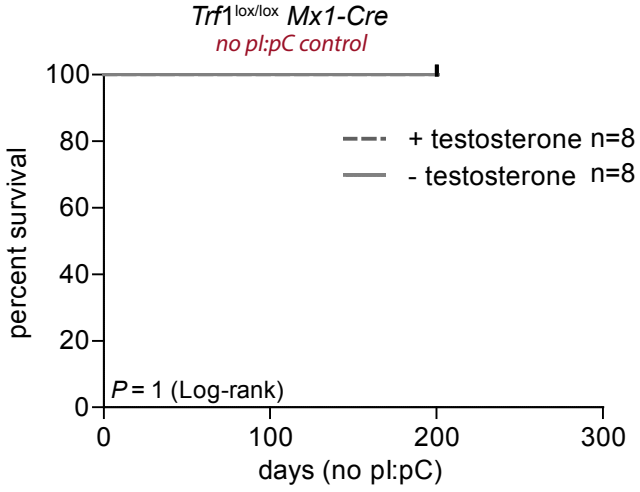
A


Trf1lox/lox Mx1-Cre
3x pI:pC / week until death
+ testosterone n=8
- testosterone n=8
percent survival
P = 0.23
(Log-rank)
days (after first pI:pC injection)

B


Trf1lox/lox Mx1-wt
3x pI:pC / week
+ testosterone n=8
- testosterone n=8
percent survival
P = 1 (Log-rank)
days (after first pI:pC injection)

C


Trf1lox/lox Mx1-Cre
no pI:pC control
+ testosterone n=8
- testosterone n=8
percent survival
P = 1 (Log-rank)
days (no pI:pC)

Supplementary Figure 3 | Survival of pI:pC treated control mice. (A) Kaplan-Meier survival curve of mice treated with pI:pC 3 times per week until death. In addition, mice were treated with or without testosterone as indicated. (B) Kaplan-Meier survival curve of *Trf1*$^{lox/lox}$ *Mx1-wt* mice treated with pI:pC 3 times per week. In addition, mice were treated with or without testosterone as indicated. Mice were sacrificed after 200 days. (C) Kaplan-Meier survival curve of *Trf1*$^{lox/lox}$ *Mx1-Cre* mice not treated with pI:pC. Mice were treated with or without testosterone as indicated. Mice were sacrificed after 200 days. n = number of mice. Log-rank (Mantel-Cox) test was used for statistical analysis. *P*-values are depicted.

Supplementary Tables

Group	Genotype	Treatment
E1	*Trf1*$^{lox/lox}$ *Mx1-Cre*	2 pI:pC injection / week (continuous); +testosterone
C1	*Trf1*$^{lox/lox}$ *Mx1-Cre*	2 pI:pC injection / week (continuous); no testosterone
E2	*Trf1*$^{lox/lox}$ *Mx1-Cre*	3 pI:pC injection / week (continuous); +testosterone
C2	*Trf1*$^{lox/lox}$ *Mx1-Cre*	3 pI:pC injection / week (continuous); no testosterone
E3	*Trf1*$^{lox/lox}$ *Mx1-Cre*	3 pI:pC injection / week (for 4 weeks); + testosterone after pI:pC
C3	*Trf1*$^{lox/lox}$ *Mx1-Cre*	3 pI:pC injection / week (for 4 weeks); no testosterone
C4	*Trf1*$^{lox/lox}$ *Mx1-Cre*	no pI:pc; +testosterone
C5	*Trf1*$^{lox/lox}$ *Mx1-Cre*	no pI:pc; no testosterone
C6	*Trf1*$^{lox/lox}$ *Mx1-wt*	3 pI:pC injection / week (continuous); +testosterone
C7	*Trf1*$^{lox/lox}$ *Mx1-wt*	3 pI:pC injection / week (continuous); no testosterone

Supplementary Table 1: Experimental and control groups of mice subjected to this study. After irradiation (12Gy) and bone marrow transplantation a total of 80 mice were stratified into 10 groups of 8 mice each which were subjected to different treatments as indicated. E denotes experimental group and C denotes control group.

Primer name	Primer sequence (5’-3’)
Actin-Forward	GGCACCACACCTTCTACAATG
Actin-Reverse	GTGGTGGTGAAGCTGTAG
Tert-Forward	GGATTGCCACTGGCTCCG
Tert-Reverse	TGCCTGACCTCCTCTTGTGAC

Supplementary Table 2: qPCR primers used in this work.

Supplementary Methods:

Bone marrow transplantation, pI:pC and testosterone treatment.

10 weeks old *Trf1lox/lox Mx1-Cre* and *Trf1lox/lox Mx1-wt* mice were used as bone marrow donors for transplantation into 8 weeks old lethally (12Gy) irradiated wild-type mice as previously described (Samper et al., 2002). A total of 2 million cells were transplanted via tail vein injection at a donor:recipient ratio of 1:8 and mice were left for a latency period of 30 days to allow bone marrow reconstitution. To induce *Cre* expression, mice were intraperitoneally injected with polyinosinic-polycytidylic acid (pI:pC; Sigma-Aldrich) (15 ug/g body weight). For androgen therapy mice were subcutaneously implanted with a 90-days testosterone slow release pellet (Innovative Research of America). After 90 days into treatment, testosterone pellets were renewed. To control for potential adverse affects of pI:pC and testosterone we included mice that were untreated, treated with testosterone alone or mice without *Cre*. For details see Supplementary Table_1.

Telomere measurement

For Q-FISH analysis tissues sections or metaphases were post fixed in 4% formaldehyde for 5 min, washed 3 x 5 min in PBS and incubated at 37°C for 15 min in pepsin solution (0.1% Porcine Pepsin, Sigma; 0.01M HCl, Merck). After another round of washes and fixation as mentioned above, slides were dehydrated in a 70%–90%–100% ethanol series (5 min each). Slides were 10 min air-dried and 30 μl of telomere probe mix added to each slide (10mM TrisCl pH 7, 25mM MgCl2, 9mM citric acid,

82mM Na2HPO4, 70% deionized formamide (Sigma), 0.25% blocking reagent (Roche) and 0.5 mg/ml Telomeric PNA probe (Panagene)), a cover slip added and slides incubated for 3 min at 85 °C, and for further 2 h at room temperature in a wet chamber in the dark. Slides were washed 2 x 15 min in 10mM TrisCl pH 7, 0.1% BSA in 70% formamide under vigorous shaking, then 3 x 5 min in TBS 0.08% Tween20, and then incubated in a 40,6-diamidino-2-phenylindole (DAPI) bath (4 mg/ml 1 DAPI (Sigma) in PBS) before mounting samples in Vectashield (VectorTM). Confocal image were acquired as stacks every 0.5 μm for a total of 1.5 μm using a Leica SP5-MP confocal microscope and maximum projections were done with the LAS-AF software. Telomere signal intensity was quantified using Definiens software.

HT-Q-FISH on peripheral blood leukocytes was done using 120–150 μl blood extracted from the facial vein. Red blood cells were lysed (Erythrocyte lysis buffer, Qiagen) and 30–90 k leukocytes were plated in duplicate into clear-bottom, black-walled 96-well plates pre-coated for 30 min with 0.001% poly-L-lysine. Plates were incubated at 37°C for 2 h and fixed with methanol/acetic acid (3:1, v/v) 2 x 10 min and then overnight at -20°C. Fixative was removed, plates dried for at least 1 h at 37°C and samples were rehydrated in PBS. Plates were then subjected to a standard Q-FISH protocol (see above) using a telomere-specific PNA-CY3 probe; DAPI was used to stain nuclei. Sixty images per well were captured using the OPERA (Perkin Elmer) High-Content Screening system. TL values were analysed using individual telomere spots (>10,000 telomere spots per sample). The average fluorescence intensities of each sample were converted into kilobase using L5178-R and L5178-S cells as calibration standards,

which have stable TLs of 79.7 and 10.2 kb, respectively. Samples were analysed in duplicate, or triplicate in the case of calibration standards.