High telomerase is a hallmark of undifferentiated spermatogonia and is required for maintenance of male germline stem cells

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Supplemental information includes 6 figures, 3 tables, supplemental experimental procedures and supplemental references.
Supplemental Experimental Procedures

ES cell targeting and generation of TERT-Tomato knock-in mice.

A Tdtomato fluorescent reporter was introduced into the initiating methionine of TERT by homologous recombination in JM8.F6 ES cells (C57BL/6-derived), using standard procedures. The ATG of TERT was Kozak-optimized (from AGCACAATGA to GCCACCATGG) prior to introduction of the TdTomato-pA reporter. A BamHI site in mTERT intron 1 was converted to an AscI site and used to insert a floxed resistance cassette. Correctly targeted clones were identified by long-range PCR, as well as southern blots using 5’, 3’ and copy number probes. Blastocyst injection was performed by the Mouse Biology Program at UC Davis. ES cells expressing both TERT and OCT4 reporters were generated by targeting TERT-Tomato mES cells (puromycin resistant) with an OCT4-ires-GFP targeting construct (G418 resistant). ES cells destined for blastocyst injection were routinely passaged on irradiated feeders in LIF-serum conditions. For other experiments, ES cells were cultured feeder- and serum-free in LIF+2i media (1000U/ml LIF, 3uM CHIR99021, 1uM PDO325901 ; 1% knockout serum replacement ; DMEM/F12 and Neurobasal A mixture supplemented with B27, N2, glutamax beta-mercaptoethanol and pen/strep). Directed differentiation towards the adipocyte lineage was performed as described (Dani et al., 1997). Briefly, ES cells were grown for three passages on gelatin to remove feeders prior to growth in hanging drop conditions. After two days in suspension, the embryoid bodies were collected and grown in suspension for three days in the presence of 100nM all-trans retinoic acid (Sigma). At this point, embryoid bodies were plated in the presence of 0.5 µg/ml bovine insulin (Gemini Bioproducts), 2nM triiodothyronine (Sigma) and 0.5µM rosiglitazone (Cayman Chemicals) for the remainder of the experiment.

Animals
TERT-Tomato mice were either maintained on a C57BL/6 background or outcrossed with B6/129, with no differences in reporter expression. Mice were maintained in a heterozygous state and genotyped by 3-primer PCR of tail genomic DNA (5’AGCTTCTTGAATCGGGGATGT, 5’ACACCCTTGCATCTTGGTTC, 5’GCAACCAAAGTGCGGTAGAT; 189bp band for the wild-type locus, 312bp band for the TERT-Reporter). The puromycin-positive selection cassette was flanked by loxP sites, and was removed by breeding to an EIIA-cre mouse line (Jackson Labs). Deletion was confirmed by 3-primer PCR. Deletion minimally affected the reporter expression pattern (data not shown), therefore experiments were performed mostly on mice retaining the resistance cassette. cKit\textsuperscript{W/Wv} mice (stock #100410) and Tg(OCT4\textDelta PE-EGFP) mice (stock #004654) were purchased from Jackson Labs. Late generation telomerase knockout-mice were generated as previously described (Blasco et al., 1997), using cousin mating schemes to prevent the generation of substrains. All mice were treated in accordance with AAALAC-approved guidelines at Stanford University.

**Antibodies**

The following antibodies were used for immunostaining: RFP (Abcam ab124754; rabbit polyclonal), cKit-PE (Ebioscience 2B8; rat monoclonal), PLZF (Millipore OP128, mouse monoclonal; or Santa Cruz SC-22839, rabbit polyclonal), GFR\alpha1 (R&D Systems AF560, goat polyclonal), MSI2 (Abcam ab76148; rabbit monoclonal), SDC4-biotin (BD Pharmigen 550351; rat monoclonal), ALCAM (Biolegend ME-9F1; rat monoclonal) SCP1 (Novus NB300-229; rabbit polyclonal), SCP3 (Abcam ab97672; mouse monoclonal), GFP (Aves Laboratories GFP-1020; chicken polyclonal) and SOHLH1 (gift of A. Rajkovic).

The following antibodies were used for flow cytometry: cKit-Pe-Cy7 (Ebioscience 2B8; rat monoclonal), CD44-APC-Cy7 (Ebioscience IM7), CD45, EPCAM-PE (Ebioscience G8.8; rat
monoclonal), alpha6-integrin-APC (BD Pharmigen goh3; rat monoclonal), Sca1-APC (Ebiosciences D7), cKit-PE (Ebiosciences 2B8), B220- Pe-Cy7 (Ebiosciences #103221), Cd4-Pe-Cy7 (Ebioscience GK1.5), Cd8-Pe-Cy7 (Ebioscience 53-6.7), Gr1-Pe-Cy7 (Ebioscience 8C5), Mac1-Pe-Cy7 (Ebioscience M1/70) and Ter119-Pe-Cy7 (Ebiosciences Ter119).

**Testes dissociation and FACS analysis**

Testes were dissociated based on standard two-step protocols (Brinster and Zimmermann, 1994). Briefly, testes were removed and detunicated using sharp forceps, and placed into HBSS (137mM NaCl, 5.4mM KCL, 0.44mM KH2PO4, 0.25mM Na2HPO4, 4.2mM NaHC03, 1G/L glucose, 9mM HEPES ph7.4) + 2% fetal bovine serum (FBS; Hyclone). Tubules were placed into 5ml/testis of 1mg/ml collagenase IV (Worthington) for 5 min at room-temperature for the removal of interstitial cells. Detangling was assisted using sharp forceps as well as trituration with a bulb pipette. The tubules were collected in a 100um filter basket, washed with HBSS, and incubated for 15min at 32C in 5ml/testis of 0.25% trypsin (EDTA-free) + 1mg/ml collagenase IV+Dnase1, with frequent trituration with a bulb pipette. The cell preparation was filtered through a 40µm basket, and in some experiments untrypsinized cell were subjected to additional 10min room-temperature incubation in HBSS/FBS +5mM EDTA. The two dissociation fractions were washed and pooled.

For immunophenotyping, cells were incubated in anti-cKit antibodies conjugated to Pe-Cy7 for 30min at 4oC prior to FACS analysis. High side-scatter properties and DAPI staining were used to exclude dead cells. All FACS experiments were performed on a single BD Aria II machine, containing a 405nM violet laser, 488nM blue laser, 561nM yellow-green laser an a 640nM red laser. Of note, the 561nM yellow-green laser with a 582/15 bandpass filter enabled optimal detection of the lowly-expressed TERT-TdTomato fluorophore. Cells were sorted using a 100um nozzle in “purity” mode. Data was analyzed with FlowJo software (Tree Star, San Carlos, CA).
FACS of somatic stem/progenitor populations

To isolate LSK stem/progenitors from the bone marrow, standard procedures were used. Briefly, long bones were flushed with PBS and subjected to red blood cell lysis. Cells were incubated with a cocktail of antibodies against cKit-PE, Sca1-APC and PeCy7-conjugated lineage markers (B220, Cd4, Cd8, Gr1, Mac1 and Ter119). To isolate stem/progenitors from the small intestine, a preparation of single cells from an enriched crypt fraction was generated by serial chelation. Briefly, the small intestine was cut open and feces/mucus removed by incubation in PBS+1mM DTT. The intestine was cut into 1cm long-pieces, and placed into 40mls of HBSS+5mM EDTA, 20mM Na-Citrate, 1% FBS. The solution was shaken horizontally at 37C at 100rpm for 15min. The intestines were collected and the supernatant discarded. The intestines were incubated in 15mls HBSS+5mM EDTA+0.5mM DTT+1% FBS in a 50ml conical, and shaken at 100rpm at 37C. This process was repeated 8 times for 10,6,5,5,10,10,15 and finally 25minutes, respectively. At the end of each cycle, the dissociation media was replaced. The supernatant from the eighth fraction contained a single-cell suspension highly enriched for crypt cells. This fraction was stained with antibodies against CD45, EPCAM-PE and CD44-APC-Cy7.

Telomerase enzymatic assay (TRAP)

Identical numbers of cells (typically 20,000-100,000) were sorted into HBSS+FBS, spun down, and resuspended in 10ul of CHAPS lysis buffer supplemented with Rnase inhibitor, protease inhibitor cocktail and 0.5 mg/ml Rnase-free BSA (Ambion). Lysates were used to program a standard 25-cycle TRAP reaction, following the manufacturer’s protocol (TRAPeze, Chemicon).

Germ Cell Transplantation

Testes cell suspensions were prepared from two adult mice, pooled, and sorted as described previously. For each cell type queried, three to four independent transplantations were
performed. Donor cells were genetically marked using either a R26-lacZ allele (Jackson Labs), or a ubiquitously expressed EGFP allele, driven by the CAG promoter (Lin and Artandi, unpublished). Cells were sorted in HBSS+5%FBS. Sort purity was confirmed by re-analysis post-sort. Cell yields were confirmed post-sort by hemocytometer. Donor cells were introduced into infertile cKit\textsuperscript{W/Wv} recipients (Jackson Labs) via efferent duct injection (Ogawa et al., 1997). The number of cells injected for the different cell populations was adjusted to obtain a countable number (~10) of colonies. Colonization was determined eight weeks after injection, either by X-gal staining or by examining EGFP epifluorescence under a low-power microscope. Colony numbers were normalized to 100,000 cells transplanted. Statistics were calculated using Prism (GraphPad Software, La Jolla, CA).

**Wholemount analysis of seminiferous tubules**

Wholemount analysis was performed as described with slight modifications (Nakagawa et al., 2010). Briefly, tubules were collected and collagenase treated (as described previously) to remove interstitial cells. Throughout the entire staining procedure, tubules were kept in small filter baskets (as opposed to being dried onto glass slides). Tubules preparations were fixed, NP40-cleared and methanol-treated as in Nakagawa et al, but with each methanol treatment step lasting only 5-10min. Blocking was performed in PBST +5% normal goat serum + 10mg/ml BSA. Primary antibodies were incubated 2 days at 4°C. After extensive PBST washes, cells were incubated 1.5 hr at room-temperature in cross-absorbed secondary antibodies diluted 1:1000 in block (Jackson Immunoresearch). After extensive PBST washes, tubules were laid onto a glass slide using fine forceps, and allowed to dry completely. The slides were mounted in Prolong Gold with DAPI. Images were captured on a Zeiss LSM510Meta confocal microscope and processed in Photoshop. For some wholemounts, the NP40 and methanol treatments were replaced by treatment with ice-cold acetone for 10min.
Histological analysis of telomerase-knockout mice

5 µm paraffin sections were deparaffinized and rehydrated, and then boiled in Target Retrieval Solution (DAKO, S1699) for antigen retrieval for 10min. After blocking with 0.5% BSA, 0.1% tween20 in PBS at room temperature for 1 hour, sections were incubated with rabbit anti-PLZF antibody at 4C over night. For signal detection, horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody and DAB substrate were used. Sections were counterstained with hematoxylin and rinsed with 0.02% ammonium water, and then PLZF+ cells were counted. Tubules including less than 10 PLZF-negative differentiating germ cells were defined as degenerated tubules.

Immunofluorescence of FACS-sorted cells

Sorted germ cells were adhered to positively-charged glass slides by cytospin. After spinning for 3 min at 500rpm in a Shandon Cytospin 4 machine, cells were briefly air-dried, then fixed in 4% PFA in PBS for 10 min at room-temperature. The subsequent steps of staining mirrored those used in whole mount analysis. For quantification of PLZF+ cells in various cell fractions, proper exposure times for PLZF were first determined on unsorted cells. Slides from the sorted cell fractions were scanned for fields containing nuclei, and images taken without examining PLZF staining results. Images were quantified in ImageJ. Cells with compromised nuclear morphology (generally large, diffuse nuclei that appear to have lysed) were excluded from the analysis. Images were captured on a Leica wide-field fluorescence microscope and processed in Photoshop.

Cell cycle Analysis

Adult mice received single intra-peritoneal injections of 50mg EdU (5-ethynyl-2’-deoxyuridine; Life Technologies) per kg of bodyweight 2 hours prior to sacrifice. After methanol permeabilization, reactive Alexa-Fluor488 was conjugated to the EdU using Click chemistry as
per the manufacturer’s protocol (Life Technologies Click-It Plus Imaging Kits). Samples were briefly washed with PBS and then processed for anti-PLZF immunostaining as mentioned previously.

RNA-seq library preparation

Dissociated testes cells were prepared and sorted from both testes of adult mice, as described previously. Four biological replicates were sorted. Cells were sorted directly into Trizol LS (Invitrogen), and RNA extracted following the manufacturer’s recommendation. RNA was further purified on Rneasy Micro columns (Qiagen), with a column-based method of gDNA removal. Yield and RNA integrity were confirmed by Bionalyzer (Agilent). cDNA was prepared and amplified using the Nugen Ovation V2 kit, starting from 5 to 10ng of total RNA. The cDNA was sonicated to 200bp size using a Covaris S2 machine, and 25ng of cDNA was used to make the libraries, following standard Illumina Truseq V2 protocols. Samples were sequenced on an Illumina Hiseq2500 machine, with paired-end 101bp reads. RNA sequencing data are available at the NCBI Short Read Archive.

RNA-seq data analysis

Paired-end 101bp RNA-seq reads were quality trimmed using Trim_Galore in paired mode with a Phred cutoff of 15 and a length cutoff of 50bp. Trimmed paired reads were aligned to the mm9 mouse genome build using command line versions of TopHat v2.0.4 and Bowtie2 v2.0.0-6 (Trapnell et al., 2012). Multi-hit mapping was restricted to five genome locations at most (-g 5) with a segment length of 45bp, discordant pairs were filtered out, and de novo junction discovery was disabled. All other mapping options were set to default values. Quality-controlled reads ranged from 15-40million reads per library, with ∼75% of reads mapping to the genome on average.
Subsequent data processing and analysis from the transcriptomes was performed in the R software (http://www.r-project.org/). For analysis in DESeq2, .bam files output from Bowtie were used to generate a matrix of genomic features and read counts using the GenomicRanges and GenomicFeatures packages available from Bioconductor. A transcript annotation file was generated from the UCSC database and counts for each gene were summed from reads aligning to gene exons.

Genes were filtered based on expression levels and all genes with zero read counts were excluded from the analyses. To estimate the robustness and quality of biological replicates after the processing, samples were subject to PCA analysis using the ‘ade4’ R package, and unsupervised clustering was performed using the R package ‘pvclust’ (Dray and Dufour, 2007). Parameters chosen for the clustering step were Pearson’s correlation as a similarity measure, and the use of complete linkage hierarchical clustering. To estimate robustness and P-values, experiments were conducted with 1,000 bootstrap replications of the clustering step.

Differential expression analysis was performed using the DESeq2 R package using pairwise comparisons. We defined significantly differentially expressed genes between cell populations as having greater than 2-fold differences in expression and a q-value lower than 0.01 (0.05 for some comparisons).

Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) was performed using version 2.0.13. Genes were rank-ordered by their T-test value. Genes that could not be converted to a HUGO gene name were excluded. GSEA was run on this pre-ranked list of genes, using default parameters.
**Supplemental Fig.1. TERT-Tomato knock-in construction and validation in mES cultures.**

A. Southern-blot strategy to confirm correction integration of TdTomato construct into the TERT locus.

B. Representative Southern blots for a correctly targeted TERT-Tomato mES line.

C. Targeting strategy to create mES cells containing both TERT and OCT4 reporter constructs.

D. Southern-blot strategy to confirm correction integration of ires-GFP construct into the OCT4 locus.

E. Representative Southern blots for a correctly targeted OCT4 allele within TERT\(^{Tomato/+}\) mES cells.

**Supplemental Fig.2. FACS and wholemount analysis of TERT-Tomato expression in the male germline.**

A. FACS analysis of dissociated tubules from postnatal day6 mice of the indicated genotypes. Cells have been gated by scatter and DAPI exclusion. Fluorescent properties of mice containing only the OCT4 or the TERT reporter were used to draw the gates.

B. Double immunostaining with anti-RFP and anti-PLZF antibodies of seminiferous tubules from TERT\(^{Tomato/+}\) and TERT\(^{+/+}\) mice. Scale bar, 50\(\mu\)m.

**Supplemental Fig.3. Telomerase expression exists as a gradient, correlated with differentiation state.**

A. FACS gating strategy for selecting live, singlet cells from the adult testis. Initial events are selected with low side-scatter values to select against dead/dying cells. Non-singlets are removed based on forward-scatter properties. Dead/dying cells are also removed by DAPI staining.

B. TERT-Tomato expression was determined in live singlet cells by FACS analysis with mice of the indicated genotypes.
C. Populations TERT$^{-\text{Neg1}}$ and TERT$^{-\text{Neg2}}$ were sorted and cytospun, and nuclear morphology determined by DAPI staining. Scale bar, 25µM. Quantification of nuclear staining results from N=3-4 mice and 650-3500 cells. Error bar shows SEM.

D. Population TERT$^{-\text{Low-meiotic}}$ was sorted, cytospun, and immunostained for synaptonemal complex markers SCP1 and SCP3. Scale bar, 25µM. Quantification of synaptonemal complex staining is from N=3 mice and 3800 cells. Error bar shows SEM.

E. Flow cytometry measurement of TERT-Tomato signal intensity in mature sperm harvested from the caudal epididymis. Representative result from at least 3 mice.

F. Indicated cell types were sorted, cytospun, and immunostained for SOHLH1. Scale bar, 25µM. Quantification reflects data from N=4 mice, N=1700-1900 cells. Error bar shows SEM.

**Supplemental Fig.4. Transcriptional analysis of adult spermatogonial subtypes.**

A. Hierarchical clustering of samples with approximately unbiased (au) p-values for each cluster, as calculated by multiscale bootstrap resampling. Number beneath the samples reflect the biological replicates.

B. Wholemount analysis of tubules triple stained for Musashi2 (MSI2), PLZF and cKit. Scale bar, 50µM.

C. FACS analysis of live singlets from adult testes, stained with cKit antibodies and either streptavidin-APC (SAV-APC) alone (left panel) or SDC4-biotin and SAV-APC (right panel).

D. FACS analysis of expression of SDC4 and cKit in the indicated TERT-Tomato populations.

E. Wholemount of adult seminiferous tubules, stained with fluorophore-conjugated ALCAM and cKit antibodies. A single seminiferous tubule was scanned, and two different locations on the tubule (in different seminiferous stages) were imaged with the same exposure conditions. Scale bar, 50µM.
F. FACS analysis of live singlets from adult testes, stained with just cKit antibodies (left panel) or cKit and ALCAM antibodies (right panel).

G. FACS analysis of expression of ALCAM and cKit in the indicated TERT-Tomato populations.

**Supplemental Fig.5. Purity of populations used for transplant.**

A. Determination of purity of cells used for transplantation. The left column indicates the FACS gating strategy used to isolate the target cell population of interest. After sorting, an aliquot of 500-1000 cells was re-analyzed on the machine to measure purity of the cell fraction. Greater than 98% purity was routinely achieved.

**Supplemental Fig.6. Mice homozygous for the TERT reporter are telomerase-knockout mice.**

A. TRAP measurement of telomerase activity of FACS-sorted DAPI-negative testes cells from the indicated genotypes. Mice homozygous for the reporter had dramatically diminished telomerase activity. Dilutions represent the activity of 16,000 and 5,000 cell equivalents, respectively.

B. Fertility of mice of the indicated genotypes. The number of pups resulting from a plug event is shown. Data reflects N=6-10 mice per genotype. Mean value is shown. P-value is calculated by the Mann-Whitney test. Inset box shows macroscopic appearance of adult G1 and G6 adult testis. Scale bar, 2mM.

C. H&E staining of testes cross-sections from early and late TERT\textsuperscript{Tomato/Tomato} knockout mice. Indicated normal and degenerate tubules are highlighted.

D. H&E staining of testes cross-section from postnatal day6 TERT\textsuperscript{Tomato/Tomato} knockout mice. Scale bar, 50µM. Representative histology from two independent litters of late-generation mice.

E. anti-PLZF immunofluorescence on postnatal day6 testicular cross-sections. N=3-5 mice, N=250-450 tubules. Mean and SEM is shown. Statistics are from unpaired T-test.
Table S1. Transcriptome analysis of undifferentiated spermatogonia. Differential expression analysis (DeSeq2) of TERT$^{\text{High}}$ cKit$^-$ vs TERT$^{\text{Low}}$ cKit$^+$ cells. Differentially expressed genes are ranked by Q-value. A negative value for “log2foldchange” represents genes up-regulated in TERT$^{\text{Low}}$ cKit$^+$ cells.

Table S2. GSEA analysis of signaling pathway signatures enriched in TERT$^{\text{High}}$ cKit$^-$ cells compared to TERT$^{\text{Low}}$ cKit$^+$ cells.

Table S3. Genes significantly over-expressed in TERT$^{\text{Low}}$ cKit$^+$ cells that were identified by Chip-seq as being bound by E2F1 or E2F4.
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Pech_Fig. S2

A

Posnatal day 6 Testis

B

Adult

Tert

Tg(Oct4-ΔPE-GFP)

Tg(Oct4-ΔPE-GFP)

Tert

Tomato/+Tg(Oct4-ΔPE-GFP)

Tg(Oct4-ΔPE-GFP);Tert

Tomato/+Tg(Oct4-ΔPE-GFP)

Tg(Oct4-ΔPE-GFP);Tert

Tomato/+Tg(Oct4-ΔPE-GFP)

Tg(Oct4-ΔPE-GFP);Tert

Tomato/+Tg(Oct4-ΔPE-GFP)

Tg(Oct4-ΔPE-GFP);Tert

Tomato/+
### Oncogenic Signature: Top 25 Signatures Up-regulated in TERT-High cKit+ cells

| NAME | SIZE | NES | NOM p-val | FDR q-val | PWR p-val | RANK AT MEAN EDGE | EDGE |
|------|------|-----|-----------|-----------|-----------|------------------|------|
| TERT | 157  | 0.839195 | 0.001596 | 0.001596 | 0.002601 | 0.12 | 1914.26 |
| TERT | 158  | 0.839195 | 0.001596 | 0.001596 | 0.002601 | 0.12 | 1914.26 |
| TERT | 159  | 0.839195 | 0.001596 | 0.001596 | 0.002601 | 0.12 | 1914.26 |
| TERT | 160  | 0.839195 | 0.001596 | 0.001596 | 0.002601 | 0.12 | 1914.26 |

### Oncogenic Signature: Top 25 Signatures Up-regulated in TERT-Low cKit+ cells

| NAME | SIZE | NES | NOM p-val | FDR q-val | PWR p-val | RANK AT MEAN EDGE | EDGE |
|------|------|-----|-----------|-----------|-----------|------------------|------|
| VEGF | 157  | 0.839195 | 0.001596 | 0.001596 | 0.002601 | 0.12 | 1914.26 |
| VEGF | 158  | 0.839195 | 0.001596 | 0.001596 | 0.002601 | 0.12 | 1914.26 |
| VEGF | 159  | 0.839195 | 0.001596 | 0.001596 | 0.002601 | 0.12 | 1914.26 |
| VEGF | 160  | 0.839195 | 0.001596 | 0.001596 | 0.002601 | 0.12 | 1914.26 |
ANNEXIN A2
MHC-CLASS II
V55733
-0.4657507
-2.212633
0.005281462
0.009
196
16.64562%
17.1698%
46.2%
0.0056313
-0.17061
1.73113
0.001341722
0.032499236
0.042
182
0.62014654
0.62014654
0.62014654
0.62014654
10.8
1000.00%
1000.00%
1000.00%
1000.00%
| Cell Cycle Regulation | DNA Replication | Checkpoints | DNA Repair | Chromatin assembly/ modification / condensation / segregation | Mitosis | Miscellaneous |
|-----------------------|-----------------|-------------|------------|----------------------------------------------------------|---------|---------------|
| CDC25A                | MCM3            | CHEK1       | PCNA       | SMC2L1                                                   | PLK     | MAP3K7        |
| CCNA2                 | CDC6            | RAD51       | E3NPA      | SMC4L1                                                   | CALR    |               |
| E2F2                  | RRM1            | BARD1       | SMC4L1     | SMC2L1                                                   | ANLN    |               |
| MCM5                  | PCNA            | BRCA1       | H2A-FX     | KIF4A                                                    | MKI67   |               |
| TOP2A                 |                 |             | H2A-FZ     | CALM2                                                    |         |               |
| RPA3                  |                 |             |            | NCL                                                       |         |               |
| TK1                   |                 |             |            | EZH2                                                     |         |               |