Telomere dysfunction impairs epidermal stem cell specification and differentiation by disrupting BMP/pSmad/P63 signaling

Na Liu1,2,3*, Yu Yin1,4*, Haiying Wang1*, Zhongcheng Zhou1, Xiaoyan Sheng1, Haifeng Fu1, Renpeng Guo1, Hua Wang1, Jiao Yang1, Peng Gong1, Wen Ning1, Zhenyu Ju5, Yifei Liu6*, Lin Liu1,2*

1 State Key Laboratory of Medicinal Chemical Biology, Nankai University, Tianjin, China, 2 Key Laboratory of Bioactive Materials, Ministry of Education, Department of Cell Biology and Genetics, College of Life Sciences, Nankai University, Tianjin, China, 3 School of Medicine, Nankai University, Tianjin, China, 4 Yunnan Key Laboratory of Primate Biomedical Research; Institute of Primate Translational Medicine, Kunming University of Science and Technology, Kunming, China, 5 Key Laboratory of Regenerative Medicine of Ministry of Education, Guangzhou Regenerative Medicine and Health Guangdong Laboratory, Institute of Aging and Regenerative Medicine, Jinan University, Guangzhou, China, 6 Yale Fertility Center and Department of OB/GYN, Yale University School of Medicine, New Haven, CT, United States of America

☯ These authors contributed equally to this work.

* yifei.liu@yale.edu (YL); liulin@nankai.edu.cn (LL)

Abstract

Telomere shortening is associated with aging and age-associated diseases. Additionally, telomere dysfunction resulting from telomerase gene mutation can lead to premature aging, such as apparent skin atrophy and hair loss. However, the molecular signaling linking telomere dysfunction to skin atrophy remains elusive. Here we show that dysfunctional telomere disrupts BMP/pSmad/P63 signaling, impairing epidermal stem cell specification and differentiation of skin and hair follicles. We find that telomere shortening mediated by Terc loss up-regulates Follistatin (Fst), inhibiting pSmad signaling and down-regulating P63 and epidermal keratins in an ESC differentiation model as well as in adult development of telomere-shortened mice. Mechanistically, short telomeres disrupt PRC2/H3K27me3-mediated repression of Fst. Our findings reveal that skin atrophy due to telomere dysfunction is caused by a previously unappreciated link with Fst and BMP signaling that could be explored in the development of therapies.

Author summary

Patients with mutations in the telomerase component (eg, Dyskerin, TERT, TERC) are frequently accompanied by symptoms of abnormal epidermis, such as hyperpigmentation, premature skin degradation, hair follicle shedding, skin atrophy, and dry skin. Mice with mutations in telomere-associated proteins or telomerase genes also show similar phenotypes, associated with telomere shortening. However, the underlying molecular signaling and mechanisms remain elusive. Here, we show that the differentiation of epidermis is disrupted resulting from short telomeres. Epidermal differentiation abnormalities can be
rescued as the telomere length is extended. Furthermore, we uncover that Fst-BMP-Smad pathway is implicated in regulation of epidermal differentiation by telomeres length.

Introduction

Telomeres consist of (TTAGGG)n DNA repeats and associated proteins that locate at chromosome ends, maintaining chromosomal stability and cell proliferation. The telomerase complex consists of a telomerase RNA component (TERC) and the reverse transcriptase catalytic subunit (TERT), and adds telomere repeats to chromosome ends to offset the loss of telomere sequences that occurs due to the end-replication problem, the inability of DNA polymerase to replicate fully the lagging DNA strand [1]. In the absence of telomerase, telomeres shorten progressively with cell division, ultimately leading to loss of telomere protection and a DNA damage response that induces senescence or cell death. Telomere shortening is closely tied to organism aging and premature aging and associated diseases [2–5]. Skin atrophy and hair loss are general phenomena associated with age [6]. Moreover, patients with the mutation of telomerase components (e.g. Dyskerin, TERT, TERC) exhibit telomere shortening and skin atrophy [7]. It has been shown that short telomeres impair differentiation and development of the epidermis, and cause skin atrophy and loss of hair follicles, in association with epidermal stem cell dysfunction with aging [8–10]. However, the molecular signaling underlying short telomeres-associated skin atrophy or degeneration and hair follicle loss remains elusive.

Embryonic stem (ES) cells are able to spontaneously differentiate into three embryonic germ layers ectoderm, mesoderm, and endoderm by standard test of embryoid body (EB) formation. This method has been extensively used to investigate signaling pathways that control ES cell differentiation towards various cell lineages [11–13], including epidermis [14–16]. Telomere length is critical for developmental pluripotency and differentiation capacity of ES cells or iPS cells [17–20]. We attempted to investigate how short telomere compromises epidermal lineage specification and differentiation initially by using ES cell lines with different telomere lengths, derived from Terc knockout (Terc−/−) mice [17]. We showed that telomere lengths affected differentiation of ES cells into epidermis. We further validated that short telomeres impeded epidermal differentiation in the adult telomerase-deficient, telomere shortened mice. Moreover, we investigated potential regulatory mechanisms of telomere length on epidermis differentiation.

Results

Short telomere impairs epidermal stem cell specification and differentiation in vitro

To investigate the differentiation defects associated with short telomeres, we initially performed in vitro differentiation experiments by standard EB formation test using mouse ES cells with various telomere lengths due to telomerase (Terc−/−) deficiency (Fig 1A and 1B, S1A Fig). Telomeres were longest in wild-type (WT) ES cells, shorter in heterozygous (Terc+/−) and early generation (G1) Terc−/− ES cells, and critically short or lost in late generation (G3 and G4) Terc−/− ES cells (Fig 1C and 1D), as we previously reported [17]. Late generation (G3 and G4) Terc−/− cells also exhibited short telomeres by day15 of differentiation (Fig 1C and 1D). Upon differentiation, WT ES cells showed significantly reduced expression of pluripotency marker genes such as Oct4 and Nanog (S1B and S1C Fig). However, G3 and G4 Terc−/− ES cells maintained expression of Nanog and Oct4 at relatively high levels, and low methylation at Nanog
promoter (S1D Fig), consistent with the finding using $Tert^{-/-}$ ES cells also with critically short telomeres [18]. Expression levels of genes related to endoderm, mesoderm and neuro-ectoderm did not differ between WT ES cells and ES cells with short telomeres, suggesting that shortening of telomeres does not significantly affect the differentiation of these germ layers (Fig 1E and 1G).

Notably, expression levels of genes important for epidermal ectoderm differentiation were consistently reduced in telomere shortened (G3/G4 $Terc^{-/-}$) ES cells following differentiation.

Fig 1. Short telomeres impair differentiation of ES cells into epidermis lineage in vitro. (A) Breeding strategy for generating G1, G3, G4 $Terc^{-/-}$ mice and isolation of their ES cell lines from the corresponding mice. ES cell lines used include WT ES cells (N33), $Terc^{-/-}$ ES cells (H1), G1 (F19), G3 (F35), and G4 (A49) $Terc^{-/-}$ ES cells with long to shortest telomeres, respectively. (B) Schematic illustration of in vitro differentiation protocol of ES cells. ES cells were cultured in medium without LIF as hanging drop for 4 days, and then transferred to microwell plates for 11 days. Samples were collected at day 0, day 8, and day 15 following differentiation for various analysis. (C) Telomere length shown as T/S ratio and relative expression levels of $Tert$ and $Terc$ analyzed by real-time qPCR at day 0, day 4, and day 15 of differentiation. Bars = Mean ± SEM (n = 4), ***, $p<0.001$, compared to WT ES cells at the same time point. (D) Telomere length distribution shown as TRF by Southern blot analysis of ES cells at day 0 and day 15 of differentiation. (E) Protein levels of epidermal ($\beta$II-Tubulin), mesodermal ($\alpha$-Sma), and endodermal (Afp) markers in ES cells with different telomeres length verified by Western blot analysis at day 15 of differentiation. ($\beta$-actin served as loading control. (F) Immunofluorescence of epidermal markers K14 and P63 at day 15 of differentiation, displaying areas with defective expression of K14 and P63 in G4 $Terc^{-/-}$ cells, compared with WT cells. Scale bar = 20 μm. (G) Immunofluorescence of neural ectodermal ($\beta$III-Tubulin), mesodermal ($\alpha$-Sma), and endodermal (Afp) markers at day 15 of differentiation in G4 $Terc^{-/-}$ cells and WT cells. Scale bar = 50 μm. ES cells, embryonic stem cells; WT, wild type; K14, Keratin 14.

https://doi.org/10.1371/journal.pgen.1008368.g001
as compared to WT ES cells (S1E and S1F Fig). During mouse embryo development, epider-
mal progenitors are specified at around embryonic day 8–12 (E8-12), later than that of neural
induction. Expression of Keratin 14 (K14) was at very low level on day 8 of differentiation
(Day 8) and was sharply increased on day 15 in WT cells. K14 was also low on day 8 in G3/G4
Terc−/− cells, but dramatically reduced on day 15, as compared to WT cells. Consistently,
expression levels of K5 (epidermal basal cell marker), K1 (epidermis marker of skin) and K4
(epidermis marker in stratified epithelia) [21], in the differentiated G3/G4 Terc−/− cells were
also significantly lower than that in WT cells (S1F Fig).

*p63* as one of the earliest genes for epidermal lineage is expressed as early as E7.5, identifies
epidermal keratinocyte stem cells, and is required for epidermal differentiation [22–24]. *p63*
also is expressed earlier than does *K14* during differentiation of human ES cells into keratino-
cytes [15]. Consistently, *p63* expression was detectable in WT, Terc+/−, and G1 Terc−/− cells by
day 7–8 of differentiation, earlier than that of Keratins, but only minimal in G3/G4 Terc−/−
cells. *p63* level was further increased by day 15 in WT, Terc+/−, and G1 Terc−/− cells, but much
lower in G3 and G4 Terc−/− cells (S1E Fig). Consistent with the qPCR data, protein levels of
both K14 and P63 at day 15 were also greatly reduced in G3/G4 Terc−/−cells as compared to
WT cells (Fig 1E). Immunofluorescence microscopy showed specific staining of P63 in the
nuclei and K14 in cytoplasm and membrane in WT cells but much reduced staining in some
G4 Terc−/− cells (Fig 1F). These data indicated that short telomeres lead to decreased expression
of P63 and K14 and that telomere-shortened stem cells may fail to stratify in the differentiation
into epidermal lineage.

**Short telomere impairs epidermis in vivo**

To examine the impacts of short telomeres on the differentiation capacity in vivo, we initially
performed standard teratoma formation test [12,25]. Both WT and G4 Terc−/− ES cells were
able to differentiate into three germ layers, including endoderm, mesoderm, and neural ecto-
derm revealed by histology (Fig 2A). However, epidermis lineage was reduced in teratomas
differentiated from ES cells with short telomeres, in contrast to that of WT ES cells (Fig 2A
and 2B, S2A Fig). Structures in size or number identified by epidermis marker K14 or epid-

ermal stem cell marker P63 were reduced in the sections of teratomas from G4 Terc−/− ES cells,
comparied with those from WT ES cells (Fig 2B). Relative mRNA levels of *p63* and K14 in tera-
omas derived from G4 Terc−/− ES cells also were lower than those from WT ES cells (Fig 2C).

Similar phenotypes also can be observed in the adult G3 Terc−/− mouse skin. Epidermis
marked by co-immunostaining of P63 and K14 and by histology was thinner on average in
skin of two-three month old G3 Terc−/− mice, compared with age-matched WT mice (Fig 2F
and 2G, S2B and S2C Fig). Additionally, hair follicles were readily seen in dermis of WT mice
but fewer in G3 Terc−/− mice (only 50% of WT mice) (Fig 2D and 2E, S3B Fig). Number of hair
follicles was calculated based on at least 10 fields of view under microscopy. In WT mouse
skin, the hair follicles are structurally intact with an average of 4 to 5 per field of view. How-
ever, hair follicles drop sharply in their numbers and loses the typical structure in the G3
Terc−/− mouse skin (Fig 2D and 2E). Both in vitro and in vivo results validated that short telo-

**Short telomere leads to excessive expression of Fst and represses BMP/
pSmad signaling**

To understand the mechanisms underlying short telomeres-affecting ES cell differentiation
towards epidermal lineage, we performed microarray analysis of G4 Terc−/− ES cells compared
with WT ES cells. Interestingly, *Follistatin* (*Fst*), a negative regulator of Smad pathway which is
Fig 2. Short telomeres impair epidermal differentiation in vivo. (A) Three embryonic germ layers shown by histology following H&E staining of teratomas formed from WT and G4 Terc<sup>−/−</sup> ES cells. Scale bar = 50 μm. (B) Immunofluorescence of epidermis markers shown by K14 and nuclear P63 in teratomas formed from WT and G4 Terc<sup>−/−</sup> ES cells. Nuclei are stained in blue with Hoechst. Scale bar = 50 μm. (C) Expression levels by qPCR of basal layer markers K14 and p63 in teratomas formed from WT and G4 Terc<sup>−/−</sup> ES cells. Bars = Mean ± SEM (n = 3). *, p<0.05; **, p<0.01, compared with WT teratomas. (D) Representative immunofluorescence images showing co-staining of P63 with K14 in the sections of mouse skin epidermis. WT mouse skin displays many hair follicles underneath and G3 Terc<sup>−/−</sup> mouse skin shows fewer and smaller hair follicles. Scale bar = 25 μm. (E) Number of hair follicles in WT and G3 Terc<sup>−/−</sup> mouse skin per field view. Ten field view was counted. **, p<0.01. (F) Representative images showing skin (back) of WT and G3 Terc<sup>−/−</sup> mice revealed by immunofluorescence of K14.
and P63 and histology by H&E staining. Scale bar = 20 μm. (G) Thickness of skin epidermis in WT and G3 Terc−/− mice estimated from H&E histology. *p<0.05.

https://doi.org/10.1371/journal.pgen.1008368.g002

critical in epidermis commitment, was expressed at higher level in G4 Terc−/− than in WT ES cells at day 0 (Fig 3A). qPCR analysis validated that expression levels of Fst were higher in G3 and G4 Terc−/− than in WT ES cells (Fig 3B). Western blot also confirmed that Fst protein level was indeed higher in G4 Terc−/− ES cells than in WT ES cells during differentiation (Fig 3C, left panel). Given that Fst is a secreted protein, we also examined Fst protein levels in the culture media for both cell lines. Fst protein was highly abundant in the culture media of G4 Terc−/− ES cells, but barely detectable in that of WT ES cells (Fig 3C, right panel). Furthermore, robust cytoplasmic and membrane staining of K14 and nuclear P63 were observed at day 15 in differentiated WT cells, but their expression levels were markedly reduced in differentiated G4 Terc−/− cells, where higher Fst fluorescence signals with dotted staining still were readily visible in the cytoplasm of or around the differentiated cells, compared with lower Fst fluorescence in WT cells (Fig 3D). Additionally, Fst protein level was higher in G4 Terc−/− teratomas than in control teratomas (WT and Terc+/− ) (Fig 3E). Compared to WT teratomas, G4 Terc−/− teratomas exhibited strong Fst immunofluorescence spotted inside or outside the cells, coincided with less and weak fluorescence staining of K14 and P63 (Fig 3F and 3G). These data provide further evidence that higher expression level of Fst is linked to short telomere.

Similar results were obtained from the skin of adult mice. Two-three month old G3 Terc−/− mice displayed thinner epidermis and skin atrophy compared to the age-matched WT mice, consistent with previous studies [8,26]. K14 level also was reduced in the epidermis of G3 Terc−/− adult mice, accompanied by increased expression of spotty Fst as compared to WT mouse epidermis (S3A Fig). Immunofluorescence staining of Fst in teratomas or tissues appears to be dotted in pattern, somewhat different from the immunostaining in cultured cells, probably because Fst can be locally confined with specific structure in tissues, whereas it diffuses in and around the cultured cells. In addition, G3 Terc−/− mice displayed defective hair follicle development as evidenced by notably reduced number of hair follicles, with reduced expression of K14 and increased Fst, as well as impaired bulb and bulge at the basal follicles where progenitor cells reside, in contrast to the intact bulb (hair germ) and bulge in WT mice (S3B Fig). Taken together, short telomeres lead to excessive expression of Fst, which is incompatible with epidermal stem cell specification and stratification of skin and hair follicles.

Fst negatively regulates pSmad1/5/8 and p63

Collectively, these findings suggested that short telomere specifically prevents the transition from the common ectodermal progenitor state into the epidermis fate. Bone morphogenesis protein 4 (BMP4) signaling is known to be activated in the embryo at the time of ectodermal fate determination, inhibits premature neural differentiation while inducing epidermis development, and can act through phosphorylation and nuclear accumulation of Smad1/5/8 [27–30]. Differentiation of epidermal cells appears to be controlled, in part, by BMP4 [31]. Fst is an antagonist of BMP4. We next asked if the Fst-BMP-Smad1/5/8 signaling pathway plays a critical role in epidermal differentiation. In the wild-type ES cells, the up-regulation of BMP4, BMP7, Smad1, and the down-stream target (Gata1) during differentiation indicates that this pathway plays an important role in normal differentiation and development of epidermis (S4 Fig).

Compared with those of WT, Terc−/−, or G1 Terc−/− ES cells, levels of phosphorylated Smad1/5/8 were reduced in G3/G4 Terc−/− ES cells during differentiation (Fig 3H), suggesting that this pathway is suppressed by short telomeres. Gene expression upstream of this pathway
seemed to be not affected by short telomeres. Downstream target genes of this signaling pathway such as Gata1 were expressed at lower levels in G4 Terc−/− than in WT cells by day 8 and day 15 of differentiation (S4 Fig). These data suggested that short telomeres suppress BMP/pSmad signaling following differentiation.

Above data imply that elevated expression of Fst resulting from short telomere might lead to reduction of pSmad1/5/8, P63 and K14, and thus defective epidermal stem cell specification and differentiation. To further validate this concept, we generated Fst overexpression (OE) ES cell line (Fig 4A) and performed EB differentiation test using WT ES cell line as control. Western blot showed that Fst OE ES cells expressed p63 and K14 at reduced levels on day 8 and day 15 of differentiation, which was also confirmed by immunofluorescence microscopy (Fig 4B)
Fig 4. Fst inhibits pSmad, P63 and K14. (A) Relative expression levels by qPCR of Fst during differentiation of WT ES cells stably overexpressing Fst (OE), compared with WT ES cells transfected with empty vector served as controls (Con). Bars = Mean ± SEM (n = 3). (B) Protein levels of Fst, Smad, pSmad, P63 and K14 by Western blot in Fst overexpressed ES cells compared with controls. Right panel, quantification of proteins level using ImageJ software, normalized to β-actin. * p<0.05; ** p<0.01; *** p<0.001, compared to controls. (C)
Notably, in the differentiated Fst OE cell culture, areas with intensive Fst fluorescence indicative of high expression level exhibited minimal K14 staining, and yet areas with low Fst fluorescence displayed strong K14 or p63 staining (Fig 4C). Hence, high Fst level is discordant with expression of P63 and K14. Consistently, pSmad1/5/8 was decreased in Fst OE cells (Fig 4B). These data suggest a conserved but new role of Fst in negatively regulating pSmad-signal-pathway during epidermal ectoderm induction.

To test whether reducing Fst can de-repress down-stream genes/signaling for epidermis, we knocked down Fst by RNA interference in the differentiated G4 Terc–/– cells. Effective knock-down of Fst by shRNA in differentiated G4 Terc–/– cells up-regulated the levels of pSmad1/5/8 and P63 (Fig 4D and 4E). Fst downregulation by RNA interference in the differentiated G4 Terc–/– cells rescued P63 but not fully rescued K14 expression. This may be explained by three potential reasons. Changes in the expression level of K14 could be delayed following P63 expression during epidermal differentiation. Factors other than Fst alone also might be involved in regulation of K14 expression. Alternatively, the regulation of Fst-P63-K14 may slightly differ in differentiated ES cells compared with undifferentiated ES cells as model. Nevertheless, these data further support the notion that excessive expression levels of Fst negatively regulate pSmad1/5/8 signaling and p63, weakening epidermal stem cell specification and differentiation.

Repair of Terc rejuvenates telomeres and rescues Fst/P63/K14 signaling

Then, we tested whether rejuvenating telomeres in ES cells with short telomeres can repress Fst. Using CRISPR/Cas9 technology, we successfully knocked in Terc in G4 Terc–/– ES cells and obtained several Terc-repaired ES cell lines (two lines are shown in Fig 5A). These Terc repaired (TR) ES cell lines exhibited much longer telomeres than did their parental G4 Terc–/– ES cell line after culture for 10 passages. Yet, their telomeres were still shorter than those of WT cells as revealed by qPCR and QFISH (Fig 5B and 5C), presumably because of inadequate passages, even though the telomerase activity was recovered (Fig 5D). Frequency of telomere loss was significantly reduced in Terc repaired ES cell lines, in contrast to that of G4 Terc–/– ES cells (Fig 5C). We repeated the in vitro differentiation assay with WT, G4 Terc–/–, and Terc repaired ES cell lines. On day 15 of differentiation, telomere length was also rescued in Terc repaired cells compared with Terc–/– cells (Fig 5B). Terc repaired cells showed reduced level of Fst and noticeably increased protein levels of P63 and K14 as compared to those of G4 Terc–/– cells (Fig 5E), which were confirmed by immunofluorescence microscopy (Fig 5F and 5G). These results suggested that epidermal differentiation could be rescued by repairing Terc and restoration of telomere length.

Fst is regulated by PRC2-mediated repression

The critical question was how short telomeres result in excessive Fst expression. Fst gene is located at the subtelomere region of the long arm of chromosome 13, whose expression might be regulated by telomere position effect (TPE) [32]. To reveal the telomere state of chromosome 13, we performed immunofluorescence microscopy to detect the chromosome 13 using the
chromosome specific probe followed by telomere FISH. Notably, one pair of chromosome 13 in G4 Terc–/– ES cells constantly displayed telomere signal-free ends, indicative of telomere loss, in contrast to four intact telomere signals of WT ES cells (Fig 6A). Moreover, chromosome fusion
Fig 6. *Fst* is regulated by epigenetic modification. (A) Frequency of telomere signal-free ends and fusion of chromosome 13 in G4 Terc−/− ES cells, compared with WT ES cells. Telomere FISH by PNA probe and chromosome identification by XMP13 probe of WT and G4 Terc−/− ES cells. Arrows indicate chromosome 13 stained with XMP13. Chr13A and Chr13B are a pair of chromosome 13 in the same spread. Loss of telomeres near *Fst* gene locus and fusion of chromosome 13 are
compared between WT and G4 Terc−/− ES cells. (B) ChIP-qPCR analysis of abundance of H3K9me3, H3K9me2, H3K9Ac, and H3K27me3 at Fst promoter loci in WT and G4 Terc−/− ES cells. Mean ± SEM (n = 3). * p < 0.05; ** p < 0.01, compared to WT cells. (C) A simplified model showing regulation by telomere length of Fst/BMP4-Smad/P63 signaling in epidermal stem cell specification and differentiation. With functional telomeres, enrichment of PRC2/H3K27me3 at Fst promoter foci represses Fst, maintaining normal BMP4-Smad signaling and proper expression levels of P63 and Keratins (e.g. K14), in the specification and differentiation of epidermis and hair follicles. In the event of telomere shortening or loss, abundance of H3K27me3 at Fst promoter loci in WT and G4 Terc−/− ES cells, compared with WT, heterozygous, or G1 Terc−/− ES cells exhibited decreased H3K9me3 immunofluorescence and foci at heterochromatin and telomeres prior to and after differentiation, compared with WT ES cells (S5B Fig). Also, G4 Terc−/− ES cells exhibited decreased H3K9me3 abundance at telomeres/subtelomeres [33]. We tested whether epigenetic modifications are implicated in regulation of Fst. Both DNA methyltransferases Dnmt3a and 3b were expressed at lower levels in G4 Terc−/− ES cells than in WT cells, but Dnmt3b expressed at higher levels following differentiation (S5A Fig). By ChIP-qPCR analysis using specific primers and Dnmt3b antibody, levels of Dnmt3b at Fst loci did not differ between G4 Terc−/− and WT ES cells (S5B Fig). Also, Fst promoter showed only low methylation levels in G4 Terc−/− and WT ES cells like that of MEF (S5C Fig). Methylation levels at subtelomeres of chromosome 13 were greatly reduced in G4 Terc−/− ES cells, but markedly increased in G4 Terc−/− cells following differentiation, compared with WT ES cells (S5D Fig). These data suggest that Fst promoter methylation may not directly contribute to excessive Fst expression due to short telomere.

We analyzed the abundance of histone modifications of H3K4me3, H3K9me3 and H3K27me3 by western blot. H3K4me3 abundance seemed not to differ between G4 Terc−/− and WT ES cells, while H3K9me3 and H3K27me3 abundance were slightly reduced in G3/G4 Terc−/− ES cells, compared with WT, heterozygous, or G1 Terc−/− ES cells (S6A Fig). Also, G4 Terc−/− ES cells exhibited decreased H3K9me3 immunofluorescence and foci at heterochromatin and telomeres prior to and after differentiation, compared with WT ES cells (S6B Fig).

Furthermore, we performed ChIP-qPCR analysis to examine the abundance of H3K9me3/2, H3K9Ac and H3K27me3 at Fst promoter loci using β-actin as a control. Enrichment of H3K9me3, H3K9Ac, and H3K9me2 at Fst promoter was low and showed no significant difference between WT and G4 Terc−/− ES cells (Fig 6B). However, H3K27me3 was highly enriched at Fst promoter. Importantly, H3K27me3 level was markedly reduced at all five loci of Fst promoter in G4 Terc−/−, compared with that of WT ES cells (Fig 6B). By luciferase Reporter assay, the Fst promoter activity was higher in G4 Terc−/− than in WT ES cells (S7A Fig). We further examined expression levels of Eed, Suz12, Ezh1 and Ezh2 which are catalytic components of Polycomb repressive complex PRC2 and potentially tri-methylate H3K27 to repress gene expression and that are shown to play important roles in skin stem cell function and differentiation [34,35]. Expression levels of Ezh1 and Ezh2 are reduced in G4 Terc−/− ES cells as compared to WT ES cells (S7B Fig). Telomere-repaired ES cells partially restored Ezh1/2 expression, together with increased H3K27me3 enrichment at Fst promoter (S7C and S7D Fig). Pluripotent marker genes Nanog and Oct4 were also down-regulated during differentiation of Terc repaired G4 ES cells, like those of WT ES cells (S7E and S7F Fig). It is interesting to note that Ezh2 expression level in one Terc-repaired ES cell line (A49 TR3) was not recovered well, and coincidently this clone had relatively shorter telomere than that of WT ES cells (Fig 5C). These results further suggest that short telomeres reduce H3K27me3 enrichment at Fst promoter, likely together with reduced Ezh1 and Ezh2 levels, de-repress Fst, and these together may contribute to excessive expression of Fst. Excessive Fst further down-regulates p63/K14 through disrupting BMP4/pSmad signaling (Fig 6C).
Discussion

Based on the data obtained from both ES cell differentiation \textit{in vitro} and \textit{in vivo}, we propose that functional telomere is important for suppressing $Fst$ to prevent its overexpression and to maintain normal expression of P63 and K14 during epidermal stem cell specification and differentiation. Short telomere disrupts PRC2- H3K27me3-mediated repression of $Fst$, which leads to excessive $Fst$ expression. Consequently, excessive $Fst$ suppresses BMP/pSmad signaling, reducing P63 and keratins and resulting in epidermal differentiation defects and skin atrophy. This model links dysfunctional telomeres to skin atrophy and hair follicle loss by disrupting $Fst$/BMP/pSmad/P63/K14 signaling.

This study also provides additional evidence in supporting that ES cell differentiation model is a powerful alternative tool to discover novel signaling and mechanisms that are involved in \textit{in vivo} cell lineage specification at very early developmental stages that might not be readily revealed in live mouse model and particularly in humans [11]. The differentiation assay used in our study shows that the dynamics of P63 and K14 in mouse ES cell is similar to that of human ES cells and mouse embryonic skin development [14]. Our results also confirmed that P63 is a master regulator for K14, K5 and other epidermal genes [36,37], and that BMP4/pSmad signaling pathway can activate P63 [38]. BMP4 negatively regulates neural induction and promotes epidermogenesis during differentiation of mouse ES cells [31], whereas blocking BMP signaling facilitates differentiation of human ES cells into neural lineages [28].

Mounting evidence supports the notion that telomere dysfunction is accompanied by symptoms of abnormal epidermis [10,39–42]. Mice with critically short telomeres exhibit symptoms, including epidermal abnormalities such as poor wound healing, ulcerative skin lesions, early hair loss and early hair graying [2,8,10,43]. We show that short telomeres lead to reduced expression of P63 and declined epidermal stratification and formation, linking to skin atrophy. Study of P63-null mice demonstrates important roles of P63 in orchestrating first epidermal stratification [44,45], p63-null mice exhibit striking defects in embryonic epidermal morphogenesis [46], and also suffer from diminished stem cell renewal capacity [47]. Moreover, TAp63 serves to maintain adult skin stem cells and prevents premature tissue aging [45,48,49]. Hence, P63 is required to maintain epidermal stem cell renewal while allowing K14 expression and epidermal differentiation [24]. Short telomeres cause stem cell failure [50], and also impair the ability of epidermal stem cells to mobilize out of the hair follicle niche, and thus skin and hair growth [26]. On the other hand, hyper-long telomeres are advantageous for skin regeneration compared with normal length telomeres [51]. Our data provide novel molecular mechanisms of linking short telomeres to reduced pSmad signaling and P63 and thus declined epidermal differentiation.

Moreover, excessive $Fst$ expression resulting from short telomere negatively regulates BMP/pSmad/P63 pathways in the epidermal stem cell specification and differentiation. It has been reported that $Fst$ is an antagonist of BMPs by blocking binding of BMP with its receptor [52]. Excessive $Fst$ may compete with BMPs and inhibit BMP-pSmad signaling. Telomere re-elongation successfully achieved by CRISPR/Cas9-mediated knock-in of $Terc$ represses $Fst$ and recovers expression of P63 and K14. Consistently, telomerase reintroduction into mice with critically short telomeres is sufficient to elongate telomeres in skin keratinocytes and to correct epidermal hair follicle stem cell defects, and rescues skin and hair growth defects [26]. These data also may explain the early findings that $Fst$-knockout mice die within hours of birth but show thicker epidermis [53]. Likewise, deletion of $Fst$ results in enhanced keratinocyte proliferation in the tail epidermis of these animals and an earlier onset of keratinocyte
hyperproliferation at the wound edge after skin injury, suggesting that Fst regulates epidermal homeostasis and also wound repair [54].

In agreement, Fst-overexpression transgenic mice are characterized by a thinner dermis and epidermis, reduced density of the dermis and smaller hair follicles, indicative of skin atrophy, and a severe delay in wound healing observed after injury [55]. Moreover, mice that overexpress Fst are smaller compared with their control littermates, and their body weight is significantly reduced. This phenotype is similar to that of late generation Terc−/− mice [2,56] (also shown in this study), and these mice also exhibit severely impaired wound healing [2]. Coincidently, p63−/− mice have an impaired wound-healing response as well [48]. Together, these data support the idea that abnormal Fst/p63 signaling is implicated in short telomere-associated skin atrophy and wound healing. Excitingly, mouse ES cells with hyper-long telomeres generate healthier chimera mice that also have longer telomeres and exhibit delayed aging and high capacity for skin wound healing [51].

Short telomeres can change expression of many genes and signaling pathways particularly with cell differentiation, as shown by the transcription profile data. We identified unique alterations of down-stream genes under regulation by the major TGFβ superfamily during differentiation of ES cells into epidermal lineage. Fst happens to be an evident negative regulator upstream in this pathway, and is up-regulated when telomere is short. We searched for mechanisms underlying short telomere-induced activation of Fst, and tested the hypothesis that repressive histone modification or DNA methylation may underlie telomere suppression of Fst. By ChIP-qPCR assay with selective related antibodies, we show that PRC2-mediated repression involving Ezh1/2 and H3K27me3 makes a major contribution to suppressing Fst. In fact, the regulatory region of Fst gene is characteristic of bivalent genes whose promoters are enriched for both activating mark by H3K4me3 and repressing mark by H3K27me3 and Ezh2, primed for differential expression upon differentiation [57–59]. In mice, PRC2 has been found to be enriched in the progenitor cells of developing epidermis, regulates epidermal specification in mouse embryos and maintains hair follicle homeostasis [60,61]. H3K27me3 marks are enriched in a subset of epidermal differentiation gene promoters in undifferentiated cells and disappear on a subset of epidermal gene promoters upon differentiation [62]. Moreover, Ezh1 and Ezh2 repress premature differentiation and H3K27me3 is involved in early lineage specification of embryonic epidermis differentiation [60]. Interestingly, Ezh1/2 null skin progenitors show reduced H3K27me3 abundance and significant up-regulation of Fst [61]. Identification of hair follicle stem cell signature genes showed that Fst also is one of genes involving transit-amplifying (TA) progeny repressed by H3K27me3, whereas BMP4 signaling is activated during this process likely induced by epigenetic shift to control by H3K4me3 and H3K79me2 [63]. Consistently, short telomere reduces H3K27me3 enrichment at Fst promoter, which leads to elevated Fst expression and defective epidermal specification and differentiation. Terc-repaired G4 Terc−/− ES cells rejuvenate telomere length to various degrees and partly restore H3K27me3-mediated suppression of Fst. Nanog and Oct4 are down-regulated following differentiation of Terc-repaired G4 Terc−/− ES cells like WT cells. Coincidentally, Tert−/− ES cells also have critically short telomeres and disrupted PRC2 function and low H3K27me3 enrichment at Nanog promoter, leading to defective suppression of Nanog during differentiation [18]. Fst-BMP4 signal pathway is known as a critical regulator for epidermal differentiation initiation and induced expression of p63, which may coordinate with BMP4 to accelerate epidermal specification by regulating accumulation of H3K27me3 [64]. Deletion of p63 resulted in a significant decrease in signal of H3K27me3 mark [64]. We show that short telomeres can up-regulate Fst via reducing H3K27me3 at Fst promoter and decrease pSmad, resulting in declined expression of p63. These findings suggest a complex feedback...
mechanism between H3K27me3 and Fst-BMP4-P63. Fst, BMP4, P63, and H3K27me3 are key players in the orchestra that regulates epidermal differentiation.

Another interesting phenomenon for Fst promoter is its hypomethylated state. Additionally, methylation levels at subtelomeres of chromosome 13 where Fst gene is located are also drastically reduced in G4 Terc−/− ES cells, compared with those of WT ES cells. Coincidently, Fst and other subtelomeric genes such as Tcstv1/3 in chromosome 13 are expressed at higher levels in G4 Terc−/− ES cells, but down-regulated in Terc-repaired G4 Terc−/− ES cells like WT cells (S8 Fig). DNA hypomethylation could lead to decreased levels of H3K27me3 in ordinarily unmethylated regions [18,65]. Our data suggests that H3K9me3-mediated gene silencing does not play a direct role in repressing Fst. We indeed find a global reduction of H3K9me3 in G4 Terc−/− ES cells in which H3K9me3 also shows reduced co-localization with telomeres. These data suggest that telomere shortening-induced reduction of H3K9me3 at telomeres/subtelomeres may have a general impact on gene de-repression instead of a direct impact on Fst gene.

Taken together, short or loss of telomere disrupts PRC2 function involving H3K27me3 and de-represses Fst. Elevated Fst inhibits pSmad/P63 signaling, leading to defective epidermal stem cell specification, stratification and differentiation. Rejuvenating telomere length can rescue these defects. We do not exclude the possibility that additional signaling pathways may also be involved in and/or cooperate with aberrant Fst/pSmad/P63 signaling in defective epidermal differentiation resulting from telomere dysfunction. Targeting Fst/pSmad/P63 pathway may have implications in ameliorating skin and hair degeneration associated with aging and telomere shortening.

Materials and methods

Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee at Nankai University (License number 20140006). All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Nankai University. All efforts were made to minimize the number of animals used by the experimental design.

Mice

Two-three month old Terc deficient (Terc−/−) mice and wild-type mice in C57Bl/6 background, and immunodeficient mice were used in this study. Mice were housed and cared for in a pathogen-free facility at Nankai University.

ES cells and culture

Terc−/− ES cells were generated from Terc deficient mice and cultured as previously described [17]. N33 ES cell line was derived from wild-type mice, heterozygous (H1) ES cells from Terc−/+ mice, and F19, F35, and A49 ES cell lines from G1, G3, G4 Terc−/− mice, respectively. These ES cells were maintained on mitomycin-C treated mouse embryonic fibroblasts as feeders in ES cell culture medium containing knockout Dulbecco’s modified Eagle medium (KO-DMEM) (Invitrogen) added with 20% fetal bovine serum (Hyclone), 1000 U/ml LIF, 0.1 mM β-mercaptoethanol, 1 mM L-glutamine, 0.1 mM non-essential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin.
**Terc repair in Terc<sup>−/−</sup>ES cell line by CRISPR/Cas9**

pSpCas9(BB)-2A-Puro (PX459) was a gift from Feng Zhang (plasmid # 48139, Addgene). Guide RNAs were designed using the online design tool available at [http://crispr.genome-engineering.org/](http://crispr.genome-engineering.org/). PX459 was digested with BbsI and then gel purified. Two pairs of oligos including targeting sequences were annealed and cloned into the BbsI-digested PX459 vector. The Terc donor sequences were obtained based on mouse genomic sequence and the information provided in the original paper [43]. The Terc donor vector contained Terc flanked by 5'(Left) and 3'(Right) homology arms. The DNA fragments are individually amplified by proper primers and then cloned into the vector with proper enzymes. G4 Terc<sup>−/−</sup>ES cell line A49 was transfected with two PX459 and Terc donor plasmids using lipofectamine 2000 transfection reagent (Invitrogen). Twenty-four hours later, 2 μg/ml puromycin was added into the culture medium for 7 days, clones were picked and the genomic DNA was extracted. PCR was performed with several pairs of primers to detect and obtain the genomic knock-in Terc repaired cell lines.

**In vitro differentiation of ES cells**

ES cells are allowed to aggregate and form three-dimensional colonies known as embryoid bodies (EBs) [66]. Differentiation of ES cells was accomplished in a two-step process: (1) Embryoid body (EB) formation was obtained by using cell suspension and hanging drop method. Undifferentiated ES cells were trypsinized to obtain a single cell suspension, and EBs were formed in ES cell culture medium without LIF, in a definite number of cells in “hanging drops” for 4 days. (2) Then, EB were transferred to 24-well microwell plates with one EB per well. Daily microscopic observations were conducted to detect beating EBs. 10~15 EBs were transferred to 6-well microwell plates per well for protein, RNA, and DNA sample collection.

**Teratoma formation assay and histological analysis**

Approximately 2×10<sup>6</sup> ES cells with different telomere length were injected subcutaneously into dorsal flanks of immunodeficient mice. Four weeks after the injection, the mice were humanely sacrificed and the teratomas were surgically dissected from the mice. Samples were weighed, fixed in PBS containing 3.7% formaldehyde, and embedded in paraffin. Sections were stained with hematoxylin and eosin for histological examination.

**RNA extraction and qPCR**

The total RNA was isolated from samples using TriZol (Invitrogen) or RNeasy mini kit (Qiagen) according to the manufacturer’s protocol. The purity and concentration of RNA were checked using Nanodrop technology (Agilent). 2μg RNA was subjected to cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen). Quantitative real-time PCR reactions were set up in duplicate with the FastStart Universal SYBR Green Master (ROX) (Roche) and run on the iCycler iQ5 2.0 Standard Edition Optical System (Bio-Rad). Each sample was repeated at least twice and analyzed with Gapdh served as the internal control. Quantification of gene expression was based on the Ct (Cycle threshold) value. Melting curve analysis and electrophoresis were performed to control PCR products specificities and exclude nonspecific amplification. PCR Primers, designed using Primer5 and Gene Runner software, are listed in [S1 Table](#).

**Western blot**

Cells were collected and washed with cold phosphate buffered saline (PBS), then resuspended in cell lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM
EGTA, 1 mM NaF, 20 mM Na₃P₂O₇, 1 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.25% deoxycholate and 0.1% SDS. 20 μg of proteins were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF, Millipore) membrane. Nonspecific binding was blocked by incubation in 5% nonfat dry milk in TBST at room temperature. Blots were then probed overnight at 4°C with primary antibodies against K14 (ab7800, Abcam), P63 (ab124762, Abcam), H3 (ab1791, Abcam), H3K4me3 (ab1012, Abcam), H3K9me3 (07–442, Millipore), H3K27me3 (07–449, Millipore), Smad1 (#9743, CST), pSmad1/5/8 (#9511, CST), pSmad2/3(#8828, CST), Smad2/3(#5678, CST), Fst (ab64490, Abcam), Dnmt3a (ab13888, Abcam), Dnmt3b (ab13604, Abcam), or β-actin (sc1616R, Santa Cruz), washed and incubated for 2 h with secondary antibodies HRP conjugated donkey anti-Rabbit IgG (NA934v GE Healthcare) or goat anti-mouse IgG (H+L) (ZB2305). Protein bands were detected using ECL western blotting detection reagents (WBKLS0100 Millipore). The band intensity was measured by software ImageJ and normalized to the intensity of β-actin. The relative expression level was calculated from the results of at least three independent experiments or samples and presented as mean ± SEM [67].

**Telomere measurement by qPCR**

Cells were washed in PBS and stored at -20°C until subsequent DNA extraction. Genome DNA was prepared using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Average telomere length was measured from total genomic DNA using a real-time PCR assay, as previously described [68], but modified for measurement of mouse telomere [69]. PCR reactions were performed on the iCycler iQ5 2.0 Standard Edition Optical System (Bio-Rad, Hercules, CA), using telomeric primers, primers for the reference control gene (mouse 36B4 single copy gene) and PCR settings as previously described [70]. For each PCR reaction, a standard curve was made by serial dilutions of known amounts of DNA. The telomere signal was normalized to the signal from the single copy gene to generate a T/S ratio indicative of relative telomere length. Equal amounts of DNA (20 ng) were used for each reaction. The primers for telomere measurement by qPCR are listed in [S2 Table](#).

**Telomere quantitative fluorescence in situ hybridization (QFISH)**

Telomere length and function (telomere integrity and chromosome stability) were estimated by telomere quantitative FISH [17,43]. Briefly, cells were incubated with 0.5 μg/ml nocodazole for 1.5 h to enrich cells at metaphases. Chromosome spreads were made by standard method. Metaphase-enriched cells were exposed to hypotonic treatment with 75 mM KCl solution, fixed with methanol:glacial acetic acid (3:1) and spread onto clean slides. Telomeres were denatured at 80°C for 3 min and hybridized with FITC-labeled telomere (CCCTAA) peptide nucleic acid (PNA) probe (0.5 μg/ml) (Panagene, Korea). Chromosomes were stained with 0.5 μg/ml DAPI. Fluorescence from chromosomes and telomeres was digitally imaged on a Zeiss microscope with fluorescein isothiocyanate (FITC)/DAPI filters, using AxioCam and AxioVision software 4.6. Telomere length shown as telomere fluorescence intensity was integrated using the TFL-TELO program (a gift kindly provided by Peter Lansdorp).

**Telomerase activity by TRAP assay**

Telomerase activity was measured by the Stretch PCR method according to the manufacturer’s instruction using TeloChaser Telomerase assay kit (T0001, MD Biotechnology). Briefly, about 2.5 × 10⁶ cells from each sample were lysed. Lysis buffer served as negative controls. PCR products of cell lysates were separated on non-denaturing TBE-based 12% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining.
Telomere Restriction Fragment (TRF) measurement

TRF analysis was performed using a commercial kit (TeloTAGGG Telomere Length Assay, catalog no. 12209136001, Roche Life Science). Cells were pretreated with RNaseA and Proteinase K (PCR Grade, 03115879001, Roche Life Science), followed by extraction using phenol:chloroform:isoamyl alcohol, digested with MboI (R0147, NEB) at 37 °C overnight and electrophoresed through 1% agarose gels in 0.5 × TBE at 14 °C using a CHEF Mapper pulsed field electrophoresis system (Bio-rad). Auto algorithm was used to separate DNA samples with a size range from 5 to 150 kb. The gel was blotted and probed using reagents in the kit.

Immunofluorescence microscopy

Tail or back skin tissues obtained from wild-type (WT) or G3 TerC deficient mice, or teratomas were fixed overnight in 3.7% paraformaldehyde at 4˚C, dehydrated through graded alcohols and xylene, and embedded in paraffin. After deparaffinizing, rehydrating and washing in PBS, sections were incubated with 3% H2O2 for 10 min at room temperature to block endogenous peroxidase, subjected to high pressure antigen recovery sequentially in 0.01% citrate buffer for 3 min, blocked with 5% goat serum in PBS for 2 h at room temperature, and then incubated with the primary antibodies against K14 (ab7800, Abcam), Fst (ab64490, Abcam) or P63 (ab124762, Abcam) overnight at 4˚C, washed and incubated for 2 h with appropriate fluorescence-conjugated secondary antibodies (Goat anti Mouse IgG (H+L), FITC, 115-095-003, Jackson; Goat anti Rabbit IgG (H+L), Alexa Fluor 594, 111-585-003, Jackson). For immunostaining of ES cells and in vitro differentiated cells, they were washed twice in PBS, then fixed in freshly prepared 3.7% paraformaldehyde in PBS (pH 7.4), permeabilized in 0.1% Triton X-100 (Sigma–Aldrich, Saint Louis, MO) in blocking solution (3% goat serum plus 0.5% BSA in PBS) for 30 min, washed and left in blocking solution for 1 h. Cells were then incubated overnight at 4˚C with primary antibodies and then secondary antibodies as described above. Blocking solution without the primary antibody served as negative control. Nuclei were counterstained with 0.5 μg/ml Hoechst 33342 in Vectashield mounting medium. Fluorescence was imaged using a Zeiss fluorescence microscope (Axio Imager Z1) and using the same exposure time for each group. ImageJ software (https://imagej.net/) was used for relative quantity measurement of fluorescence intensity. Region-of-interest (ROI) tool was used to select the cell or background, and the fluorescence intensity of ROIs achieved. Background with the same threshold was subtracted for each image.

Immunofluorescence-telomere FISH (IF-FISH)

IF-FISH was performed based on an established protocol [71]. Briefly, immunostaining of the cells was performed as described above. After washing the excess of secondary antibody with PBS, cells were fixed in 4% formaldehyde for 2 min, dehydrated with ethanol, and incubated with FITC-telomeric PNA probe as described earlier for telomere QFISH. Fluorescence was imaged using the Zeiss fluorescence microscope.

Bisulfite genomic sequencing

DNA methylation by bisulfite sequencing Genomic DNA was extracted from cells using DNeasy & Blood Tissue Kit (Qiagen) according to the manufacturer’s instructions. Bisulfite treatment of DNA was performed with the EpTect Bisulfite Kit (Qiagen). Bisulfite converted DNA was amplified by seminested PCR, using HS EX Taq DNA Polymerase (Takara). Primer sequences are detailed in S3 Table. PCR products were recovered from stained gels (EasyPure
Quick Gel Extraction Kit (Transgen), cloned into a pEASY-T1 Simple Cloning vector (Transgen) and then sequenced.

**Overexpression of Fst or p63**

The plasmid pEASY-T1-Fst- overexpression (OE) and pEASY-T1-p63-OE were constructed by amplification of Fst or p63 cDNA by PCR and cloning it into pEASY-T1 simple cloning vector (TransGen). Following digestion with XhoI and NotI, Fst or p63 sequences were inserted into Plch37 plasmid. Then the recombinant plasmids were transfected into J1 ES cells or MEF. At 48 h after transfection with 2 μg plasmid using lipofectamine 2000 (Invitrogen), cells were collected for protein and RNA extraction. For obtaining stably transfected cell lines with Fst overexpression, cells were transfected with 2 μg plasmid using lipofectamine 2000 (Invitrogen) and selected by 1.5 μg/ml puromycin for 7–10 days, and clones were picked.

**Fst RNAi**

shRNA sequences were synthesized (S4 Table), and cloned into pSIREN-RetroQ, according to manufacturer’s instructions. The shRNAs without sequence homology to mouse genes served as a negative control. The RNAi retrovirus was packaged using Plat-E cells and then infected cells during differentiation.

**ChIP-qPCR assay**

ChIP-qPCR analysis was performed as described previously [72], with slight modification. Briefly, 5 × 10^7 cells were fixed with 1% paraformaldehyde, lysed, and sonicated to achieve the majority of DNA fragments with 100–1000 bp. DNA fragments were then enriched by immunoprecipitation with 5 μg H3K9me3 antibody (ab8898, Abcam), 7 μg H3K9Ac antibody (ab4441, Abcam), 5 μg Dnmt3b antibody (ab13604, Abcam), 5 μg H3K9me2 antibody (ab1220, Abcam) or 5 μg H3K27me3 (ab6002, Abcam). The eluted protein:DNA complex was reverse-crosslinked at 65 °C overnight. DNA was recovered after proteinase and RNase A treatment. Real-time PCR was performed to compare the histone modification at the Fst promoter region using primers provided in S5 Table. Normal rabbit IgG (#2729S, Cell Signaling) or Mouse (G3A1) mAb IgG1 Isotype Control (5415S, Cell Signaling) served as negative control.

**Genome-wide gene expression by microarray analysis**

Microarray was performed by CapitalBio Corporation (Beijing, China) using Affymetrix 430 2.0 oligonucleotide mouse arrays designed from GenBank, dbEST, and RefSeq sequences based on the UniGene database. The analysis was carried out based on the software and method provided by CapitalBio (http://www.capitalbio.com). Only probe sets showing at least 1.8-fold change were retained in the final list. The detection call indicates whether a transcript was reliably detected (P, Present) or not (A, Absent). We performed hierarchical clustering with the differentially expressed genes using cluster software (version 3) and by applied mean centering and normalization of genes and arrays prior to average linkage clustering.

**Luciferase reporter assay**

The Fst promoter (~2000bp) was cloned into pGL3-basic vector, following digestion with XhoI and HindIII. 2×10^5 ES cells per 12 well were transfected with 1 μg pGL3-basic vector containing Fst promoter and 10 ng pRL-SV40 vector as control using lipofectamine 2000 (Invitrogen) according to manufacturer’s instruction. 24 hours after transfection, ES cells were
lysed with 1×PLB (positive lysis buffer, Promega), shaken for 15 min, and then centrifuged at 13000 rpm for 10 min at 4˚C. The supernatants were collected and analyzed for luciferase activity by dual reporter assay according to manufacturer’s instructions.

**Chromosome XMP13 FISH and telomere FISH**

XMP13 probe (D-1413, Metasystems) specific for mouse Chromosome 13 was used for chromosome identification. FISH on chromosome spread was performed according to manufacturer’s instructions. The probe was added and coverslip placed, sealed with rubber cement, denatured by heating slide at 75˚C for 2 min, and incubated in humidified chamber at 37˚C overnight. Slides were washed and stained with 0.5 μg/ml DAPI in VectaShield antifade medium. Digital images were captured using a CCD camera on a Zeiss Imager Z2 microscope. The coordinates of the chromosome were recorded with the venire scale along the top and side of the microscope stage. The slides were washed and performed with telomere FISH as described above. After staining with DAPI again, fluorescence from chromosomes and telomeres was digitally imaged using the same microscope according to the recorded coordinates. The telomeres of chromosome 13 were revealed by comparison of the images from the same coordinates.

**Statistical analysis**

The data from multiple groups were analyzed by ANOVA, and means were compared by Fisher’s protected least significant difference (PLSD) using the StatView software from SAS Institute. T-test was used to analyze statistical significance of the two-paired groups. Significant differences were defined as p < 0.05, 0.01, or lower.

**Supporting information**

**S1 Fig. In vitro differentiation of ES cells with various telomere lengths.** (A) Morphology of colonies of ES cells (WT, Terc+/–, G1, G3, and G4 Terc–/– ES cells), embryoid body (EB) at day 4, and differentiated cells by day 8 and day 15. (B&C) Relative expression level of pluripotent marker genes Oct4 (B) and Nanog (C) at indicated time points of differentiation. Bars = Mean ± SEM (n = 3). (D) Methylation level of Nanog promoter analyzed at day 0 and day 15 of differentiation in WT and G4 Terc–/– ES cells. Genomic DNA was treated with bisulfite, followed by PCR amplification and sequencing. Circles, CpG sites within the regions analyzed; filled circles, methylated cytosines indicated by percentages underneath; open circles, unmethylated cytosines. (E&F) Relative mRNA levels by qPCR analysis of epidermal stem cell marker p63 (E), and epidermis basal layer markers K14, K5, K4, and K1 (F) at day 0, day 8, and day 15 of in vitro differentiation. Bars = Mean ± SEM (n = 3). *, p<0.05; **, p<0.01, compared to WT ES cells at the same time point. ES cells, embryonic stem cells; WT, wild type; K14, Keratin 14; K5, keratin 5; K4, keratin 4; K1, keratin 1; EB, embryoid body.

**S2 Fig. Short telomeres impair epidermal differentiation in vivo.** (A) Epidermal differentiation in teratomas from WT and G4 Terc–/– ES cells as shown by immunofluorescence (IF) of P63 and K14. Scale bar = 50 μm. (B) Representative images showing skin (tail) of WT and G3 Terc–/– mice revealed by immunofluorescence of K14 and P63 and histology by H&E staining. Scale bar = 50 μm. (C) Thickness of skin epidermis in WT and G3 Terc–/– mice estimated from H&E histology. *, p<0.05.
S3 Fig. Co-immunofluorescence of P63 or K14 with Fst expression. (A) Representative immunofluorescence images showing co-staining of K14 (green) with Fst (red) in sections of mouse back skin. Scale bar = 20 μm. (B) Representative immunofluorescence images showing co-staining of K14 with Fst in the sections of mouse skin epidermis. WT mouse skin displays many hair follicles underneath and G3 Terc−/− mouse skin shows fewer and smaller hair follicles. Scale bar = 25 μm.

S4 Fig. Genome-wide gene expression profile showing differential gene expression in WT cells with long telomeres and G4 Terc−/− knockout (KO) cells with shortest telomeres. (A) Heatmap illustrating relative expression pattern of G4 Terc−/− cells compared to WT cells in duplicates. The genes with changes > = 1.8-fold between two groups were chosen for heatmap. The number of differentially expressed genes increased during the differentiation. (B) Heatmap highlighting relative expression pattern of genes related to DNA methylation, pluripotency, BMP/TGF-β signaling pathway and epidermis in G4 Terc−/− cells compared to WT cells in duplicates. During differentiation, WT ES cells exhibit significant reduction in expression of pluripotency genes, but G4 Terc−/− ES cells still maintain relatively high expression levels of pluripotency genes. On the contrary, BMP/TGF-β signaling genes are expressed at higher levels during differentiation of WT (wild type) ES cells, but at reduced levels in G4 Terc−/− ES cells. (C) Relative expression levels of genes related to BMP4/Smad1 pathway analyzed by qPCR in ES cells with various telomere lengths. BMP, bone morphogenetic protein.

S5 Fig. Comparison of DNA methylation levels in ES cells and following differentiation. (A) Protein levels of both Dnmt3a and Dnmt3b are lower in G3/G4 Terc−/− ES cells than in WT ES cells by Western blot. However, Dnmt3b levels are higher and Dnmt3a lower in G3/G4 Terc−/− cells than in WT cells by day 15 of differentiation. β-actin served as loading control. (B) Real-time PCR based ChIP analysis of Dnmt3b abundance at Fst promoter region in WT and G4 Terc−/− ES cells. Bars = Mean ± SEM (n = 4). (C) Methylation status of Fst in WT ES cells, G4 Terc−/− ES cells, and MEF. (D) Methylation level of subtelomere region of Chr13 in ES cells and at day 15 following differentiation. Genomic DNA was treated with bisulfite, followed by PCR amplification and sequencing. Circles, CpG sites within the regions analyzed; filled circles, methylated cytosines indicated by percentages underneath; open circles, unmethylated cytosines. MEF, mouse embryonic fibroblasts cells.

S6 Fig. Histone levels in ES cells with various telomere lengths and following differentiation. (A) Histone levels by Western blot analysis of WT, Terc+/−, G1, G3, and G4 Terc−/− ES cells prior to differentiation (day 0) and at day 8, day 15 of differentiation. Histone H3 served as loading control. (B) Immunofluorescence and co-localization of H3K9me3 distribution and foci and telomere FISH in WT and G4 Terc−/− ES cells at day 0 or at day 15 of differentiation. Relative H3K9me3 immunofluorescence intensity was estimated by Image J software. ***, P<0.001. (JPG)

S7 Fig. Regulation of Fst by Ezh1, Ezh2 and H3K27me3. (A) Fst promoter activity is higher in G4 Terc−/− than in WT ES cells. Mean ± SEM (n = 3). **, P<0.01. (B) Relative expression levels of genes associated with PRC2 and H3K27me3 by qPCR analysis. (C) Expression levels by qPCR of Ezh1 and Ezh2 in WT, G4 Terc−/− and G4 Terc-repaired ES cells. Bars = Mean ± SEM (n = 3). *, p<0.05; **, p<0.01, compared with WT ES cells. (D) ChIP-qPCR analysis of H3K27me3 abundance at Fst promoter region in WT, G4 Terc−/− and Terc repaired ES cells, showing decreased level of H3K27me3 at Fst promoter in cells with short telomere.
Mean ± SEM (n = 3). β-actin served as control. *, p<0.05; **, p<0.01. (E&F) Immunofluorescence of Nanog and Oct4 at day 0 (E) and day 15 (F) of differentiation in WT, G4 Terc−/− and G4 Terc-repaired cells. Scale bar = 20 μm.

S8 Fig. Heatmap illustrating relative expression pattern of representative genes located between Fst and telomere in WT ES cells, G4 Terc−/− knockout ES cells and Terc repaired G4 Terc−/− ES cells. The genes near the end of long arm of chromosome 13 with expression levels with FPKM more than 1 by RNA-seq are shown.

(TIF)

S1 Table. Primers for quantitative real-time PCR analysis.
(DOCX)

S2 Table. Primers used for telomere length measurement by qPCR.
(DOCX)

S3 Table. Primers for methylation analysis.
(DOCX)

S4 Table. shRNA sequences targeting to Fst.
(DOCX)

S5 Table. Primers for ChIP-qPCR.
(DOCX)

Acknowledgments

We thank Fang Wang, Kai Liu, Rongfei Zhang, Jiaojiao Li, Yingying Dong, Yan Li, Qian Zhang, Zhenrong Zhao, Ming Zeng, and Jiameng Dan for providing materials, assisting experiments or discussion.

Author Contributions

Conceptualization: Lin Liu.
Data curation: Yu Yin.
Investigation: Na Liu, Yu Yin, Haiying Wang, Zhongcheng Zhou, Xiaoyan Sheng, Haifeng Fu, Renpeng Guo, Hua Wang, Jiao Yang, Peng Gong, Lin Liu.
Methodology: Yu Yin, Zhongcheng Zhou, Xiaoyan Sheng, Haifeng Fu, Hua Wang, Yifei Liu.
Resources: Wen Ning, Zhenyu Ju, Yifei Liu.
Supervision: Lin Liu.
Validation: Haiying Wang, Yifei Liu.
Writing – original draft: Na Liu.
Writing – review & editing: Zhenyu Ju, Yifei Liu, Lin Liu.

References

1. Martinez P, Blasco MA (2011) Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. Nat Rev Cancer 11: 161–176. https://doi.org/10.1038/nrc3025 PMID: 21346783
2. Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, et al. (1999) Longevity, stress response, and cancer in aging telomerase-deficient mice. Cell 96: 701–712. https://doi.org/10.1016/s0092-8674(00)80580-2 PMID: 10089885

3. Chang S, Multani AS, Cabrera NG, Naylor ML, Laud P, et al. (2004) Essential role of limiting telomeres in the pathogenesis of Werner syndrome. Nat Genet 36: 877–882. https://doi.org/10.1038/ng1389 PMID: 15235603

4. Armanios M, Alder JK, Parry EM, Karim B, Strong MA, et al. (2009) Short telomeres are sufficient to cause the degenerative defects associated with aging. Am J Hum Genet 85: 823–832. https://doi.org/10.1016/j.ajhg.2009.10.028 PMID: 19944403

5. Blackburn EH, Epel ES, Lin J (2015) Human telomere biology: A contributory and interactive factor in aging, disease risks, and protection. Science 350: 1193–1198. https://doi.org/10.1126/science.aab3389 PMID: 26785477

6. Hsu YC, Li L, Fuchs E (2014) Emerging interactions between skin stem cells and their niches. Nat Med 20: 847–856. https://doi.org/10.1038/nm.3643 PMID: 25100530

7. Vulliamy T, Marrone A, Szydlo R, Walne A, Mason PJ, et al. (2004) Disease anticipation is associated with progressive telomere shortening in families with dyskeratosi congenita due to mutations in TERC. Nat Genet 36: 447–449. https://doi.org/10.1038/ng1346 PMID: 15098033

8. Flores I, Cayuela ML, Blasco MA (2005) Effects of telomerase and telomere length on epidermal stem cell behavior. Science 309: 1253–1256. https://doi.org/10.1126/science.1115025 PMID: 16037417

9. Flores I, Canela A, Vera E, Tejera A, Cotsarelis G, et al. (2008) The longest telomeres: a general signature of adult stem cell compartments. Genes Dev 22: 654–667. https://doi.org/10.1101/gad.451008 PMID: 18283121

10. Buckingham EM, Klingelhutz AJ (2011) The role of telomeres in the ageing of human skin. Exp Dermol 20: 297–302. https://doi.org/10.1111/j.1600-0625.2010.01242.x PMID: 21371125

11. Keller G (2005) Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes Dev 19: 1129–1155. https://doi.org/10.1101/gad.1303605 PMID: 15905405

12. Tsankov AM, Akopian V, Pop R, Chetty S, Gifford CA, et al. (2015) A qPCR ScoreCard quantifies the differentiation potential of human pluripotent stem cells. Nat Biotechnol 33: 1182–1192. https://doi.org/10.1038/nbt.3387 PMID: 26501952

13. De Los Angeles A, Ferrari F, Xi R, Fujiwara Y, Benvenisty N, et al. (2015) Hallmarks of pluripotency. Nature 525: 469–478. https://doi.org/10.1038/nature15515 PMID: 26399828

14. Tadeu AM, Horsley V (2013) Notch signaling represses p63 expression in the developing surface ectoderm. Development 140: 3777–3786. https://doi.org/10.1242/dev.093948 PMID: 23924630

15. Green H, Easley K, Iuchi S (2003) Marker succession during the development of keratinocytes from cultured human embryonic stem cells. Proc Natl Acad Sci U S A 100: 15625–15630. https://doi.org/10.1073/pnas.0307226100 PMID: 14693151

16. Guenou H, Nissan X, Larcher F, Feteira J, Lemaitre G, et al. (2009) Human embryonic stem-cell derivatives for full reconstruction of the pluristratified epidermis: a preclinical study. Lancet 374: 1745–1753. https://doi.org/10.1016/S0140-6736(09)61496-3 PMID: 19932355

17. Huang J, Wang F, Okuka M, Liu N, Ji G, et al. (2011) Association of telomere length with authentic pluripotency of ES/iPS cells. Cell Res 21: 779–792. https://doi.org/10.1038/cr.2011.16 PMID: 21283131

18. Pucci F, Gardano L, Harrington L (2013) Short telomeres in ESCs lead to unstable differentiation. Cell Stem Cell 12: 479–486. https://doi.org/10.1016/j.stem.2013.01.018 PMID: 23561444

19. Marion RM, Strati K, Li H, Tejera A, Schoeftner S, et al. (2009) Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells. Cell Stem Cell 4: 141–154. https://doi.org/10.1016/j.stem.2008.12.010 PMID: 19200803

20. Aguado T, Gutierrez FJ, Aix E, Schneider RP, Giovinazzo G, et al. (2017) Telomere Length Defines the Cardiomyocyte Differentiation Potency of Mouse Induced Pluripotent Stem Cells. Stem Cells 35: 362–373. https://doi.org/10.1002/stem.2497 PMID: 27612935

21. Albers KM (1996) Keratin biochemistry. Clin Dermatol 14: 309–320. PMID: 8862908

22. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, et al. (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398: 708–713. https://doi.org/10.1038/19531 PMID: 10227293

23. Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, et al. (2001) p63 identifies keratinocyte stem cells. Proc Natl Acad Sci U S A 98: 3156–3161. https://doi.org/10.1073/pnas.061032098 PMID: 11248048

24. Fuchs E (2008) Skin stem cells: rising to the surface. J Cell Biol 180: 273–284. https://doi.org/10.1083/jcb.200708185 PMID: 18209104
25. Avior Y, Biancotti JC, Benvenisty N (2015) TeratoScore: Assessing the Differentiation Potential of Human Pluripotent Stem Cells by Quantitative Expression Analysis of Teratomas. Stem Cell Reports 4: 967–974. https://doi.org/10.1016/j.stemcr.2015.05.006 PMID: 26070610

26. Siegl-Cachedener I, Flores I, Klett P, Blasco MA (2007) Telomerase reverses epidermal hair follicle stem cell defects and loss of long-term survival associated with critically short telomeres. J Cell Biol 179: 277–290. https://doi.org/10.1083/jcb.200704141 PMID: 17954610

27. Oshimori N, Fuchs E (2012) Paracrine TGF-beta signaling counterbalances BMP-mediated repression in hair follicle stem cell activation. Cell Stem Cell 10: 63–75. https://doi.org/10.1016/j.stem.2011.11.005 PMID: 2226356

28. Gerrard L, Rodgers L, Cui W (2005) Differentiation of human embryonic stem cells to neural lineages in adherent culture by blocking bone morphogenetic protein signaling. Stem Cells 23: 1234–1241. https://doi.org/10.1634/stemcells.2005-0110 PMID: 16002783

29. Qiao Y, Zhu Y, Sheng N, Chen J, Tao R, et al. (2012) AP2gamma regulates neural and epidermal development downstream of the BMP pathway at early stages of ectodermal patterning. Cell Res 22: 1546–1561. https://doi.org/10.1038/cr.2012.122 PMID: 22945355

30. Di-Gregorio A, Sancho M, Stuckey DW, Crompton LA, Godwin J, et al. (2007) BMP signalling inhibits premature neural differentiation in the mouse embryo. Development 134: 3359–3369. https://doi.org/10.1242/dev.005967 PMID: 17699604

31. Kawasaki H, Mizuseki K, Nishikawa S, Kaneko S, Kuwana Y, et al. (2000) Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron 28: 31–40. https://doi.org/10.1016/s0896-6273(00)00083-0 PMID: 11086981

32. Robin JD, Ludlow AT, Batten K, Magdiner F, Stadler G, et al. (2014) Telomere position effect: regulation of gene expression with progressive telomere shortening over long distances. Genes Dev 28: 2464–2476. https://doi.org/10.1101/gad.251041.114 PMID: 25403178

33. Benetti R, Garcia-Cao M, Blasco MA (2007) Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. Nat Genet 39: 243–250. https://doi.org/10.1038/ng1952 PMID: 17277871

34. Dauber KL, Perdigoto CN, Valdes VJ, Santoriello FJ, Cohen I, et al. (2016) Dissecting the Roles of Polycomb Repressive Complex 2 Subunits in the Control of Skin Development. J Invest Dermatol 136: 1647–1655. https://doi.org/10.1016/j.jid.2016.02.809 PMID: 26994968

35. Bird AT, Zhang J, Perdigoto CN, Nicolis S, et al. (2013) Polycomb subunits Ezh1 and Ezh2 regulate the Merkel cell differentiation program in skin stem cells. EMBO J 32: 1990–2000. https://doi.org/10.1038/embj.2013.110 PMID: 23673358

36. Truong AB, Kretz M, Ridky TW, Kimmel R, Khavari PA (2006) p63 regulates proliferation and differentiation of developmentally mature keratinocytes. Genes Dev 20: 3185–3197. https://doi.org/10.1101/gad.1463206 PMID: 17114587

37. Romano RA, Ortt K, Birkaya B, Smalley K, Sinha S (2009) An active role of the DeltaN isoform of p63 in regulating basal keratin genes K5 and K14 and directing epidermal cell fate. PLoS One 4: e5623. https://doi.org/10.1371/journal.pone.0005623 PMID: 19461998

38. Laronda MM, Unno K, Ishi K, Serna VA, Butler LM, et al. (2013) Diethylstilbestrol induces vaginal adenosis by disrupting SMAD/RUNX1-mediated cell fate decision in the Mullerian duct epithelium. Dev Biol 381: 5–16. https://doi.org/10.1016/j.ydbio.2013.06.024 PMID: 23830984

39. Munoz P, Blanco R, Flores JM, Blasco MA (2005) XPF nuclease-dependent telomere loss and increased DNA damage in mice overexpressing TRF2 result in premature aging and cancer. Dev Biol 381: 5–16. https://doi.org/10.1016/j.ydbio.2013.06.024 PMID: 23830984

40. Tejera AM, Stagno d’Alcontres M, Thanasoula M, Marion RM, Martinez P, et al. (2010) TPP1 is required for TERT recruitment, telomere elongation during nuclear reprogramming, and normal skin development in mice. Dev Cell 18: 775–789. https://doi.org/10.1016/j.devcel.2010.03.011 PMID: 20493811

41. Martinez P, Thanasoula M, Munoz P, Liao C, Tejera A, et al. (2009) Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice. Genes Dev 23: 2060–2075. https://doi.org/10.1101/gad.543509 PMID: 19679647

42. Stout GJ, Blasco MA (2009) Genetic dissection of the mechanisms underlying telomere-associated diseases: impact of the TRF2 telomeric protein on mouse epidermal stem cells. Dis Model Mech 2: 139–156. https://doi.org/10.1242/dmm.002121 PMID: 19259387

43. Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, et al. (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 91: 25–34. https://doi.org/10.1016/s0092-8674(01)80006-4 PMID: 9335332
44. Shalom-Feuerstein R, Lena AM, Zhou H, De La Forest Divonne S, Van Bokhoven H, et al. (2011) DeltaNp63 is an ectodermal gatekeeper of epidermal morphogenesis. Cell Death Differ 18: 887–896. https://doi.org/10.1038/cdd.2010.159 PMID: 21127502

45. Senoo M, Pinto F, Crum CP, McKeon F (2007) p63 Is essential for the proliferative potential of stem cells in stratified epithelia. Cell 129: 523–536. https://doi.org/10.1016/j.cell.2007.02.045 PMID: 17482546

46. Vanbokhoven H, Melino G, Candi E, Declercq W (2011) p63, a story of mice and men. J Invest Dermatol 131: 1196–1207. https://doi.org/10.1038/jid.2011.84 PMID: 21471985

47. Romano RA, Smalley K, Magraw C, Serna VA, Kurita T, et al. (2012) DeltaNp63 knockout mice reveal its indispensable role as a master regulator of epithelial development and differentiation. Development 139: 772–782. https://doi.org/10.1242/dev.071191 PMID: 22274697

48. Su X, Gi YJ, Tsai KY, Cho MS, et al. (2009) TAp63 prevents premature aging by promoting adult stem cell maintenance. Cell Stem Cell 5: 64–75. https://doi.org/10.1016/j.stem.2009.04.003 PMID: 19570515

49. Sethi I, Romano RA, Gluck C, Smalley K, Vojtesek B, et al. (2015) A global analysis of the complex landscape of isoforms and regulatory networks of p63 in human cells and tissues. BMC Genomics 16: 584. https://doi.org/10.1186/s12864-015-1793-9 PMID: 27252083

50. Hao LY, Armanios M, Strong MA, Karim B, Feldser DM, et al. (2005) Short telomeres, even in the presence of telomerase, limit tissue renewal capacity. Cell 123: 1121–1131. https://doi.org/10.1016/j.cell.2005.11.020 PMID: 16360040

51. Varela E, Munoz-Lorente MA, Tejera AM, Ortega S, Blasco MA (2016) Generation of mice with longer and better preserved telomeres in the absence of genetic manipulations. Nat Commun 7: 11739. https://doi.org/10.1038/ncomms11739 PMID: 27252083

52. Fainsod A, Deissler K, Yelin R, Marom K, Epstein M, et al. (1997) The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. Mech Dev 63: 39–50. https://doi.org/10.1016/s0925-4773(97)00673-4 PMID: 9178255

53. Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR, et al. (1995) Multiple defects and perinatal death in mice deficient in follistatin. Nature 374: 360–363. https://doi.org/10.1038/374360a0 PMID: 7885475

54. Antsiferova M, Klatte JE, Bodo E, Paus R, Jorcano JL, et al. (2009) Keratinocyte-derived follistatin regulates epidermal homeostasis and wound repair. Lab Invest 89: 131–141. https://doi.org/10.1038/labinvest.2008.120 PMID: 19079322

55. Wankell M, Munz B, Hubner G, Hans W, Wolf E, et al. (2001) Impaired wound healing in transgenic mice overexpressing the activin antagonist follistatin in the epidermis. EMBO J 20: 5361–5372. https://doi.org/10.1093/emboj/20.19.5361 PMID: 11574468

56. Herrera E, Samper E, Martin-Caballero J, Flores JM, Lee HW, et al. (1999) Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. EMBO J 18: 2950–2960. https://doi.org/10.1093/emboj/18.11.2950 PMID: 10357808

57. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, et al. (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448: 553–560. https://doi.org/10.1038/nature06008 PMID: 17603471

58. Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, et al. (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441: 349–353. https://doi.org/10.1038/nature04733 PMID: 16625203

59. Shen X, Liu Y, Hsu YJ, Fujiwara Y, Kim J, et al. (2008) EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. Mol Cell 32: 491–502. https://doi.org/10.1016/j.molcel.2008.10.016 PMID: 19026780

60. Ezhkova E, Pasolli HA, Parker JS, Stokes N, Su IH, et al. (2009) Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. Cell 136: 1122–1135. https://doi.org/10.1016/j.cell.2008.12.043 PMID: 19303854

61. Ezhkova E, Lien WH, Stokes N, Pasolli HA, Silva JM, et al. (2011) EZH1 and EZH2 cogovern histone H3K27 trimethylation and are essential for hair follicle homeostasis and wound repair. Genes Dev 25: 485–498. https://doi.org/10.1101/gad.2019811 PMID: 21317239

62. Sen GL, Webster DE, Barragan DI, Chang HY, Khavari PA (2008) Control of differentiation in a self-renewing mammalian tissue by the histone demethylase JMJD3. Genes Dev 22: 1865–1870. https://doi.org/10.1101/gad.1673508 PMID: 18628393

63. Lien WH, Guo X, Polak L, Lawton LN, Young RA, et al. (2011) Genome-wide maps of histone modifications unwind in vivo chromatin states of the hair follicle lineage. Cell Stem Cell 9: 219–232. https://doi.org/10.1016/j.stem.2011.07.015 PMID: 21885018
64. Pattison JM, Melo SP, Piekos SN, Torkelson JL, Bashkirova E, et al. (2018) Retinoic acid and BMP4 cooperate with p63 to alter chromatin dynamics during surface epithelial commitment. Nat Genet 50: 1658–1665. https://doi.org/10.1038/s41588-018-0263-0 PMID: 30397335

65. Brinkman AB, Gu H, Bartels SJ, Zhang Y, Matarese F, et al. (2012) Sequential ChIP-bisulfite sequencing enables direct genome-scale investigation of chromatin and DNA methylation cross-talk. Genome Res 22: 1128–1138. https://doi.org/10.1101/gr.133728.111 PMID: 22466170

66. Keller GM (1995) In vitro differentiation of embryonic stem cells. Curr Opin Cell Biol 7: 862–869. PMID: 8608017

67. Hartig SM (2013) Basic image analysis and manipulation in ImageJ. Curr Protoc Mol Biol Chapter 14: Unit14 15.

68. Cawthon RM (2002) Telomere measurement by quantitative PCR. Nucleic Acids Res 30: e47. https://doi.org/10.1093/nar/30.10.e47 PMID: 12000852

69. Callicott RJ, Womack JE (2006) Real-time PCR assay for measurement of mouse telomeres. Comp Med 56: 17–22. PMID: 16521855

70. Liu L, Bailey SM, Okuka M, Munoz P, Li C, et al. (2007) Telomere lengthening early in development. Nat Cell Biol 9: 1436–1441. https://doi.org/10.1038/ncb1664 PMID: 17982445

71. Sfeir A, Kabir S, van Overbeek M, Celli GB, de Lange T (2010) Loss of Rap1 induces telomere recombination in the absence of NHEJ or a DNA damage signal. Science 327: 1657–1661. https://doi.org/10.1126/science.1185100 PMID: 2039076

72. Dan J, Liu Y, Liu N, Chiourea M, Okuka M, et al. (2014) Rif1 maintains telomere length homeostasis of ESCs by mediating heterochromatin silencing. Dev Cell 29: 7–19. https://doi.org/10.1016/j.devcel.2014.03.004 PMID: 24735877