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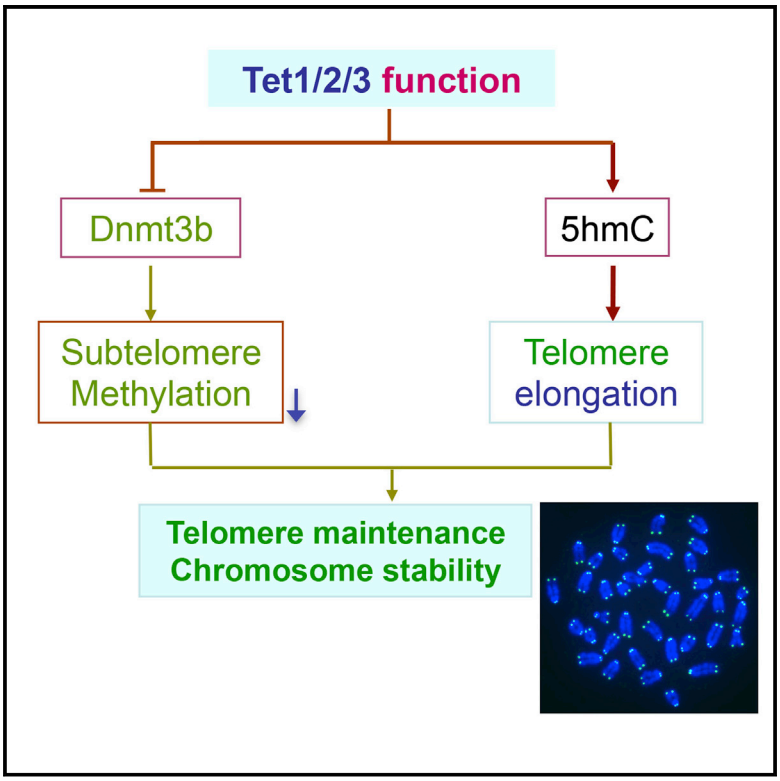
Tet Enzymes Regulate Telomere Maintenance and Chromosomal Stability of Mouse ESCs

Graphical Abstract



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In Brief

Sub-telomeric DNA methylation negatively regulates telomere recombination and telomere length. Here, Yang et al. report that Tet enzymes maintain telomere length and chromosomal stability by modulating both Dnmt3b expression and methylation levels.

Highlights

- Tet enzymes maintain telomere length
- Tet enzymes maintain chromosomal stability of ESCs
- Dnmt3b and 5hmC are involved in Tet-signaling-mediated telomere maintenance
- Excessive Zscan4 and Dnmt3b lead to heterogeneous telomere elongation and loss

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Tet Enzymes Regulate Telomere Maintenance and Chromosomal Stability of Mouse ESCs

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SUMMARY

Ten-eleven translocation (Tet) family proteins convert 5-methylcytosine to 5-hydroxymethylcytosine. We show that mouse embryonic stem cells (ESCs) depleted of *Tet1* and/or *Tet2* by RNAi exhibit short telomeres and chromosomal instability, concomitant with reduced telomere recombination. *Tet1* and *Tet2* double-knockout ESCs also display short telomeres but to a lesser extent. Notably, *Tet1/2/3* triple-knockout ESCs show heterogeneous telomere lengths and increased frequency of telomere loss and chromosomal fusion. Mechanistically, *Tets* depletion or deficiency increases *Dnmt3b* and decreases 5hmC levels, resulting in elevated methylation levels at sub-telomeres. Consistently, knockdown of *Dnmt3b* or addition of 2i (MAPK and GSK3β inhibitors), which also inhibits *Dnmt3b*, reduces telomere shortening, partially rescuing *Tet1/2* deficiency. Interestingly, *Tet1/2* double or *Tet1/2/3* triple knockout in ESCs consistently upregulates *Zscan4*, which may counteract telomere shortening. Together, Tet enzymes play important roles in telomere maintenance and chromosomal stability of ESCs by modulating sub-telomeric methylation levels.

INTRODUCTION

Ten-eleven translocation (Tet) family proteins oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), an intermediate that can lead to DNA demethylation (Kohli and Zhang, 2013; Tahiliani et al., 2009). TET proteins are implicated in diverse biological processes, including epigenetic regulation of gene transcription, embryonic development, stem cell function and pluripotency, and cancer, but the underlying mechanisms still remain to be defined (review Pastor et al., 2013; Costa et al., 2013; Dawlaty et al., 2013; Ficz et al., 2011; Ito et al., 2010; Koh et al., 2011). Tet proteins regulate genome-wide reprogramming by modulating DNA methylation levels (Wu et al., 2011; Xu et al., 2011) and affect embryonic stem cell (ESC) pluripotency and differentiation (Costa et al., 2013; Ficz et al., 2011; Ito et al., 2010).

Roles of Tets in vivo were tested by generating *Tets* knockout (KO) mice. Intriguingly, if they survive from early development, *Tets*-deficient mice mostly do not show observable phenotypes (Dawlaty et al., 2013; Li et al., 2011). Mice mutant for either *Tet1* or *Tet2* are viable or show only neuronal deficiencies (Dawlaty et al., 2011; Ko et al., 2011; Li et al., 2011; Zhang et al., 2013). Some *Tet1* mutant mice have a slightly smaller body size at birth (Dawlaty et al., 2011), which might reflect a developmental delay, and also are subfertile (Yamaguchi et al., 2012). These enzymes may have overlapping roles in development. KO of both *Tet1* and *Tet2* suggests that *Tet1/2* do not play a significant role in embryonic development (Dawlaty et al., 2013; Hu et al., 2014). This seems to be difficult to reconcile with the roles of *Tets* in self-renewal and pluripotency of ESCs/induced pluripotent stem cells (iPSCs) (Costa et al., 2013; Ficz et al., 2011; Ito et al., 2010).

Telomeres maintain genomic stability and are critical for unlimited self-renewal and pluripotency of ESCs and iPSCs (Huang et al., 2011; Marion et al., 2009). Mouse telomeres are quite long, and telomerase *Terc*- deficient mice following several generations of breeding acquire striking phenotypes associated with telomere dysfunction, including developmental defects, aging, and cancer (Blasco et al., 1997; Herrera et al., 1999; Rudolph et al., 1999). *Tet1* and *Tet2* double-knockout (DKO) mice, if survived from embryonic lethal, show partially penetrant perinatal lethality or reduced fertility with small ovaries (Dawlaty et al., 2013). These defects mimic those of telomere-shortened, late-generation, telomerase-deficient mice that also exhibit small body, defective neurogenesis, and infertility (Hao et al., 2005; Herrera et al., 1999).

Nevertheless, ESCs isolated from the first generation (G1) telomerase-deficient mice already exhibit telomere dysfunction

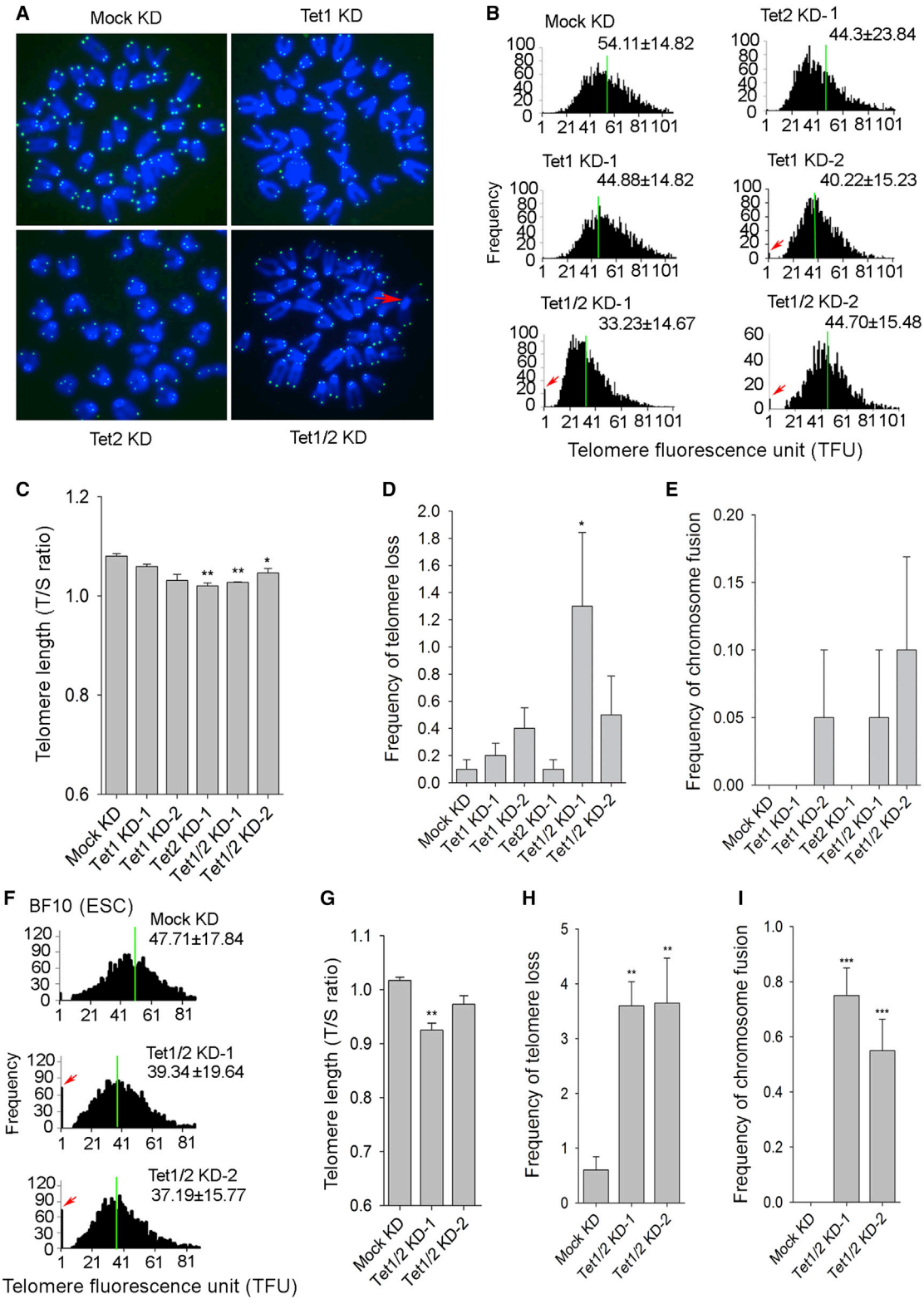

Figure 1. Telomere Shortening and Chromosomal Instability of Mouse ESCs Depleted of Tet1 and/or Tet2

(A) Representative telomere FISH images of *Tet1* or *Tet2* stable KD and *Tet1* and *Tet2* double KD (abbreviated as *Tet1/2* KD) ESCs (NF2 Nanog-GFP ESCs). Blue dots, chromosomes stained with DAPI; green dots, telomeres. Red arrow indicates telomere loss and chromosomal fusion.

(legend continued on next page)

and significantly reduced developmental potency (Huang et al., 2011), indicating that the high proliferation rates of cultured ESCs can accelerate telomere shortening. Functional telomeres are required for the genome-wide epigenetic regulation of ESC differentiation (Pucci et al., 2013). *Tet* depletion also skews ESC differentiation (Dawlaty et al., 2014; Koh et al., 2011). It remains unclear whether Tet1 or/and Tet2 regulate telomere lengths. Here, we report that Tet1 and Tet2 enzymes are required to maintain telomere function and chromosomal stability of mouse ESCs.

RESULTS

Depletion of *Tet1* and *Tet2* in ESCs Leads to Telomere Shortening and Chromosomal Instability

Tet1 and *Tet2*, but not *Tet3*, are highly expressed in ESCs (Ito et al., 2010; Koh et al., 2011) and iPSCs, in contrast to mouse embryonic fibroblasts (MEFs) or most somatic tissues (data not shown). *Tet3* is highly expressed in MEFs and various somatic tissues but low in ESCs/iPSCs. To test whether *Tet1* and *Tet2* play a role in telomere maintenance, we depleted *Tet1* and/or *Tet2* in mouse ESCs by RNAi using at least two sequences specifically targeting *Tet1*, *Tet2*, or both genes (Ficz et al., 2011; Ito et al., 2010; Experimental Procedures). The RNAi effectively depleted *Tet* mRNA and protein levels (Figures S1A–S1E), and *Tet1* or *Tet2* depletion alone slightly or minimally affected expression of pluripotency genes *Oct4* and *Nanog* (Figures S1F–S1H). Depletion of both *Tet1* and *Tet2* reduced expression of pluripotency genes, particularly Nanog, but these ESCs maintained colony formation without morphologic signs of differentiation (Figures S1I–S1K).

We found that telomeres were shortened in ESCs depleted of either *Tet1* or *Tet2* and more profoundly so in double-knockdown (DKD) ESCs as estimated by telomere quantitative fluorescence in situ hybridization (FISH) assay (Figures 1A and 1B) and independently validated by quantitative real-time PCR method (Figure 1C). Moreover, frequency of telomere loss and chromosome fusion increased in ESCs depleted of both *Tet1* and *Tet2* (Figures 1D and 1E). These observations were confirmed in another independent ESC line (BF10) following depletion of both *Tet1* and *Tet2* (Figures 1F–1I). Cell proliferation was slightly reduced by double knockdown (KD) of *Tet1* and *Tet2* (Figure S1L).

To assess the effect of *Tet1*/*Tet2* depletion on development, we injected *Tet1*/*Tet2* DKD or control ESCs into blastocysts and compared rates of chimera formation. Chimeric pups with black and albino coat were obtained only from mock control ESCs, also confirmed by microsatellite genotyping (representative gel image data shown), but not from *Tet1* and *Tet2* DKD ESCs (Figures S2A–S2E). Our data support previous findings that *Tet1 and Tet2* regulate ESC self-renewal and pluripotency (Costa et al., 2013; Ficz et al., 2011; Ito et al., 2010; Koh et al., 2011). Furthermore, we show that telomere shortening is a general phenomenon resulting from depletion of *Tet1* and/or *Tet2*, suggesting that *Tet1* and *Tet2* regulate telomere maintenance in ESCs.

Tet1 and *Tet2* Regulate Telomere Recombination

To explore the mechanisms underlying telomere dysfunction arising from depletion of *Tet1* and *Tet2*, we initially analyzed expression of genes related to telomerase, a primary pathway for extension of telomeres. Transient KD of *Tet1*, *Tet2*, or both did not immediately reduce expression of major telomerase genes *Tert* and *Terc* (Figure S1A). Despite telomere shortening, stable KD of *Tet1* or *Tet2* alone also did not cause appreciable changes in expression of telomerase genes *Tert* and *Terc* or in telomerase activity. However, we noticed that stable KD of both *Tet1* and *Tet2* caused variations in expression of *Tert* and *Terc* and in telomerase activity (Figures 2A–2D). Extension of telomeres by telomerase usually takes place at a slow rate. Hence, minor reduction in telomerase activity would be an unlikely explanation for the rapid telomere loss associated with *Tet1* and *Tet2* depletion.

Telomeres also can be elongated by alternative lengthening of telomere (ALT) pathways involving telomere recombination (Bailey et al., 2004; Bechter et al., 2004; Conomos et al., 2013; Londoño-Vallejo et al., 2004). Telomere elongation by recombination is characterized by telomere sister chromatid exchange (TSCE) revealed by chromosome orientation (CO)-FISH analysis (Bailey et al., 2004; Bechter et al., 2004; Liu et al., 2007; Zalzman et al., 2010). Frequency of TSCE was reduced upon *Tet1* or *Tet2* KD, and particularly upon *Tet1/2* double KD, compared to mock controls in two independent ESC lines (Figures 2E and 2F). Moreover, expression of genes associated with telomere recombination (Conomos et al., 2013; Zalzman et al., 2010), particularly *Dmc1* and *Smc1*β, was consistently decreased following depletion of *Tet1* and/or *Tet2* (Figures 2G and 2H). These data suggest that *Tet1* and *Tet2* play a role in telomere recombination and elongation.

Telomere Shortening in *Tet1* or *Tet1/Tet2* DKO ESCs

KD experiments by short hairpin RNA (shRNA) could produce incomplete silencing or off-target effects. Thus, we attempted to knock out *Tet1* gene in ESCs by CRISPR/Cas9 and analyzed telomeres. *Tet1* was effectively knocked out, as evidenced by sequencing and qPCR analysis, which showed only minimal

(B) Histogram displays distribution of relative telomere length shown as TFU by telomere quantitative fluorescence in situ hybridization (QFISH) following depletion of *Tet1* and/or *Tet2*. Heavy black bars on y axis indicated by red arrows show frequency of telomere signal-free ends. Telomere length was measured at passage 6 following stable KD. The medium telomere length (green bars) is shown as mean ± SD above each panel. More than 15 chromosome spread was quantified for each group.

(C) Relative telomere length shown as T/S ratio by qPCR. Three replicates are shown.

(D) Frequency per chromosome spread of telomere signal-free ends, indicative of telomere loss after *Tet1* and *Tet2* KD.

(E) Frequency per chromosome spread of chromosomal fusion after *Tet1* and *Tet2* KD.

(F–I) Double KD of *Tet1* and *Tet2* in another mouse ESC line (BF10) leads to telomere shortening and dysfunction, shown as telomere length distribution histogram by QFISH (F), T/S ratio by qPCR (G), frequency of telomere signal-free ends (H), and chromosomal fusion (I). Telomere length was measured at passage 11 following stable double KD of *Tet1/2*. Data represent mean ± SEM.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared with mock ESC controls. See also Figure S1.

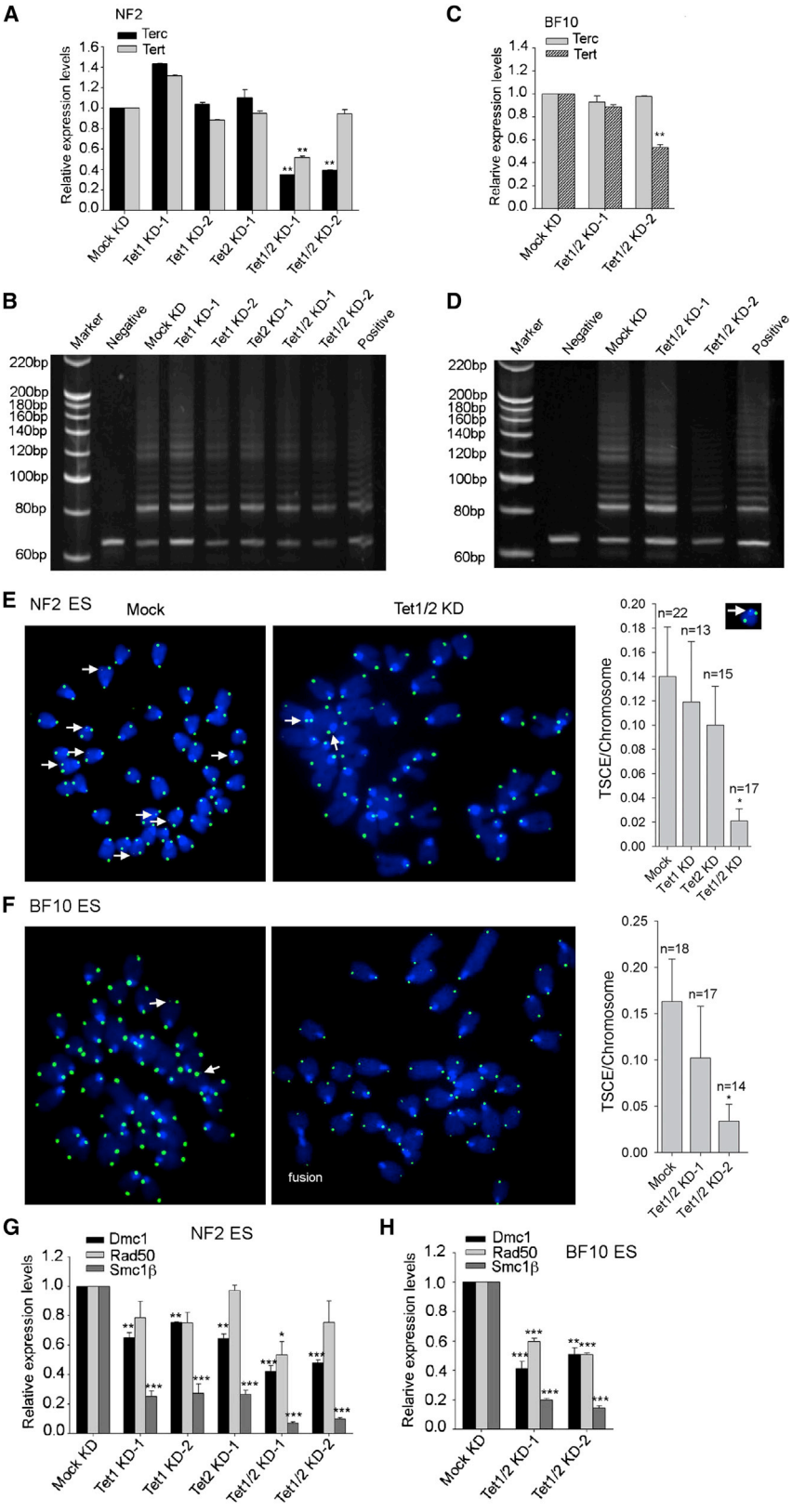

Figure 2. Effects of *Tet1* or *Tet2* KD or *Tet1/2* Double KD on Telomerase and Telomere Sister Chromatic Exchange

(A–D) Expression of telomerase genes and telomerase activity following KD of *Tet1*/*Tet2*. (A and C) qRT-PCR analysis of expression levels of *Tert* and *Terc* in NF2 (A) and BF10 ESCs (C). (B and D) Telomerase activity by TRAP assay of NF2 (B) and BF10 ESCs (D). Lysis buffer served as negative controls.

(E and F) TSCE (indicated by white arrows) revealed by CO-FISH analysis of independent ESC lines from two mouse strains following *Tet1/2* depletion. (Right panel) Frequency of TSCE in *Tet1/2* DKD ESC, compared with mock controls, based on the number of TSCE dot/per chromosome spread (n) is shown.

(G and H) qRT-PCR analysis of expression levels of genes for telomere recombination, *Dmc1*, *Rad50*, and *Smc1β*. *p < 0.05; **p < 0.01; ***p < 0.001, compared with Mock KD controls. Data represent mean ± SEM.

See also Figure S2.

Tet1 expression (Figures S3A–S3C). Telomeres were shortened in *Tet1* KO ESCs, compared with wild-type (WT) ESCs, as shown by quantitative FISH (QFISH) and Southern blot analysis (Figures S3D and S3E).

We also measured telomere length in *Tet1* and *Tet2* DKO ESC lines, generated by gene targeting method (Hu et al., 2014). The *Tet1/2* DKO ESCs enabled study of effects of complete and prolonged *Tet* deficiency on telomeres. 2i (mitogen-activated protein kinase [MAPK] inhibitor and GSK3β inhibitor), which decrease MAPK and activate β-catenin/Wnt signaling, provided transcriptional and epigenomic foundations for naive ground state pluripotency (Ficz et al., 2013; Marks et al., 2012; Ying et al., 2008) and were used to maintain self-renewal of *Tet1/Tet2* DKO ESCs (Hu et al., 2014). *Tet1/Tet2* DKO ESCs manifested increased levels of DNA methylation and dramatically reduced levels of 5hmC by immunofluorescence and thus high 5mC/5hmC ratio (Figures 3A and 3B), consistent with the null status of *Tet1* and *Tet2*. *Tet1/Tet2* DKO ESCs harbored shorter telomeres than did the WT ESCs, regardless of 2i (Figure 3C). However, contrary to our expectation that telomeres would shorten more in *Tet1/2* DKO ESCs than in DKD ESCs, telomeres of DKO ESCs shortened to lesser extent compared to those of KD ESCs (Figure 1; see below). However, we need to emphasize that the KD and KO ESCs were derived from different backgrounds and origins. In addition, telomere loss and chromosomal fusions did not differ between *Tet1/Tet2* DKO and WT ESCs cultured in the presence of 2i and slightly increased in *Tet1/Tet2* DKO compared with WT ESCs cultured in the absence of 2i (Figures 3D and 3E). The average shorter telomeres in *Tet1/Tet2* DKO ESCs compared to WT ESCs were confirmed by qPCR method (Figure 3F). Furthermore, *Tet1/Tet2* DKO ESCs exhibited increased frequency of TSCE (Figures 3G and 3H), suggesting that telomere recombination might be involved in telomere length regulation in these cells.

Triple KO of *Tet1*, *Tet2*, and *Tet3* in ESCs Induces Telomere Loss and Chromosomal Fusion

Recently, *Tet1/2/3* triple-knockout (TKO) ESCs generated by CRISPR/Cas9 were shown to exhibit longer telomeres compared with WT ESCs (Lu et al., 2014). We sought to assess telomere functions from two additional *Tet1/2/3* TKO ESC lines (Hu et al., 2014; Dawlaty et al., 2014). *Tet1/2/3* TKO ESCs from both M.M.D. and G.X. labs formed nice colonies similar to those of *Tet1/2* DKO and WT ESCs (Figure S4A). Remarkably, telomere loss and chromosome fusion were more elevated in *Tet1/2/3* TKO ESCs than in *Tet1/2* DKO ESCs and WT ESCs (Figures 4A–4C). Over 90% of chromosome spreads from the TKO ESCs provided by the M.M.D. lab and 15% from the G.X. lab contained at least one chromosome fusion (Figures 4A–4C). Coincidently, the TKO ESCs from M.M.D. lab do not contribute to chimeras, whereas the TKO ESCs from the G.X. lab do. However, the average telomere lengths of *Tet1/2* DKO ESCs from M.M.D. lab did not differ from those of WT ESCs, and telomeres were slightly shorter in *Tet1/2/3* TKO ESCs than in *Tet1/2* DKO ESCs and WT ESCs (Figures 4A and 4D). High frequency of telomere loss can contribute to the average shorter telomeres in the TKO ESCs. *Tet1/2* DKO ESCs from G.X. lab exhibited shorter telomeres compared to WT ESCs, but telomeres of *Tet1/2/3* TKO ESCs were longer than those of *Tet1/2* DKO ESCs (Figures 4A and 4D). The average telomere length obtained by QFISH method was validated by Southern blot analysis, which measures telomeres shown as terminal restriction fragment (TRF) (Figures 4E and S4B). Also, TRF confirmed the shorter average telomeres in *Tet1/2* DKD ESC lines (NF2 and BF10). It is important to note that QFISH can measure even rare short telomeres, whereas TRF measures primarily mean telomere length. Notably, heterogeneous telomere lengths and distributions were observed in TKO ESCs compared with WT ESCs (Figures 4A and 4D), suggesting that an ALT pathway might have been activated.

Tet1 and *Tet2* Regulate Sub-telomeric DNA Methylation

Genome-wide mapping of 5hmC in ESCs shows enrichment at start sites of genes whose promoters carry histone 3 lysine 27 trimethylation (H3K27me3) and histone 3 lysine 4 trimethylation (H3K4me3) marks, but not at sites of heterochromatic H3K9 or H4K20 trimethylation, where 5mC preferentially occupies (Mikkelsen et al., 2007; Pastor et al., 2011). Fluorescence microscopy using antibodies to 5mC or 5hmC also identified that 5mC foci co-localized with heterochromatic region marked by Hoechst, whereas 5hmC distributed non-specifically in the nuclei, and notably more intensively stained 5mC foci with heterochromatin were found in *Tet1/2* DKO ESCs (Figure 3A).

To reveal the enrichment of 5mC or 5hmC at sub-telomeres, we performed methylated DNA immunoprecipitation (MeDIP) using primers specific for sub-telomeres of chromosomes 7 and 13. The 5mC levels did not increase at these sub-telomeric loci following double KD of *Tet1/2* in ESCs, compared with mock KD controls (Figure 5A), but were elevated following double KO of *Tet1/2* in ESCs (Figure 5B). Likewise, heterochromatic telomere regions were co-localized with fewer 5mC foci in WT ESCs, and the frequency of telomere regions co-localized with more strongly stained 5mC foci nearly doubled in *Tet1/Tet2* DKO ESCs (Figure 5C). Levels of 5hmC were reduced at sub-telomeres of chromosome 13 following double KD of *Tet1/2* and more reduced at both chromosomes in the DKO ESCs (Figures 5A and 5B). These data corroborate with findings of high 5mC/5hmC ratio in the DKO ESCs with minimal levels of 5hmC (Figures 3A and 3B).

Moreover, we analyzed methylation levels by bisulfite sequencing using primers specific for sub-telomeres of selected chromosomes 1, 7, and 13 (Figure 5D). DNA methylation levels at sub-telomeres of chromosomes 1 and 7 were elevated in *Tet1/Tet2* DKO ESCs, compared with WT ESCs. Moreover, 2i noticeably reduced DNA methylation levels at sub-telomeres of all three chromosomes tested, consistent with reduced Dnmt3a/b protein levels (Figure S7A; immunofluorescence of Dnmt3b data not shown). Hence, Tet1 and Tet2 play a critical role in regulating methylation levels at sub-telomeres, impacting telomere recombination.

To examine whether telomerase also is involved in telomere length regulation by Tets, we knocked out *Terc* in *Tet1/2* DKO ESCs by CRISPR/Cas9. *Terc* deficiency further shortened telomeres in *Tet1/2* DKO ESCs (Figure S5), suggesting that lack of both *Tet1* and *Tet2* shortens telomeres independently of telomerase *Terc*.

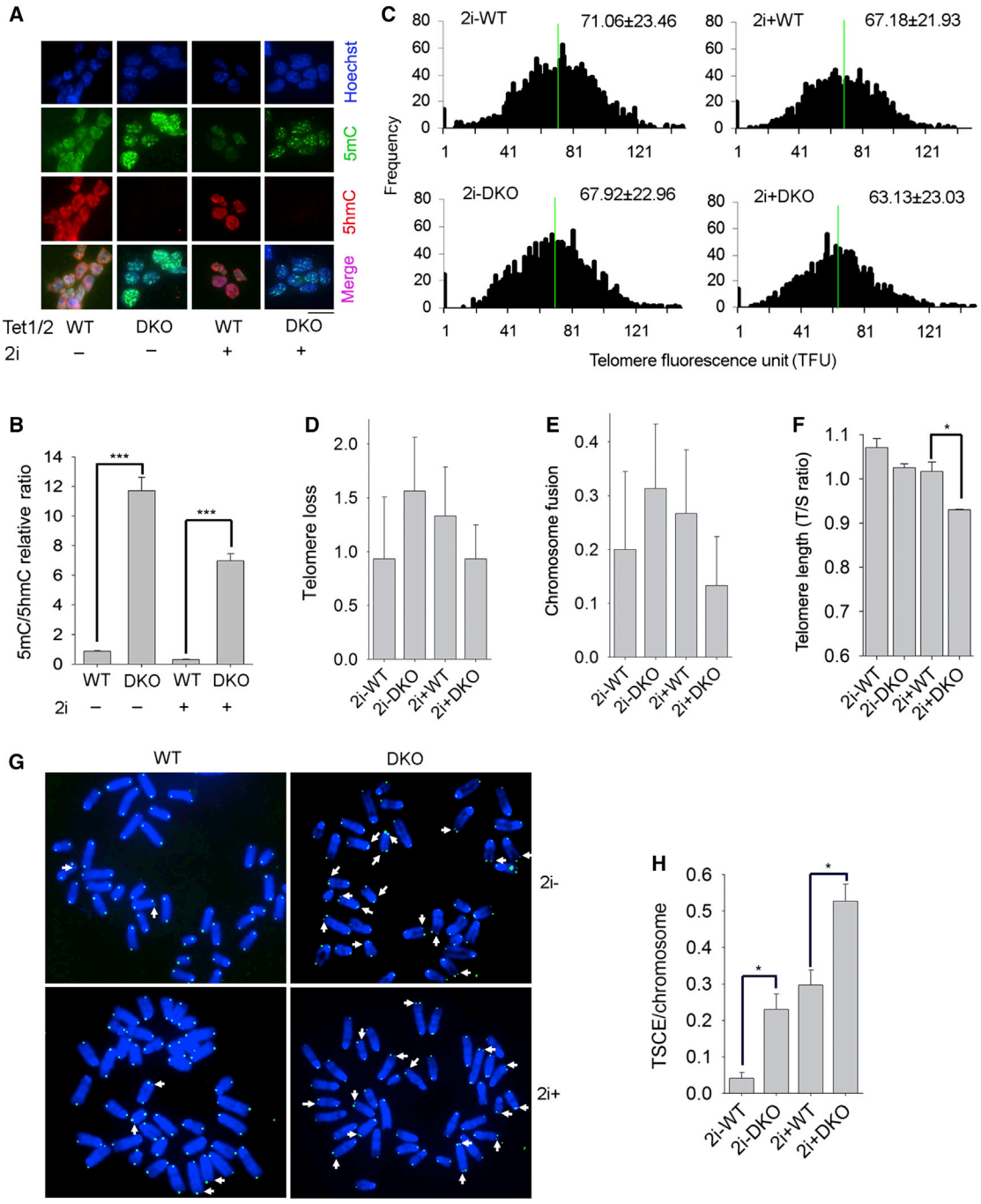

Figure 3. *Tet1* and *Tet2* DKO ESCs Exhibit Reduced Telomere Shortening, in Association with Increased TSCE

(A) Immunofluorescence of DNA methylation (5mC) and DNA hydroxymethylation (5hmC) in *Tet1* and *Tet2* DKO ESCs. The scale bar represents 20 μm.

(B) 5mC/5hmC relative fluorescence intensity ratio estimated using ImageJ software.

(C) Histogram shows distribution of relative telomere length as TFU by QFISH. Heavy black bars on y axis show frequency of telomere signal-free ends. The medium telomere length (green bars) also is shown as mean ± SD above each panel.

(D and E) Frequency of telomere loss and chromosome fusion in WT and DKO ESCs cultured in the presence or absence of 2i (2i+ or 2i−).

(F) Relative telomere length of WT and DKO ESCs shown as T/S ratio by qPCR.

(G) Micrographs showing TSCE (indicated by white arrows) by CO-FISH analysis.

(H) Frequency of TSCE, based on the number of TSCE dot/per chromosome spread. *$p < 0.05$; ***$p < 0.001$, compared with WT ESCs. Data represent mean ± SEM.

See also Figure S3.

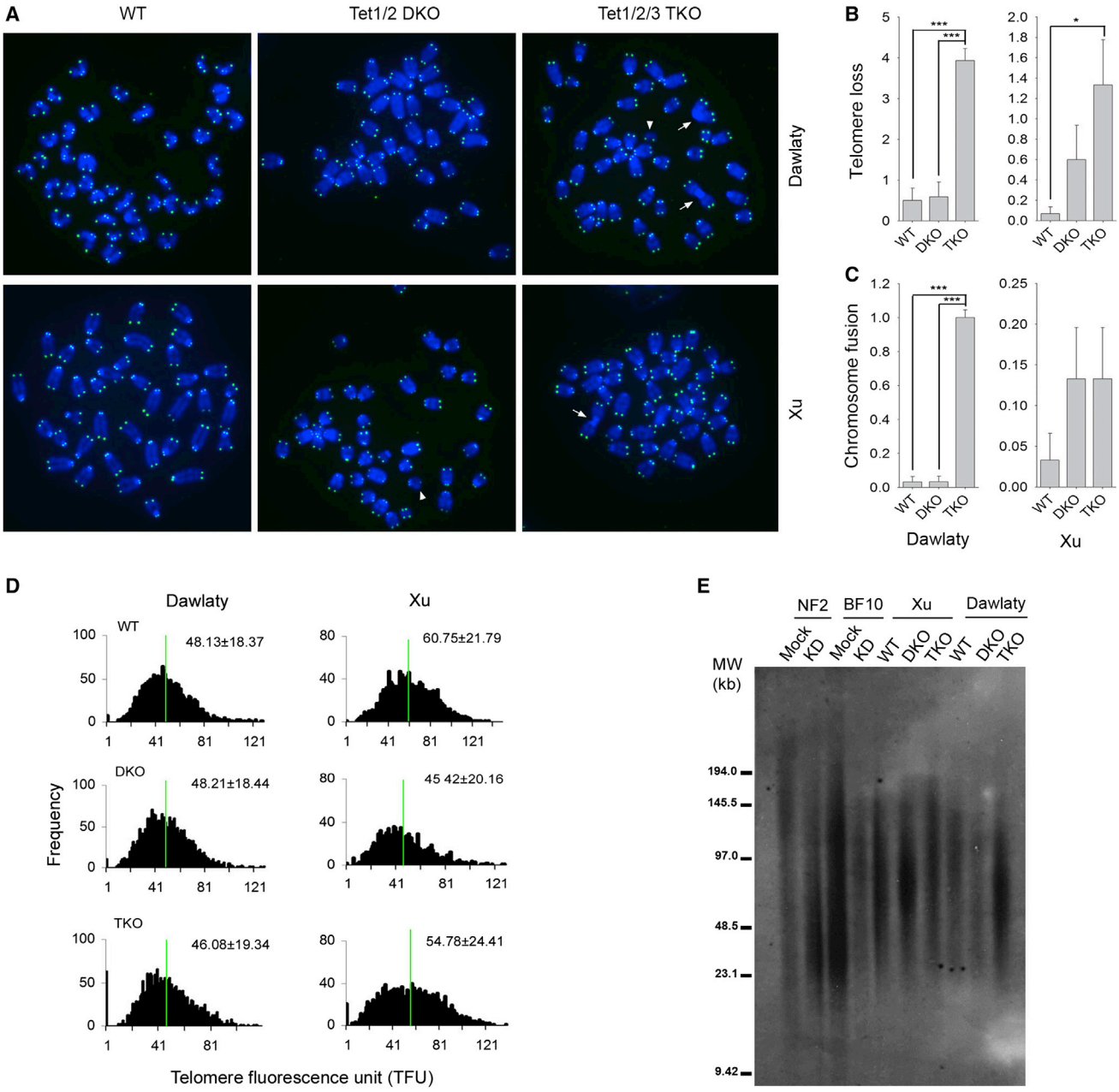

Figure 4. TKO of *Tet1*, *Tet2*, and *Tet3* in ESCs Results in Telomere Dysfunction and Prominent Chromosomal Fusion

(A) Telomere QFISH images of ESCs, showing telomere loss (arrowhead) and chromosomal fusion (arrow) in *Tet1/2/3* TKO ESCs, compared with WT and *Tet1/2* DKO ESCs.

(B) Frequency of telomere loss per chromosome spread. Approximately 30 spreads were counted for each genotype.

(C) Frequency of chromosomal fusion per chromosome spread.

(D) Histogram showing distribution of relative telomere length as TFU by QFISH. Heavy black bars on y axis show frequency of telomere signal-free ends. The medium telomere length (green bars) also is shown as mean ± SD above each panel.

(E) Telomere length distribution shown as TRF by Southern blot analysis. KD, *Tet1/2* double KD ESCs. *p < 0.05; ***p < 0.001. Data represent mean ± SEM. See also Figure S4.

Dnmt3b Is Involved in Telomere Length Regulation by *Tet1* and *Tet2*

Tet1 and Tet2 regulate genome-wide DNA methylation levels by inducing DNA hydroxymethylation, and *Tet1* deficiency results in an increase in 5mC levels at many Tet1-enriched regions (Wu and Zhang, 2011; Xu et al., 2011). Notably, Tet1 occupies the promoter region of DNA methyltransferase *Dnmt3b* and represses *Dnmt3b*, and consistently, *Tet1* depleted cells showed increased

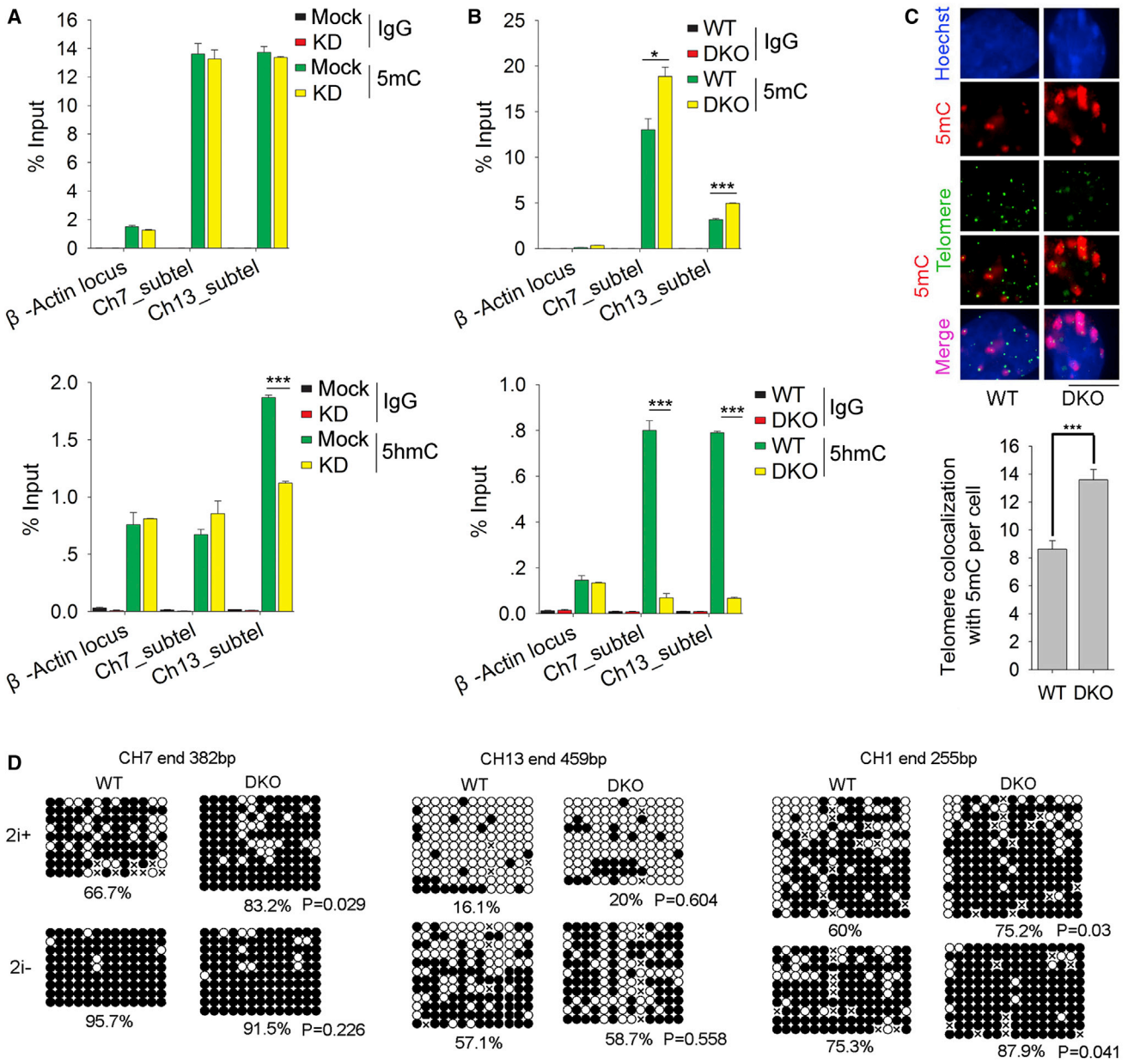

Figure 5. Tet1 and Tet2 Regulate Sub-telomeric Methylation

(A) MeDIP assay of 5mC or 5hmC levels at sub-telomeres of chr7 and chr13 in mock and *Tet1/2* DKD ESCs. The β-actin locus served as control.

(B) MeDIP assay of 5mC or 5hmC levels at sub-telomeres of chr7 and chr13 in WT and *Tet1/2* DKO ESCs. *$p < 0.05$; ***$p < 0.001$.

(C) Representative images showing telomeres (green), 5mC (red), or merged fluorescence (yellow) in wild-type (WT) and *Tet1* and *Tet2* DKO ESCs and frequency of telomeres co-localized with 5mC foci. The scale bar represents 10 μm.

(D) Bisulfite sequencing analysis of CpG methylation at sub-telomeric regions of chromosomes 1, 7, and 13, respectively. Open circles indicate unmethylated cytosines, filled circles methylated cytosines, and crosses mutation of cytosines. $p < 0.05$, considered significant differences, compared with WT ESCs. Data represent mean ± SEM.

See also Figure S5.

Dnmt3b, but not *Dnmt3a*, levels by microarray analysis (Freudenberg et al., 2012). In line with this, stable KD of both *Tet1* and *Tet2* led to increased 5mC and reduced 5hmC levels as quantified by immunofluorescence microscopy (Figure S6A) coincided with upregulation of Dnmt3b (Figures S6B–S6F).

To test whether high levels of Dnmt3b are implicated in *Tet1/2* depletion-mediated telomere shortening, we knocked down *Dnmt3b* in *Tet1* and *Tet2* DKD ESCs. The efficacy of *Dnmt3b* KD was confirmed by western blot and immunofluorescence microscopy (Figures 6A and 6B). *Dnmt3b* KD reduced DNA

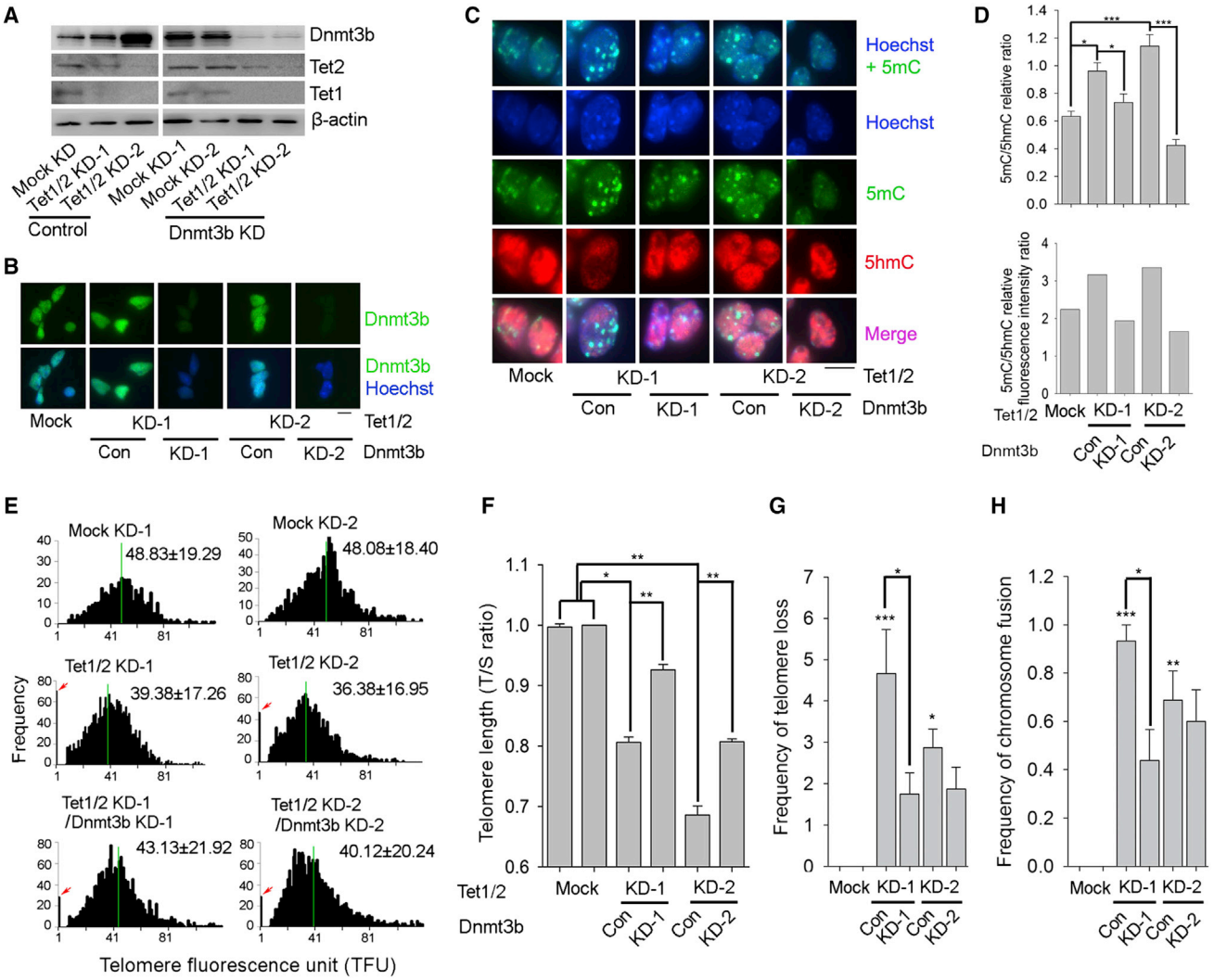

Figure 6. KD of *Dnmt3b* Elongates Telomeres of ESCs Depleted of both *Tet1* and *Tet2*

(A) Western blot analysis of Tet1, Tet2, and Dnmt3b protein levels in control and KD ESC lines (BF10). β-actin served as a loading control. Two different shRNAs sequences against *Dnmt3b* were used to achieve stable *Dnmt3b* KD ESCs.

(B) Immunofluorescence microscopy of Dnmt3b in control and *Tet1/Tet2* KD and *Dnmt3b* KD ESCs. The scale bar represents 10 μm.

(C and D) KD of Dnmt3b reduces 5mC levels in *Tet1*- and *Tet2*-depleted ESCs. (C) Immunofluorescence microscopy of DNA methylation (5mC) and DNA hydroxymethylation (5hmC) relative levels between control KD, *Tet1/2* double KD, and *Dnmt3b/Tet1/2* triple KD ESCs (BF10) is shown. The scale bar represents 10 μm. (D) Relative methylation quantification by 5mC/5hmC ratio estimated using ImageJ software (upper panel) or by FACS analysis (lower panel) is shown.

(E) Histogram showing distribution of relative telomere length as TFU by QFISH at P10 after KD of *Dnmt3b*. Heavy black bars on y axis indicated by red arrows show frequency of telomere signal-free ends. The medium telomere length (green bars) also is shown as mean ± SD above each panel.

(F) Relative telomere length shown as T/S ratio by qPCR. Three replicates are shown.

(G and H) Frequency of telomere loss per chromosome spread (G) and chromosomal fusion per chromosome spread after *Dnmt3b* KD in BF10 ESCs depleted of *Tet1* and *Tet2* (H). *$p < 0.05$; **$p < 0.01$, compared with Mock ESC controls. Data represent mean ± SEM.

See also Figures S6 and S7.

methylation levels (Figures 6C and 6D) and elongated telomeres (Figures 6E and 6F). Staining for 5mC co-localized with heterochromatic foci, contrary to diffuse nuclear staining of 5hmC (Figure 6C). Telomeres elongated in the *Tet1/2/Dnmt3b* KD ESCs cultured for ten additional passages compared to *Tet1* and *Tet2* DKD ESCs as analyzed by telomere QFISH and qPCR. Also, frequency of telomere loss and chromosomal fusion was reduced in *Tet1/2/Dnmt3b* KD ESCs, in comparison with *Tet1* and *Tet2* double KD alone (Figures 6G and 6H). Together, these data further support the notion that *Tet1* and *Tet2* suppress *Dnmt3b* and regulate telomere maintenance.

Implication of Zscan4 and Dnmt3b in Tet Signaling

It is intriguing to note that *Tet1/Tet2* DKO ESCs show telomere lengthening compared with *Tet1/Tet2* DKD ESCs. Above data suggest that telomere recombination as shown by TSCE could

be the major pathway leading to rapid and remarkable changes in telomere lengths. *Zscan4* is involved in telomere recombination and elongation (Zalzman et al., 2010). Additionally, the sub-telomeric DNA methylation mediated by Dnmt3a and Dnmt3b negatively regulates telomere recombination and telomere lengths (Gonzalo et al., 2006). *Tet1/Tet2* DKO ESCs exhibited increased frequency of TSCE (Figures 3G and 3H). Therefore, we examined whether and how Zscan4 and Dnmt3a/3b are implicated in Tet signaling. Dnmt3a protein levels seemed to be stable, but Dnmt3b was noticeably elevated in *Tet1/Tet2* DKO ESCs, and 2i decreased Dnmt3a and Dnmt3b protein (Figure S7A) and 5mC levels (Figures 3A and 3B), consistent with the notion that 2i reduces *Dnmt3a/3b* to promote hypomethylation and enhances TET1/2 activity (and consequently 5hmC levels) and "passive" loss of DNA methylation (Ficz et al., 2013; Leitch et al., 2013). *Tet1/Tet2* DKO ESCs expressed higher levels of Zscan4 by immunoblot than did WT ESCs, and 2i reduced Zscan4 protein levels in both WT and DKO ESCs (Figure S7A), also validated by immunofluorescence microscopy and fluorescence-activated cell sorting (FACS) analysis (data not shown). Increased expression of *Zscan4* and TSCE might counteract and thus reduce telomere shortening resulting from Dnmt3b-mediated sub-telomeric methylation in *Tet1/2* DKO ESCs.

To understand whether Tet1 and Tet2 directly impact Zscan4 and Dnmt3a/3b, we performed KD experiments. *Tet1* and *Tet2* transient KD for 48 hr immediately upregulated Dnmt3b, but not Dnmt3a, as confirmed by qPCR analysis (Figure S7B) and also by western blot (Figure S7C). Zscan4 protein levels also declined following *Tet1* and *Tet2* transient KD.

Also, we compared expression of Zscan4 and Dnmt3b in *Tets* DKO and TKO ESC lines. Dnmt3b and 3a levels were higher in ESCs from M.M.D lab than from G.X. lab (Figure S7D). Zscan4 was expressed at even higher levels in both TKO ESC lines by qPCR analysis and by western blot than in their corresponding DKO ESCs (Figures S7E and S7F). Additionally, Zscan4 expression levels were relatively higher in ESCs from M.M.D. lab than those from G.X. lab. Dnmt3b levels were higher in DKO ESCs than in WT ESCs from G.X. lab and declined in TKO ESCs compared with DKO ESCs. By FACS analysis of Zscan4 immunofluorescence, WT ESCs originated from M.M.D showed higher percentage of Zscan4$^+$ ESCs than did those from G.X. lab (5.62% versus 1.76%; Figure S7G). Moreover, DKO and TKO ESCs from M.M.D. exhibited further increasing proportion of Zscan4$^+$ cells (11.15% and 21.16%, respectively) with some reduction of Zscan4 fluorescence intensity, compared with WT ESCs. DKO and TKO ESCs from G.X. lab also displayed increasing proportion of Zscan4$^+$ cells but to less extent (4.51% and 7.74%), with increased Zscan4 fluorescence intensity (Figure S7G). Zscan4 expression levels were higher in ESCs from M.M.D. than those from G.X. lab, supporting the qPCR and western blot data (Figures S7E and S7F).

These data suggest that ESC lines established from different labs can have various expression levels of Dnmt3b and Zscan4 in terms of the proportion and intensity, causing variations in the average telomere length. Regardless of 2i and the average telomere length, *Tets* TKO ESCs exhibit elevated telomere loss and chromosome instability.

DISCUSSION

We show that Tets play important roles in regulation of telomeres and maintenance of chromosomal stability. *Tet1/2* double KD rapidly induces expression of *Dnmt3b*, concomitant with reduced levels of 5hmC and TSCE, leading to telomere shortening and chromosomal instability. Complete lack of *Tet1/2* by double KO also results in elevated expression of *Dnmt3b*, accompanied with highly elevated ratio of 5mC/5hmC and minimal 5hmC. Low or minimal 5hmC levels dramatically reduce hydroxymethylation levels at sub-telomeres. Elevated levels of Dnmt3b can lead to telomere shortening, whereas increased Zscan4 induces TSCE and telomere recombination, and this partly compensates for telomere shortening in *Tet1/2* DKO ESCs, showing reduced telomere shortening on average. Excessive expression of *Zscan4* in *Ter1/2/3* TKO ESCs may contribute to heterogeneous telomere elongation by ALT. Abnormally high levels of Zscan4, together with high Dnmt3b/5mC levels, can cause heterogeneous telomere elongation and shortening, telomere loss, and high frequency of chromosome fusion arising in *Tet1/2/3* TKO ESCs. Consistently, 2i reduces levels of both Dnmt3a/3b and Zscan4, such that telomere length and function are partly recovered and chromosome fusion reduced.

Depletion of *Tet1* and *Tet2* by KD induces rapid telomere shortening, in association with reduced incidence of TSCE. Presumably, the KD experiments provide immediate and direct impact upon *Tet1/Tet2* depletion, whereas data obtained from the KO cell lines may represent indirect or long-term effects of *Tet* deficiency. High expression levels of *Tet1* and *Tet2* in mouse ESCs likely play critical roles in maintaining low methylation at sub-telomeres, favoring telomere elongation by recombination. In agreement, DNA methylation mediated by DNMT3A/3B and TETs interact preferentially at heterochromatic region and silencing repeats (Smith and Meissner, 2013). Our data on the role of Tet enzymes in telomere function also may explain the seemingly contradictory functions of Tets reported in ESC pluripotency and differentiation (more dramatic phenotypes by KD or KO of *Tet1* or *Tet2* alone in ESCs) versus development in vivo (mild or no immediate phenotypes of *Tet1* or/and *Tet2* KO mice). *Tet1*, *Tet2*, and even *Tet3* seem to have overlapping or compensatory roles in regulation of telomeres, differentiation, and pluripotency of ESCs in vitro and development and differentiation in vivo. *Tet1* and *Tet2* also are highly expressed in iPSCs and required for reprogramming and iPSC induction (Doege et al., 2012; Gao et al., 2013) and may play a similar role in telomere maintenance and chromosomal stability of iPSCs.

Telomere shortening can be partly rescued by high expression of *Zscan4* in ESCs without *Tets*. It will be interesting to test whether telomeres continue to shorten during differentiation of ESCs and fetal embryonic development and in animals without *Tets* and *Zscan4* expression. *Zscan4* is specifically expressed in two-cell embryos and sporadically in small subpopulation (1%–5%) of ESCs and inactivated in differentiated cells including fibroblasts (Dan et al., 2014; Zalzman et al., 2010). Appropriate expression of *Zscan4* is required for telomere recombination and effective lengthening of ESCs, whereas excessive *Zscan4* can disrupt telomere length homeostasis, leading to heterogeneous telomere elongation and loss and chromosome fusion

(Dan et al., 2014). *Zscan4* is inactivated with differentiation of ESCs, and *Tet1/2* DKO and *Tet1/2/3* TKO ESCs likely lose the compensatory mechanisms of telomere maintenance following differentiation.

The proportion of ESCs that express *Zscan4* and *Zscan4* levels could affect average telomere lengths. In the recent report (Lu et al., 2014), the basal levels of *Zscan4* seem to be low in ESCs, with only 0.23% of WT ESC population positive for Zscan4 by FACS and a significant increase in Zscan4$^+$ cells in TKO ESCs (1.54%; by 6.7-fold) could lead to high TSCE and longer telomeres. Moreover, methods for ESC derivation and gene deletion as well as ESC passage number could influence telomere lengths. Telomeres lengthen to some degree in normal mouse ESCs/iPSCs with increasing passages (Huang et al., 2011; Marion et al., 2009). The passage number could vary among these *Tet* DKO or TKO ESC lines from different labs. Differences in telomere lengths between *Tet* KD and DKO ESCs may also be explained by ESC passage numbers when the telomeres were analyzed. In KD experiments, the passage number was controlled and the comparison made for the same passages of KD and mock ESC controls.

Our data suggest that both Zscan4 and Dnmt3b-mediated DNA methylation are involved in telomere recombination and length regulation by *Tets*. *Dnmt3b* expression is rapidly increased and *Zscan4* reduced following transient KD of *Tet1* and *Tet2*. *Dnmt3b* levels also are increased in *Tet1/2* stable double KD and in *Tet1/2* DKO ESCs. In addition, recombination-related genes such as *Smc1β* and *Dmc1* are downregulated following *Tet1/2* double KD, consistent with the array data from *Tet1* KO in ESCs, showing reduced expression of *Smc1β* and two-cell genes including *Zscan4* (*Gm397*), *Tcstv1*, and *Tcstv3* (Dawlaty et al., 2011). We show that *Dnmt3b* KD in *Tet1/2*-depleted ESCs can partly rescue telomere shortening. In agreement, ESCs deficient for DNA methyltransferases exhibit significantly elongated telomeres facilitated by recombination (Gonzalo et al., 2006).

Telomere loss and chromosome fusion resulting from *Tets* deficiency may have implication in aging and cancer. The shortest telomere, not the average telomere length, drives chromosome instability, affecting cell viability and transformation (der-Sarkissian et al., 2004; Hemann et al., 2001). *Tet1* and *Tet2* mutation or deletion leads to tumorigenesis (Abdel-Wahab et al., 2009; Couronné et al., 2012; Hsu et al., 2012; Huang et al., 2013; Ko et al., 2010; Lemonnier et al., 2012; Li et al., 2011; Müller et al., 2012; Quivoron et al., 2011). We find that *Tet* deficiency leads to heterogeneous telomere elongation and shortening and chromosomal instability, defects that are associated with tumorigenesis and aging (Artandi et al., 2000; Blasco, 2007; Rudolph et al., 1999). Further investigation of Tet enzymes in the context of telomere biology may have significant implications for aging and cancer.

EXPERIMENTAL PROCEDURES

ESCs and Culture

NF2 ESC lines were derived from Nanog-EGFP transgenic mice and BF10 ESC lines from B6C3F1 mice (Chen et al., 2009; Liu et al., 2011). *Tet1* and *Tet2* DKO or *Tet1/2/3* TKO ESCs from G.X. lab were generated from C57BL/6X129 mouse background and validated by genotyping and absent Tet1 and Tet2 proteins or all Tets by western blot (Hu et al., 2014). *Tet1/2* DKO, *Tet1/2/3* TKO, and WT ESCs generated from M.M.D. lab (Dawlaty et al., 2013, 2014) also were used for telomere analysis. ESCs were routinely cultured on mitomycin-C-treated MEF feeder in ESC culture medium consisting of KO DMEM supplemented with 20% fetal bovine serum (FBS) (ESC quality; Hyclone), 1,000 U/ml leukemia inhibitory factor (LIF) (ESGRO; Chemicon), 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, 1 mM L-glutamine, and penicillin (100 U/ml) and streptomycin (100 μg/ml; Dan et al., 2014). J1 ESCs were cultured without MEF feeder. ESC lines from G.X. or M.M.D. lab were cultured as described (Hu et al., 2014; Dawlaty et al., 2013, 2014).

KD of *Tet1*, *Tet2*, or *Dnmt3b* by RNAi

Tet1, *Tet2*, and *Dnmt3b* shRNA sequences shown as below were synthesized and cloned into pSIREN-RetroQ, according to manufacturer's instructions. KD of *Tet1*, *Tet2*, or both by RNAi was achieved using at least two sequences specifically targeting to the *Tet1* or *Tet2* (Ficz et al., 2011; Ito et al., 2010). The mock KD shRNA without sequence homology to mouse genes served as negative control. pSIREN-RetroQ control and KD plasmid (2 μg) were introduced into Plat-E cells using lipofectamine transfection reagent according to the manufacturer's recommendation. Viruses were collected with 0.45 μm membrane filter at 48 hr and 72 hr, respectively, after transfection. ESCs (2×10^5) were infected with the virus twice, selected by 1.5 μg/ml puromycin for 7–10 days, clones were picked, and stable KD ESC lines obtained. *Tet1*/*Tet2* KD ESCs were puro resistance and, for *Dnmt3b* KD, the neo resistance was used to substitute the pSIREN-RetroQ puro resistance, ESCs then selected with 500 μg/ml G-418 for 7 days, and the stable KD clones picked.

Telomere QFISH

Telomere length and function (telomere integrity and chromosome stability) was estimated by telomere QFISH (Hande et al., 1999; Herrera et al., 1999; Huang et al., 2011; Poon et al., 1999; Zijlmans et al., 1997). Cells were incubated with 0.3 μg/ml nocodazole for 3 hr to enrich cells at metaphases. 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. Telomere FISH and quantification were performed as described previously (Herrera et al., 1999; Poon et al., 1999), except for fluorescein isothiocyanate (FITC)-labeled (CCCTAA) peptide nucleic acid (PNA) probe (Panagene) used in this study. Telomeres were denatured at 80°C for 3 min and hybridized with telomere PNA probe (0.5 μg/ml). Fluorescence from chromosomes and telomeres was digitally imaged on Zeiss Axio Imager Z1 microscope with FITC/DAPI filters, using AxioCam and AxioVision software 4.6. For quantitative measurement of telomere length, telomere fluorescence intensity was integrated using the TFL-TELO program.

Telomere CO-FISH

Strand-specific CO-FISH was performed based on original publication (Bailey et al., 2004), with minor modification (detailed in Supplemental Experimental Procedures).

Telomere Measurement by Quantitative Real-Time PCR

Average telomere length was measured from total genomic DNA using real-time PCR assay (Callicott and Womack, 2006; Cawthon, 2002; Huang et al., 2011).

MeDIP-qPCR and hMeDIP-qPCR

MeDIP-qPCR and hMeDIP-qPCR experiments and analysis were carried out as described (Weber et al., 2005).

Analysis of Sub-telomeric DNA Methylation

Analysis of DNA methylation of sub-telomeric genomic DNA regions and bisulfite sequencing were carried out as described (Gonzalo et al., 2006).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.058.

AUTHOR CONTRIBUTIONS

J.Y. conducted most experiments, analyzed the data, and prepared the manuscript. R.G., Hua Wang, X.Y., Z.Z., J. Dan, Haiying Wang, P.G., W.D., Y.Y., S.M., L.W., and J. Ding conducted part of experiments or provided materials or reagents. J.L., D.L.K., M.M.D., J.W., and G.X. interpreted the data, provided materials or reagents, and revised the manuscript. L.L. conceived the project, designed experiments, and wrote and revised the manuscript.

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