TIN2 deficiency leads to ALT-associated phenotypes and differentiation defects in embryonic stem cells

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SUMMARY

Telomere integrity is critical for embryonic development, and core telomere-binding proteins, such as TIN2, are key to maintaining telomere stability. Here, we report that homozygous Tin2S341X resulted in embryonic lethality in mice and reduced expression of Tin2 in the derived mouse embryonic stem cells (mESCs). Homozygous mutant mESCs were able to self-renew and remain undifferentiated but displayed many phenotypes associated with alternative lengthening of telomeres (ALT), including excessively long and heterogeneous telomeres, increased ALT-associated promyelocytic leukemia (PML) bodies, and unstable chromosomal ends. These cells also showed upregulation of Zscan4 expression and elevated targeting of DAXX/ATRX and H3K9me3 marks on telomeres. Furthermore, the mutant mESCs were impeded in their differentiation capacity. Upon differentiation, DAXX/ATRX and PML bodies dissociated from telomeres in these cells, where elevated DNA damage was also apparent. Our results reveal differential responses to telomere dysfunction in mESCs versus differentiated cells and highlight the critical role of TIN2 in embryonic development.

INTRODUCTION

Mammalian telomere DNA consists of tandem TTAGGG repeats and is regulated and protected by myriad factors, including telomerase and the six-protein complex composed of TRF1, TRF2, TIN2/TIN2, POT1, TPP1, and RAP1 (de Lange, 2005; Liu et al., 2004). To achieve unlimited growth, most tumor cells reactivate telomerase to maintain their telomere length. However, about 10%-15% of human cancers use the telomerase-independent alternative lengthening of telomeres (ALT) mechanism (Shay and Wright, 2019). During the two-cell stage in mouse embryonic development, embryos are telomerase inactive and use the ALT mechanism to elongate telomeres (Falco et al., 2007; Zalzman et al., 2010). Mouse embryonic fibroblasts (MEFs) and embryonic stem cells (ESCs) derived from Terc-/- mice have also been reported to activate the ALT mechanism (Niida et al., 2000). These findings support an important role for ALT in mouse embryonic development. Pathogenic mutations in telomerase subunits and telomere-binding proteins have been found to disrupt telomere maintenance and contribute to numerous human diseases, such as dyskeratosis congenita (DC) and cancer (Martinez and Blasco, 2017). In human cells, TIN2 bridges different telomere-binding activities for telomere length regulation and end protection. For example, it can stabilize TRF1/TRF2 and TPP1/POT1 complexes on telomeres (Kim et al., 1999, 2004) and negatively regulate telomere length by modulating telomerase recruitment (Frank et al., 2015; Ye and de Lange, 2004). Many TIN2 pathogenetic mutations were identified in patients with DC, pulmonary fibrosis, or rarer cases of cancer (He et al., 2020; Savage et al., 2008; Schmutz et al., 2020; Walne et al., 2008).

Telomere integrity is vital for embryonic development. Individual abrogation of Trf1 (Karlseder et al., 2003), Trf2 (Celli and de Lange, 2005), Tpp1 (Kibe et al., 2010), or Pot1a (Hockemeyer et al., 2006) led to embryonic lethality. In the case of Tin2, complete loss resulted in embryonic lethality at embryonic day 3.5 (E3.5)-E7.5 (Chiang et al., 2004). Furthermore, the derivation of mouse ESC (mESC) lines from Tin2-/- blastocytes was unsuccessful due to severe growth defects during the early stages of mESC establishment (Chiang et al., 2004). The deletion of Tin2 in human cells and mouse fibroblast cells caused severe DNA damage and cell growth defects (Celli and de Lange, 2005; Kim et al., 2008, 2017). Interestingly, recent studies showed that Trf2 knockout (KO) in mESCs did not result in cell death or end-to-end chromosome fusions (Markiewicz-Potoczny et al., 2020; Ruis et al., 2020), suggesting that ESCs and somatic cells may respond to telomere dysfunction differently and that further work is needed to investigate such differences.
The predominant human TIN2 short isoform TIN2S is more widely studied and encoded by the first six exons of the TINF2/TIN2 gene, whereas the longer TIN2L isoform utilizes all nine exons (Kaminker et al., 2009). The mouse Tin2/Tin2 locus is similarly organized as its human counterpart, where all nine exons are present without producing any apparent shorter isoforms (Nelson et al., 2018). When we introduced a premature termination mutation (Tin2S341X) into the Tin2/Tin2 locus in mice to study the contribution of Tin2 exon7–9, we found that homozygous Tin2S341X mutation resulted in embryonic lethality. The derived mESC lines showed drastically reduced Tin2 expression but were nonetheless able to self-renew and maintain their undifferentiated state. These mutant mESCs displayed many ALT-associated characteristics and increased DAXX/ATRX recruitment and H3K9me3 occupancy on telomeres. Moreover, the ability of the mutant mESCs to differentiate in vitro and in vivo was impaired. In mutant cells, differentiation induction led to the reduced association of DAXX/ATRX and promyelocytic leukemia (PML) bodies with telomeres and widespread DNA damage. Finally, both Tin2L and Tin2S could bind to telomeres efficiently, and re-introducing either isoform rescued the Tin2S341X mESCs in the assays we performed. These findings illustrate the important role of Tin2 in the regulation of telomeres and differentiation in mESCs and suggest comparable functions of TIN2S and TIN2L in mESC telomere maintenance.

**RESULTS**

**Tin2S341X mutation reduces Tin2 expression and leads to embryonic lethality in mice**

We used the CRISPR-Cas9 technology to introduce a stop codon at the 5’ end of exon 7 in the mouse Tin2 locus (Figure S1A) and herein refer to wild-type mice as Tin2WT, heterozygous mice as Tin2WT/S341X, and homozygous mutants as Tin2S341X. Of the 315 pups obtained from crossing Tin2WT/S341X mice, no Tin2S341X animals were obtained, whereas the ratio of wild-type to heterozygous progeny was roughly 1:2 (Figure 1A). Heterozygous mice developed normally with no notable differences from Tin2WT mice. We then genotyped the embryos from heterozygous intercrosses at selected time points (Figure 1B). Among E3.5 embryos, the observed ratio of the three expected genotypes was consistent with Mendel’s law of segregation. By E10.5, however, no homozygous mutant embryos could be found. Only a single dead homozygous mutant embryo was found at E7.5, suggesting that death of Tin2S341X embryos likely occurred between E3.5 and E7.5.

To probe the impact of the Tin2S341X mutation in early development, we established mESC lines of all three genotypes from blastocysts by crossing Tin2WT/S341X mice, including two homozygous Tin2S341X lines obtained in two independent experiments (Figure S1B). Both wild-type and mutant mESC lines appeared normal in morphology and grew as colonies. Since heterozygous mice developed normally, we focused on the homozygotes and the two matched Tin2WT control lines hereafter.

When we analyzed the mESCs by qRT-PCR using primers spanning exons 1–2 (primer 1) or 5–6 (primer 2), we observed decreased Tin2 transcripts in mutant cells (Figure 1C), which was corroborated by significantly reduced protein levels in immunofluorescence (IF)-fluorescence in situ hybridization (FISH) assays using an antibody that has been shown to recognize mouse TIN2 proteins in immunostaining assays (Figures 1D and 1E). Taken together with reduced Tin2 mRNA levels, this downregulation of Tin2 protein expression in Tin2S341X mESCs is likely due to cellular protective mechanisms, such as nonsense-mediated mRNA decay, and points to TIN2 deficiency in the mutant mESCs (Lykke-Andersen and Jensen, 2015). Given the importance of TIN2 in stabilizing core telomeric complexes, we also examined the telomeric targeting of TRF1 and TRF2 but found no difference between mutant and wild-type mESCs (Figures S1C and S1D), suggesting that TRF1/TRF2 binding to telomere DNA remained largely unchanged despite decreased Tin2 expression in Tin2S341X cells. Then, we ectopically expressed SFB-tagged mouse TIN2 proteins that correspond to either Tin2L or Tin2S in Tin2S341X mutant mESCs (Figure S1E) and examined their telomere binding efficiencies by telomere chromatin immunoprecipitation (ChIP) using an anti-FLAG antibody. We found similar telomere signal intensities were detected in Tin2L- and Tin2S-overexpressing cells, suggesting that lacking exons 7–9 did not affect Tin2 telomere binding (Figures 1F and 1G).

**Tin2S341X mESCs harbor longer telomeres and display ALT-associated characteristics**

To understand how the Tin2S341X mutation might affect Tin2 function, we carried out telomere restriction fragment (TRF) analysis by pulsed-field gel electrophoresis (PFGE) in various mESC lines. Compared with wild-type as well as heterozygous mESCs, the median telomere length in the two homozygous mutant mESC lines was considerably longer (Figure 2A). The quantitative FISH (Q-FISH) assay again found Tin2S341X mESCs to harbor significantly longer and more heterogeneously distributed telomeres than wild-type cells (Figures 2B and 2C). These results combined indicate abnormal telomere lengthening in Tin2S341X mESCs. To determine whether it was the result of abnormal telomerase activities, we carried out telomeric repeat amplification protocol (TRAP) assays, but Tin2S341X...
mESCs appeared similar to control cells (Figure S2A). We noticed mutant mESCs exhibited a drastic increase in telomere-associated PML bodies (APBs) (Figures 2D, 2E, and S2B). Increased APBs and extremely long and heterogeneous telomeres are two hallmarks of ALT cancer cells (Bryan et al., 1995; Yeager et al., 1999), which often contain increased telomere dysfunction-induced foci (TIF), fragile telomeres due to DNA replication stress, and higher frequencies of telomere sister chromatid exchange (T-SCE) and formation of extrachromosomal circular telomeric DNA called C-circle (Cesare and Griffith, 2004; Henson et al., 2009; Londono-Vallejo et al., 2004; Takai et al., 2003). To assess whether such phenotypes could also be observed in Tin2S341X mESCs, we first co-stained the cells for γ-H2AX and telomeres. Significantly more Tin2S341X mESCs appeared to harbor TIFs than control cells (Figures 2F and 2G). In FISH assays, both Tin2S341X mESC lines exhibited more fragile telomeres than wild-type cells as well (Figures 2H and 2I). Consistent with their heterogeneous telomere length distribution, Tin2S341X mESCs also exhibited more frequent T-SCE events, as evidenced by increased overlapping signals in cells co-stained with

**Figure 1. Homozygous Tin2<sup>S341X</sup> mutation is embryonically lethal in mice**
(A) The number and percentage of offspring with the expected versus actual genotypes from Tin2<sup>WT/S341X</sup> breeding pairs are listed.
(B) The number and percentage of early embryos at different stages from Tin2<sup>WT/S341X</sup> intercrosses are listed.
(C) Tin2 mRNA expression in various mESC lines was analyzed by qPCR. Three independent experiments were performed. Error bars indicate SD.
(D) mESCs were analyzed by IF-FISH using an antibody that can recognize mouse TIN2 in IF assays (red) and a telomere probe (green). Scale bars: 5 μm.
(E) Fluorescence intensities of TIN2 signals in samples from (D) were measured and plotted, with each dot representing one TIN2 focus. Red line indicates mean intensity of each sample. Around 20 cells for each line were examined.
(F) Tin2<sup>S341X</sup> mESCs ectopically expressing SFB-tagged TIN2S, TIN2L, or empty vector control (EV) were analyzed in telomere ChIP assays using an anti-FLAG antibody. Co-precipitated DNA was slot blotted and then hybridized with telomere or B1(Alu) probes. Representative images are shown. EV-SFB served as negative control.
(G) Data from (F) were quantified and plotted and represent results from two technical replicates. Error bars indicate SD. For (C and E), statistical significance was determined by t test. For (G), one-way ANOVA was used. For all panels, *p < 0.05 and **p < 0.01. Not significant (ns), p > 0.05.
telomere C- and G-strand probes (Figures 2J and 2K). C-circle formation assays via 29 amplification and slot blotting, however, revealed no significant differences between Tin2WT and Tin2S341X cells (Figures 2C and 2D). It has been reported that some telomerase-positive tumors with APBs contain no C-circles and that forced expression of hTERT in ALT tumors can reduce C-circles to non-ALT levels (Plantinga et al., 2013). Our results collectively showed that, despite the presence of active telomerase in the mutant mESCs, the Tin2S341X mutation led to many ALT-associated characteristics.

To further determine how Tin2 deficiency might be responsible for the ALT-associated phenotypes, we first knocked down Tin2 in wild-type mESCs and examined the level of APBs in these cells. With efficient inhibition, Tin2 foci became undetectable in IF-FISH assays, which was concurrent with an increase in the number of APBs (Figures 2L, 2E, and 2F). Next, we generated inducible Tin2 CRISPR KO mESCs. Following doxycycline (Dox) addition, which activated Cas9-mediated Tin2 KO, Tin2 foci again became undetectable in IF-FISH assays, which coincided with the increase in APBs (Figures 2M and S2G). The level of APBs in Tin2 knockdown (KD) and KO cells was similar to that in Tin2WT cells. Interestingly, in Tin2S341X mutant mESCs, re-introducing mouse Tin2 proteins that correspond to either Tin2L or Tin2S effectively reduced the number of APBs to levels in Tin2WT cells (Figures S1E and 2N versus 2E), indicating that Tin2 deficiency was responsible for the ALT-associated phenotypes in mutant cells and that the two Tin2 isoforms may have comparable roles.

Increased telomeric H3K9me3 marks and DAXX/ATRX recruitment in Tin2S341X mESCs

Global DNA demethylation has been linked to increased ALT-associated telomere elongation and recombination in mESCs (Gonzalo et al., 2006), but when we measured cytosine DNA methylation levels at subtelomeric regions on Chr1 and Chr16 (Gonzalo et al., 2006), we saw no difference between wild-type and mutant mESCs (Figure S3A). Although we cannot exclude the possibility that differential DNA methylation levels may be present in other subtelomeric regions, these results point to minimal effects on subtelomeric DNA methylation with the Tin2S341X mutation.

In mESCs, it has been reported that increased telomeric H3K9me3 may promote the appearance of ALT features (Gauchier et al., 2019). In Tin2S341X mESCs, H3K9me3 ChIP brought down more telomere DNA signals compared with control cells but showed no difference when B1 repeat sequences (human Alu homologues) were examined (Figures 3A and 3B). In comparison, signal intensities for telomeres and B1 repeats were similar between mutant and control mESCs in H3 ChIP samples (Figures 3A and 3B). The establishment of H3K9me3 marks on telomeres depends on histone methyltransferases as well as DAXX/ATRX complex that can localize to heterochromatin (Lewis et al., 2010). We showed previously that, in Dnmt1/Dnmt3a/Dnmt3b triple-KO mESCs, the DAXX/ATRX complex became enriched at telomeres and in turn recruited histone methyltransferases and upregulated H3K9me3 marks on telomeres (He et al., 2015). IF-FISH analysis of the mutant mESCs revealed more co-localization of DAXX and ATRX with...
telomere signals compared with wild-type cells (Figures 3C–3F). Higher telomere signal intensities were also detected in DAXX ChIP in mutant mESCs (Figures S3B and S3C). IF-FISH showed that telomeric DAXX foci increased to levels seen in Tin2WT mESCs when Tin2WT mESCs were treated with small interfering RNAs (siRNAs) targeting Tin2 or when Tin2 was inducibly knocked out (Figures 3G and 3H versus 3D). Ectopically expressing TIN2L or TIN2S in mutant mESCs reduced telomeric DAXX signals as in Tin2WT cells (Figure 3I), supporting the idea that TIN2 reduction caused by Tin2S341X mutation led to the enrichment of DAXX on telomeres.
**Tin2<sup>S341X</sup> mESCs upregulate the expression of subtelomeric and 2C-related genes**

To better understand the changes that occurred in Tin2<sup>S341X</sup> mESCs, we carried out RNA sequencing (RNA-seq) to analyze their transcriptome. In both mutant and wild-type mESCs, pluripotency markers were similarly highly expressed, whereas markers for differentiated germ layers were expressed at low levels (Figure 4A). Indeed, the Tin2<sup>S341X</sup> lines expressed OCT4 and NANOG at levels similar to controls (Figures 4B, 4C, S4A, and S4B) when analyzed by indirect immunoblotting and immunofluorescence and exhibited alkaline phosphatase activities as well (Figures S4C and S4D). These results indicate that Tin2<sup>S341X</sup> mESCs were able to maintain the undifferentiated state.

RNA-seq analysis also revealed an overall increase in gene expression in the mutant cells, where 87% (135/155) of the differentially expressed genes were upregulated (p.adj. < 0.05; log<sub>2</sub>(fold change) > 1; Figure 4D). Gene ontology analysis showed those genes are enriched in processes of cellular responses to heat and translation initiation (Figure S4E). Of the genes with elevated expression, 17 (12.6%) are located in subtelomeric regions (<10 Mb from chromosomal ends; Figure S4F), including Isg15 and Hoxc10, two genes respectively located in the subtelomeric regions of chr4 and chr15 (Figure 4E). In upregulated genes, the odds ratio of subtelomeric genes compared with non-subtelomeric genes is 1.696 (p = 0.042), indicating higher sensitivity of genes in subtelomeric regions to the Tin2 mutation (Figure S4G).

Notably, Zscan4 family members, including Zscan4a, Zscan4c, and Zscan4f, were upregulated in mutant mESCs (Figure 4F). High-level expression of Zscan4 is a characteristic of two-cell (2C)-stage embryos, which utilize the ALT mechanism for telomere elongation (Falco et al., 2007).
Indeed, several 2C-embryo-specific genes were upregulated in mutant mESCs as well (Figure 4F; Hung et al., 2013; Macfarlan et al., 2012). The heightened expression of Zscan4, Tmem92, and Sp110 was also validated by the qPCR assay (Figure 4G). In mESCs, telomerase deficiency or global DNA damage can induce Zscan4 expression (Huang et al., 2011; Storm et al., 2014). In turn, ZSCAN4 can facilitate telomere recombination to prevent excessive telomere shortening (Zalzman et al., 2010). ZSCAN4 can also bind to mouse endogenous retrovirus MERVL/Mt2 in mESCs and activate its expression (Zhang et al., 2019). The activation of endogenous retrovirus thus is another hallmark of 2C embryos and 2C-state mESCs (Macfarlan et al., 2012). qPCR analysis showed that MERVL_int and Mt2_mm were indeed upregulated in mutant mESCs, whereas the expression of L1Md repeats, which are not specific to the 2C stage, remained similar among different mESC lines (Figure 4G). Ectopically expressing TIN2L or TIN2S in mutant mESCs downregulated Zscan4, Tmem92, MERVL-int, and Mt2_mm expression (Figure 5A), while Tin2 inducible KO mESCs showed a moderate increase of several 2C genes after 48 h of Dox treatment (Figure S4I). These data suggest that the transcriptional changes caused by the Tin2S341X mutation are more likely a result of reduced TIN2 expression.

Tin2S341X mESCs exhibit impaired differentiation capacity

We noticed early in our study that Tin2S341X cells grew more slowly and had lower rates of EdU uptake than control cells (Figures S5A and S5B). We have previously shown that Tin2 can localize to mitochondria and regulate ATP metabolism in human cells and that Tin2 inhibition can negatively affect cell growth (Chen et al., 2012; Kim et al., 2017). Therefore, we examined ATP and reactive oxygen species (ROS) production in our mESC lines (Figures S5C and S5D). No obvious difference between wild-type and mutant cells was detected, suggesting that ATP metabolism likely remained unaffected and did not contribute to the slower growth of the mutant cells.

Despite the modest growth difference, mutant mESCs were able to proliferate for extended passages while maintaining their undifferentiated state (Figures 4A–4C). We then carried out teratoma assays in nude mice to investigate the differentiation capacity of the mESC lines. As shown in Figure 5A, teratomas were observed in five out of six nude mice in the wild-type group but none in the mutant group, suggesting that the Tin2S341X mutation impeded the differentiation capacity of mESCs. Next, we investigated these cells using the embryoid body (EB) formation assay. Both wild-type and mutant ESCs were able to form EBs with no apparent cell death during 8 days of culturing in low-attachment dishes (Figure S5E).

As expected, pluripotency markers, such as Pou5f1 and Nanog, decreased in wild-type cells as they became more differentiated, accompanied by increased expression of various germ layer markers (Figure 5B). In comparison, although Tin2S341X mutant cells also downregulated pluripotency markers as days progressed, the expression level of germ layer markers was significantly lower (Figure 5B), indicating a block in differentiation pathways. In mutant mESCs, overexpressing Tin2L and Tin2S, but not empty vector, increased germ layer marker expression significantly after EB differentiation (Figure 5C), indicating that the differentiation defects in mutant mESCs loss could be rescued by either Tin2 isoform.

Tin2S341X embryos died shortly after reaching the E3.5 blastocyst stage (Figure 1B). In mouse embryos, the neural plate forms around E5.5–E7.5 (Hitoshi et al., 2004). When we differentiated the mESCs into ectodermal lineage, both wild-type and mutant cells downregulated the expression of pluripotency markers Nanog and Pou5f1 (Figure S5F). Compared with control cells, however, the expression of neural progenitor markers Nes and Pax6 was significantly lower in mutant cells (Figure S5F), supporting the notion that Tin2S341X cells were impaired in differentiation capacity.

Tin2S341X mutation leads to widespread DNA damage upon differentiation

In undifferentiated Tin2S341X mESCs, we observed increased telomeric targeting of PML bodies and DAXX/ATRX proteins (Figures 2D, 2E, and 3C–3F). By day 12 following ectoderm differentiation induction, co-staining signals of DAXX/ATRX proteins and PML bodies with telomeres in mutant cells had dropped to levels similar to control cells (Figures 6A–6D, S6A, and S6B). PML bodies are known to play critical roles in DNA damage response and chromatin stability modulation on both telomeres and other genomic regions (Dellaire et al., 2006; Marchesini et al., 2016). Likewise, DAXX and ATRX can coordinate with other proteins to maintain genome stability in tumor cells and mESCs (He et al., 2015; Juhasz et al., 2018). We reasoned that following differentiation, with the disassociation of DAXX/ATRX proteins and PML bodies from telomeres, telomere chromatin might become more susceptible to damage. Indeed, Q-FISH revealed more fragile telomeres and chromosome fusions in differentiated cells derived from mutant mESCs (Figures S6C and S6D). When differentiated cells were co-stained for γH2AX and telomeres, overlapping γH2AX and telomere signals remained higher in mutant cells (Figures 6E and 6F), at levels similar to those observed in undifferentiated Tin2S341X mESCs (Figures 2F and 2G). Notably, a considerable proportion of the differentiated Tin2S341X cells also exhibited high levels of pan-nuclear staining of γH2AX (Figures 6G and 6H), an indication

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of severe DNA damage, replication stress, and/or pre-apoptosis (de Feraudy et al., 2010; Moeglin et al., 2019). Such pan-nuclear γH2AX signals were absent in undifferentiated mESCs, where only clustered γH2AX foci were visible (Figure 2F). These findings suggest possible links between genome-wide instability and increased telomeric dissociation of DAXX/ATRX proteins and PML bodies and led us to examine ESCs depleted of Daxx or Pml. Efficient KD of Daxx or Pml in control mESCs had little effect on the level of TIFs (Figures 6I and S6E). In contrast, depleting Daxx and Pml individually or together led to significantly more TIFs in mutant mESCs (Figure 6I). When we examined pan-nuclear DNA damage, Daxx KD alone had little effect on either wild-type or mutant cells, while Pml KD led to modest but reproducible increases in pan-nuclear γH2AX signals in mutant cells (Figure S6F). However, in mutant cells with combined Daxx/Pml KD, a significant increase in pan-nuclear γH2AX staining was apparent (Figures 6J and S6G), suggesting DAXX and PML may function synergistically with TIN2 to ensure telomere integrity and genome stability in mESCs. Collectively, our findings support the model where Tin2S341X mESCs display drastically reduced Tin2 expression, ALT-associated phenotypes, and elevated recruitment of DAXX/ATRX proteins and PML bodies to telomeres (Figure 6K). In the mutant cells, telomeres become more unstable as DAXX/ATRX proteins and PML bodies disassociate from telomeres upon differentiating, which leads to widespread chromatin instability and profound differentiation defects (Figure 6K).

**DISCUSSION**

The majority of work on telomere dysfunction has focused on cancer and somatic cells; the consequences in ESCs are much less studied and poorly understood. Recent evidence
Figure 6. Mutant mESCs exhibit decreased telomere association of DAXX/ATRX and PML upon differentiation

mESCs were differentiated to ectoderm lineage and analyzed at day 12 (D12).

(A–D) Cells were analyzed by IF-FISH using a telomere probe (green) together with an antibody against DAXX (red; A) or PML (purple; C). Representative images from each genotype are shown with scale bars of 5 µm. Telomere-localized DAXX (B) and (legend continued on next page)
from mESCs demonstrates that TRF2 is dispensable and suggests that mESCs may utilize a unique telomere protection mechanism in cases of dysfunctional telomere proteins (Markiewicz-Potoczny et al., 2020; Ruis et al., 2020). In this study, we showed that the Tin2S341X mutation reduces TIN2 deficiency and the mutant mESCs display many of the characteristics associated with ALT cells, likely due to disrupted telomere maintenance pathways and the activation of ALT-mediating factors. Likewise, Trf2−/− mESCs were reported to active Zscan4 and other 2C-related genes (Markiewicz-Potoczny et al., 2020). ALT-associated chromatin remodelers and PML bodies were found to be enriched at telomeres in Trf1 KO MEFs (Porreca et al., 2020). Taken together, these data suggest loss of core telomere regulators during mouse early embryogenesis tends to promote ALT-associated phenotypes.

Telomere instability can prevent mESCs from normal differentiation, as evidenced by Terc−/− mESCs that had short telomeres, reduced pluripotency potential, and difficulties in maintaining the differentiated state in vitro (Huang et al., 2011; Pucci et al., 2013). When we induced Tin2S341X mESCs to differentiate into neural progenitor cells, they displayed widespread DNA damage, an observation in line with findings that newly generated neurons were hypersensitive to telomere dysfunction and DNA damage (Cheng et al., 2007).

The expression of subtelomeric genes appears more affected in Tin2S341X mESCs, and several of them, such as Isg15 and Hoxc10, are developmental process related. Gene ontology analysis on genome-wide subtelomeric genes also found several developmental processes to be enriched (data not shown). Whether and how telomere defects may affect developmental genes at subtelomeric regions warrants further investigation.

We hypothesize that telomeres may be differentially regulated in undifferentiated versus differentiated mESCs. In Tin2S341X cells, chromatin remodeling factors, such as DAXX/ATRX and PML bodies, were recruited to telomeres in undifferentiated mESCs but dissociated from telomeres upon differentiation. How such dissociation occurs remains unclear. PML bodies are often enriched in gene-rich and transcriptionally active regions (Wang et al., 2004). DAXXX/ATRX mediate the deposition of the histone variant H3.3 to telomeres and other heterochromatin regions (Wong et al., 2009), while genome-wide profiling has indicated changes in H3.3-binding sites following mESC differentiation into neural progenitor cells (NPCs) (Goldberg et al., 2010). We thus speculate that perhaps DAXXX/ATRX and PML bodies can translocate to other regions of the chromatin in response to differentiation-induced gene expression and/or changes in chromatin structures.

Notably, there have been conflicting reports on telomeric histone modifications in ALT cells. For instance, while global H3K9me3 decrease is linked to ALT phenotypes (Episkopou et al., 2014), it has also been shown that typical ALT cells, such as U2OS, have relatively high levels of telomeric H3K9me3, but most non-ALT laboratory cell lines lack telomeric H3K9me3 marks under normal culture conditions (Cubiles et al., 2018). Increased telomeric H3K9me3 may even promote the appearance of ALT features in mESCs (Gauchier et al., 2019). Further studies in these fields will help uncover how dysfunction in telomere regulators affects telomere chromatin status in ESCs and how such changes impact the ESC differentiation process.

In patients, premature termination mutations are among major TIN2 pathogenic mutations. For example, TINF2/TIN2 K280RfsX36 in DC patients resulted in earlier disease onset, more severe symptoms, and reduced expression of the truncated TIN2 protein (Sasa et al., 2012). Other premature terminations are also associated with elongated telomeres in several cancers (He et al., 2020; Schmutz et al., 2020). Premature termination mutations on TIN2L-specific exons have also been noted in clinical studies. For example, ClinVar
VCV000575752 has recorded TINF2/TIN2 c.1090dup p.Leu364Profs*9 found in a pediatric patient with motor delay and hypertonia (unpublished data). In our study, the Tin2S341X mutation in exon7 reduced Tin2 expression and re-introducing either Tin2 isoform yielded comparable results in Tin2S341X mESCs. These findings suggest decreased Tin2 expression is likely the main culprit for the phenotypes and support comparable functions for the two isoforms. How Tin2S and Tin2L may function differentially in other aspects warrants further exploration. More in-depth molecular and clinical research should shed light on how Tin2S and Tin2L may function differentially and contribute to human diseases.

EXPERIMENTAL PROCEDURES

CRISPR-Cas9-mediated knockin of a stop codon in the mouse Tin2/Tin2 locus
All experiments involving animals were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University, People’s Republic of China. C57Bl/6J mice were used in this study. Embryo injection and founder line construction was done by Shanghai Bangyao BioTech. Two heterozygous animals (out of 8) with the desired mutation were set up as founders and back-crossed for six generations. Genotyping was done by PCR and Sanger sequencing. Genotyping primers, CRISPR single-guide RNA (sgRNA), and donor template sequences are shown in Table S1.

IF staining and IF-FISH
Cells were plated on 0.2% gelatin-coated glass coverslips, fixed with 2% paraformaldehyde, and permeabilized in 0.5% Triton X-100 (in PBS) before further analysis. For IF-FISH, an additional incubation with PNA-(CCCTAA)3-fluorescein isothiocyanate (FITC) probe (Panagene) was performed at 37°C for 2 h after secondary antibody incubation. Slides were preserved in Vectashield mounting media with DAPI. Fluorescence microscopy was performed on a Lightening STED SP8 (Leica) microscope. All antibodies used in this study are listed in Table S2. For IF-FISH, >120 cells from three independent experiments were analyzed unless otherwise noted. For Tin2 signal intensity analysis, the TFL-TELO program was used, and the intensities of the dots with TeloC signals but no Tin2 signals were counted as 0.

ChIP and slot blotting
We crosslinked 1 × 10⁷ cells with 1% formaldehyde at room temperature for 5 min and then quenched in 125 mM glycine. Sonicated and pre-cleared lysates were then incubated with appropriate antibodies. The eluted DNA was purified by phenol:chloroform extraction, slot blotted onto Hybond-N+ membranes (GE Amersham), and probed with biotin-labeled probes. Signal intensity was measured by ImageJ. Telo probe was biotin-TTAGGGT TAGGGTATAAGGT. B1(Alu) probe was biotin-TAATCCCAGC ACTTGGG.

Statistical analysis
For Tin2WT−1, Tin2WT−2, Tin2S341X−1, and Tin2S341X−2, Student’s t test with 95% confidence level was used to compare means of wild type and mutant. For multiple samples, one-way ANOVA followed by Dunnett’s multiple comparison test with 95% confidence level was performed. For multiple samples across multiple time points, two-way ANOVA was performed. A p < 0.05 was considered statistically significant.

Data and code availability
RNA-seq data from this study can be found in GEO: GSE156514.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.03.005.

AUTHOR CONTRIBUTIONS

Z.S., Y.H., and S.Y. conceived the project. S.Y. conducted most of the experiments and data analysis. Y.H. designed the truncation mutation. F.Z. performed the experiments about embryos and constructed mESCs. W.C. performed Q-TRAP and methylation assay and assisted with other experiments. C.W. performed C-circle experiment. Z.H. performed the TRF assay. S.L. assisted with embryo experiments and other assays. S.Y. and K.W. analyzed the RNA-seq data. S.Y. wrote the manuscript. Z.S., D.L., and Y.H. revised the manuscript. S.Y., Z.S., Y.H., Y.C., W.C., S.L., W.M., J.H., and D.L. discussed the project.

CONFLICTS OF INTERESTS

The authors declare no competing interests.

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