Zscan4 inhibits maintenance DNA methylation to facilitate telomere elongation in mouse embryonic stem cells

Jiameng Dan1,2, Philippe Rousseau3, Swanand Hardikar1,2, Nicolas Veland1,2,6, Jiemin Wong4, Chantal Autexier3,5, and Taiping Chen1,2,6,7,*

1Department of Epigenetics and Molecular Carcinogenesis, The University of Texas MD Anderson Cancer Center, Smithville, Texas 78957, USA
2Center for Cancer Epigenetics, The University of Texas MD Anderson Cancer Center, Smithville, Texas 78957, USA
3Bloomfield Center for Research in Aging, Lady Davis Institute for Medical Research, Jewish General Hospital, McGill University, Montréal, Québec H3T 1E2, Canada
4Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China
5Division of Experimental Medicine, Department of Anatomy and Cell Biology, McGill University, Montréal, Québec H3A 0C7, Canada
6The University of Texas Graduate School of Biomedical Sciences at Houston, Houston 77030, Texas, USA

SUMMARY

Proper telomere length is essential for embryonic stem cell (ESC) self-renewal and pluripotency. Mouse ESCs (mESCs) sporadically convert to a transient totipotent state similar to that of two-cell (2C) embryos to recover shortened telomeres. Zscan4, which exhibits a burst of expression in 2C-like mESCs, is required for telomere extension in these cells. However, the mechanism by which Zscan4 extends telomeres remains elusive. Here, we show that Zscan4 facilitates telomere elongation by inducing global DNA demethylation through downregulation of Uhrf1 and Dnmt1, major components of the maintenance DNA methylation machinery. Mechanistically, Zscan4 recruits Uhrf1 and Dnmt1 and promotes their degradation that is dependent on the E3 ubiquitin ligase activity of Uhrf1. Importantly, blocking DNA demethylation prevents telomere elongation associated with Zscan4 expression, suggesting that DNA demethylation mediates the effect of Zscan4. Our results define a molecular pathway that contributes to the maintenance of telomere length homeostasis in mESCs.
INTRODUCTION

Mammalian telomeres are specialized heterochromatin structures that contain TTAGGG repeats and associated proteins, which protect the ends of linear chromosomes from DNA repair and degradation activities (Blasco, 2005). Proper telomere length is required for mammalian cell proliferation, while short telomeres trigger DNA breaks, chromosome fusions, cellular senescence and aging (Blasco, 2005). Normal proliferative somatic cells undergo telomere shortening and eventually become senescent. In contrast, embryonic stem cells (ESCs) are able to elongate their telomeres and proliferate indefinitely, while maintaining pluripotency and genomic stability. ESCs with short telomeres exhibit reduced developmental pluripotency (Huang et al., 2011) and impaired differentiation capacity (Pucci et al., 2013).

Multiple mechanisms contribute to telomere elongation in ESCs. One is that ESCs have high telomerase activity, which adds telomeric repeats de novo after each cell division (Greider and Blackburn, 1985, 1989). Deletion of either the reverse transcriptase (Tert) or the RNA component (Terc) of telomerase leads to telomere shortening in mouse ESCs (mESCs) (Huang et al., 2011; Pucci et al., 2013). Telomerase-independent pathways, such as ALT (alternative lengthening of telomeres), which relies on homologous recombination between telomeric sequences, also play important roles in telomere length homeostasis in ESCs (Blasco, 2007).

Recent work identified a transient cell population within mESC cultures that exhibits a gene expression pattern similar to that of two-cell (2C) embryos. These 2C-like mESCs are totipotent, as they have the ability to contribute to both embryonic and extraembryonic tissues (Macfarlan et al., 2012). While 2C-like mESCs are rare at any given time, nearly all mESCs cycle in and out of this totipotent state in long-term cultures (Macfarlan et al., 2012). Zscan4 (zinc finger and SCAN domain-containing protein 4), which is specifically expressed at the two-cell stage during mouse development, exhibits a burst of expression in 2C-like mESCs and is associated with drastic and rapid telomere extension (Falco et al., 2007; Macfarlan et al., 2012; Zalzman et al., 2010). Two-cell embryos also undergo rapid telomere lengthening through telomere recombination or telomere sister chromatid exchange (T-SCE) (Liu et al., 2007), although it is unclear whether Zscan4 plays a role. Zscan4 knockdown in mESCs leads to telomere shortening, genomic instability, and proliferation defects (Zalzman et al., 2010). Moreover, Zscan4 expression is induced after telomere shortening in mESCs with long cell cycle (Nakai-Futatsugi and Niwa, 2016) or telomerase deficiency (Huang et al., 2011). These findings led to the notion that mESCs transiently convert to the 2C-like state to recover shortened telomeres through a Zscan4-dependent pathway (Nakai-Futatsugi and Niwa, 2016). How Zscan4 facilitates telomere elongation is unknown.
Chromatin modifications, including DNA methylation in subtelomeric regions, play critical roles in the regulation of mammalian telomeres by affecting telomere recombination (Blasco, 2007). DNA methylation is catalyzed by two classes of DNA methyltransferases (Dnmts): Dnmt3a and Dnmt3b function primarily as de novo Dnmts that establish DNA methylation patterns, whereas Dnmt1 is the major maintenance Dnmt that propagates DNA methylation through cell division (Chen and Li, 2004). Recruitment of Dnmt1 to newly replicated DNA depends on Uhrf1 (ubiquitin-like with PHD and ring finger domain 1), a multi-domain protein with DNA- and histone-binding capabilities and E3 ubiquitin ligase activity (Bostick et al., 2007; Sharif et al., 2007). Genetic studies have shown that disruption of either Dnmt1 or Dnmt3a/Dnmt3b in mESCs leads to dramatic telomere elongation by homologous recombination (Gonzalo et al., 2006). Interestingly, recent evidence suggests that 2C-like mESCs undergo drastic chromatin remodeling, including decondensation of heterochromatin and genome-wide DNA hypomethylation (Akiyama et al., 2015; Eckersley-Maslin et al., 2016; Ishiuchi et al., 2015). However, little is known about the molecular mechanisms involved in these chromatin changes, as well as the functional relevance of the changes in telomere regulation. In the present study, we provide evidence that Zscan4, by promoting Uhrf1 and Dnmt1 degradation, induces global DNA demethylation, which in turn facilitates telomere recombination and elongation.

RESULTS

Uhrf1 and Dnmt1 Degradation Leads to Global DNA Demethylation in 2C-Like mESCs

While analyzing DNA methylation in cultured mESCs with immunofluorescence (IF) using a 5-methylcytosine (5mC) antibody, we noticed that a rare population of cells shows strikingly low levels of DNA methylation. These cells appeared to have decondensed heterochromatin, as evidenced by less prominent DAPI-bright spots, reminiscent of 2C-like mESCs (Akiyama et al., 2015; Ishiuchi et al., 2015; Macfarlan et al., 2012). To confirm their identity, we generated mESC reporter lines, where GFP expression, driven by the Zscan4 promoter, occurred only in 2C-like cells, as evidenced by the expression of endogenous Zscan4 and the retroelement MuERV-L (murine endogenous retrovirus with leucine tRNA primer), another marker of 2C-like mESCs (Macfarlan et al., 2011; Macfarlan et al., 2012) (Figures S1A-E). IF analysis demonstrated that DNA methylation levels were markedly lower in GFP+ cells compared to GFP− cells (Figures 1A and 1B). To verify the results, we sorted the GFP+ and GFP− populations by fluorescence-activated cell sorting (FACS) and measured total 5mC levels by dot blot analysis and also examined methylation at the major and minor satellite repeats by Southern blot following digestion with methylation-sensitive restriction enzymes. Both assays showed that GFP+ cells are severely hypomethylated (Figures 1C-E). Indeed, transcripts of major and minor satellite repeats were dramatically elevated in GFP+ cells (Figure S1F), as these repeats are normally repressed by DNA methylation. Our results are consistent with a recent report showing that 2C-like mESCs exhibit genome-wide DNA hypomethylation (Eckersley-Maslin et al., 2016).

To determine the mechanism underlying DNA hypomethylation in 2C-like mESCs, we examined the expression of key enzymes and regulators involved in DNA methylation. Western blot analysis of FACS-sorted GFP+ and GFP− cells revealed drastic downregulation...
of Uhrf1 and Dnmt1 in the GFP+ population (Figure 1F). IF analysis verified that Uhrf1 was hardly detectable and Dnmt1 was markedly decreased in GFP+ cells (Figures 1G and 1I). To further confirm the change in Dnmt1 level, we examined a mESC line carrying a Flag-Dnmt1 knock-in (KI) allele, whereby endogenous Dnmt1 was Flag-tagged on one copy (KI+/+) (Figures S2A-C). Double IF analysis with Zscan4 and Flag antibodies showed that Flag-Dnmt1 was severely reduced in cells expressing endogenous Zscan4 (Figure S2D). We also verified Uhrf1 and Dnmt1 downregulation and DNA hypomethylation in mESC lines expressing another 2C-like cell reporter, namely GFP driven by the 5’ long terminal repeat (LTR) of MuERV-L (MuERV-L-LTRGFP) (Figure S1G-I). Dnmt3b also showed modest downregulation in cells expressing the Zscan4-GFP reporter (Figures S3A and S3B). While IF analysis showed no obvious change in total Dnmt3a level in GFP+ cells (Figure S3A), Western blot indicated a modest decrease in Dnmt3a1, but no change in Dnmt3a2 (Figure S3B). The Dnmt3a1 and Dnmt3a2 transcripts are produced from different promoters, and Dnmt3a2 is the predominant isoform in mESCs (Chen et al., 2002). All the other proteins examined exhibited no differences in GFP+ and GFP− mESCs (Figures S3A and S3B). These included the Dnmt3a/3b accessory factor Dnmt3L (Dnmt3-like) (Chen and Li, 2004), the Dnmt1 regulators PCNA (proliferating cell nuclear antigen), Usp7 (ubiquitin specific peptidase 7) and Lsd1/Kdm1a (lysine-specific demethylase 1, also known as lysine (K) demethylase 1a) (Chuang et al., 1997; Qin et al., 2011; Wang et al., 2009), and the 5mC dioxygenases Tet1 (ten-eleven translocation 1) and Tet2, which are involved in DNA demethylation (Wu and Zhang, 2014). Modest decreases of Dnmt3a1 and Dnmt3b may contribute to, but likely do not play a major role in, global DNA hypomethylation in 2C-like mESCs, as complete inactivation of Dnmt3a, Dnmt3b or both has no immediate impact on global DNA methylation in mESCs (Chen et al., 2003; Okano et al., 1999).

RT-qPCR analysis revealed a moderate decrease in Dnmt3b mRNA and a moderate increase in Dnmt3a2 mRNA in GFP+ cells (Figure S3C). Despite severe downregulation of Uhrf1 and Dnmt1 proteins, their mRNA levels were unaltered in GFP+ cells (Figures S1H and S3C), suggesting posttranscriptional regulation. Indeed, in Zscan4-GFP reporter cells treated with the proteasome inhibitor MG132, Uhrf1 and Dnmt1 levels were largely maintained in GFP+ cells (Figures 1H-J), indicating that they are degraded by the ubiquitin-proteasome system in 2C-like mESCs. While inhibition of protein synthesis may contribute to gene expression in 2C-like cells (Eckersley-Maslin et al., 2016; Hung et al., 2013), the highly variable changes of different proteins suggest that their turnover rates could be the major determinant of their levels. Taken together, our results suggest that failure to maintain DNA methylation, mainly due to Uhrf1 and Dnmt1 degradation, leads to severe hypomethylation in 2C-like mESCs.

Zscan4 Induces Uhrf1 and Dnmt1 Downregulation

Zscan4 was initially suspected to promote telomere elongation by regulating telomere recombination-related genes, such as Spo11, Dmc1 and Smc1β (Zalzman et al., 2010). However, these genes showed only mild changes in expression in mESCs overexpressing Zscan4 (Zalzman et al., 2010). Indeed, we found that these genes are expressed at comparable levels in Zscan4+ and Zscan4− mESCs (Figure S3D), consistent with transcriptional profiling data reported previously (Akiyama et al., 2015; Amano et al., 2013).
Given that Zscan4+/2C-like mESCs show DNA hypomethylation (as shown above), increased telomere recombination and telomere elongation (Zalzman et al., 2010), which are highly similar to the phenotypes of Dnmt1- or Dnmt3a/3b-deficient mESCs (Gonzalo et al., 2006), we hypothesized that Zscan4 facilitates telomere elongation by inducing Uhrf1 and Dnmt1 downregulation and DNA demethylation.

To test the hypothesis, we modulated Zscan4 expression in mESCs and assessed the effects on Uhrf1, Dnmt1 and DNA methylation levels. The Zscan4 cluster has nine paralogous genes (including pseudogenes). Three of them, Zscan4c, Zscan4d and Zscan4f, encode full-length proteins (506 amino acids), which show 95-99% sequence identity and presumably have identical functions. Zscan4d is the predominant form in two-cell embryos, whereas Zscan4c and Zscan4f are mainly expressed in 2C-like mESCs (Falco et al., 2007). We stably expressed Myc-Zscan4c in Flag-Dnmt1 KI/+ mESCs. These cells showed dramatic decreases in the protein levels of Dnmt1 (detected by both Dnmt1 and Flag antibodies) and Uhrf1 (Uhrf1 decrease was consistently more severe) (Figure 2A) but no changes in their transcript levels (Figures 2A and S4A), consistent with posttranscriptional regulation.

Concomitantly, Myc-Zscan4c-expressing cells underwent dramatic global DNA demethylation (Figures 2B and S4B). IF analysis of co-cultured Myc-Zscan4c-expressing cells and mocktransfected cells confirmed that Myc-Zscan4c expression resulted in severe downregulation of Uhrf1 and Dnmt1, as well as global hypomethylation (Figure 2C).

Expression of Flag-Zscan4c or -Zscan4f in wild-type (WT) J1 mESCs also induced DNA demethylation, as evidenced by severe loss of methylation at the major and minor satellite repeats and the intracisternal A-particle (IAP) retrotransposons (Figures S4C and S4D). The effects of Zscan4 were reversible, as shRNA-mediated knockdown of Zscan4 in a stable clone resulted in restoration of the levels of Uhrf1, Dnmt1 and DNA methylation (Figures 2D and 2E; Figures S4E and S4F).

To determine the role of endogenous Zscan4 in DNA methylation in 2C-like mESCs, we stably expressed a Zscan4 shRNA (shZscan4, which targets all Zscan4 proteins) in mESC lines transfected with the Zscan4-GFP reporter. shZscan4, but not control shRNA (shControl), efficiently depleted endogenous Zscan4 in 2C-like (GFP+) cells (Figures 3A and 3B). Consequently, DNA demethylation (Figures 3C-E) and downregulation of Uhrf1 (Figure 3F) and Dnmt1 (Figure 3G) were largely prevented. Collectively, our data suggest that Zscan4 is responsible for inducing Uhrf1 and Dnmt1 degradation and DNA demethylation in 2C-like mESCs.

**Blocking DNA Demethylation Prevents Zscan4-Mediated Telomere Elongation**

Zscan4 activates telomere recombination and extends telomeres, highly similar to the effects of DNA hypomethylation (Gonzalo et al., 2006; Zalzman et al., 2010). We postulated that Zscan4 facilitates telomere elongation by inducing DNA demethylation. To test the hypothesis, we sought to block DNA demethylation in Zscan4c-expressing cells and assess the effect on telomere length. Attempts to express Uhrf1 in these cells were unsuccessful, likely due to Zscan4c-mediated degradation. Although we were able to express Dnmt1, it failed to rescue DNA hypomethylation and telomere elongation, because Uhrf1 was depleted in these cells (Figures S5A-C). Previous work showed that, in the absence of Uhrf1, a
Dnmt1-PCNA fusion protein is able to localize to DNA replication foci and maintain DNA methylation in mESCs (Liu et al., 2013). We therefore generated stable clones in J1 mESCs expressing either Myc-Zscan4c alone or Myc-Zscan4c and Flag-tagged Dnmt1-PCNA fusion protein simultaneously (Figure 4A). In contrast to severe hypomethylation in cells expressing Zscan4c alone, DNA demethylation was largely blocked in cells expressing both Zscan4c and the Dnmt1-PCNA fusion protein (Figure 4B). Quantitative fluorescence in situ hybridization (Q-FISH) and flow cytometry and FISH (Flow-FISH) showed that telomere elongation associated with Zscan4c expression was prevented with co-expression of the Dnmt1-PCNA fusion protein (Figures 4C and 4D). Zscan4 induces telomere elongation by promoting telomere recombination (Zalzman et al., 2010). Indeed, the frequency of T-SCE events, as analyzed by chromosome orientation FISH (CO-FISH), increased substantially in cells expressing Zscan4c alone, but not in cells expressing both Zscan4c and the Dnmt1-PCNA fusion protein (Figures 4E and 4F). Thus, we conclude that global DNA demethylation plays an essential role in mediating the effects of Zscan4 on telomere recombination and elongation.

Telomeres and subtelomeres are enriched with repressive chromatin marks (e.g. DNA methylation, H3K9me3, H4K20me3 and HP1), forming condensed heterochromatin structures (Blasco, 2007). To determine the impact of Zscan4 on telomeric chromatin features, we performed MNase digestion and chromatin immunoprecipitation (ChIP) with H3K9me3, H4K20me3 and HP1γ antibodies, followed by hybridization with a telomere probe. Both MNase digestion and ChIP assays showed no obvious change in telomeric heterochromatin in cells overexpressing Zscan4c (Figures S6A-F). These results suggest that Zscan4 induces DNA demethylation and telomere elongation without affecting telomeric chromatin compaction, consistent with previous findings that telomere elongation in either Dnmt1-/- or Dnmt3a/3b-/- mESCs is not associated with changes in H3K9me3 and H4K20me3 enrichment at telomeres (Gonzalo et al., 2006).

**Zscan4 Recruits the Dnmt1-Uhrf1 Complex**

To gain insights into the mechanism by which Zscan4 leads to Uhrf1 and Dnmt1 downregulation, we asked whether Zscan4 forms a complex with Uhrf1 and/or Dnmt1. Co-immunoprecipitation (co-IP) assays with Myc- and Flag-tagged proteins expressed in HEK293 cells demonstrated that Zscan4c interacts with both Dnmt1 and Uhrf1 (Figure 5A). Similar co-IP experiments using WT mESCs, Dnmt1-deficient mESCs (Lei et al., 1996) or Uhrf1-deficient mESCs (generated by CRISPR/Cas9 technology, Figures S7A-E) revealed that Uhrf1 is required for Zscan4c-Dnmt1 interaction, whereas Dnmt1 is dispensable for Zscan4c-Uhrf1 interaction (Figure 5B and 5C). Our results, together with the known interaction between Dnmt1 and Uhrf1 (Bostick et al., 2007; Sharif et al., 2007), suggest that Zscan4, via Uhrf1, recruits the Dnmt1-Uhrf1 complex.

Uhrf1 contains multiple domains (Figure S7F), which recognize or set up specific chromatin marks (Bostick et al., 2007; Liu et al., 2013; Nishiyama et al., 2013; Qin et al., 2015; Rothbart et al., 2013; Sharif et al., 2007) and are also involved in intra-molecular interactions critical for Uhrf1 conformation (Fang et al., 2016). Mutagenesis and co-IP experiments indicated that deletion of the TTD (tandem tudor domain), PHD (plant
homeodomain) or RING (Really Interesting New Gene) domain abolished the interaction between Uhrf1 and Zscan4c, whereas deletion of the UBL (ubiquitin-like) or SRA (SET and RING associated) domain had no effect (Figure S7F). However, Uhrf1 variants with point mutations that eliminate the chromatin-binding ability of TTD or PHD (Liu et al., 2013; Rothbart et al., 2013) or the E3 ligase activity of the RING domain (Nishiyama et al., 2013) were still capable of interacting with Zscan4c (Figure S7G). Likely, proper folding of Uhrf1, rather than its role as a “reader” and “writer” of chromatin marks, is important for interaction with Zscan4.

Both Uhrf1-Binding and Self-Association abilities of Zscan4 Are Required for Its Effects on DNA Methylation and Telomere Length

Zscan4 has an N-terminal SCAN domain and a C-terminal zinc finger (ZNF) domain with four C2H2-type ZNF motifs (Figure 5D). We used mutagenesis and co-IP experiments to map its Uhrf1-interaction region(s). Deletion of the ZNF domain (all four ZNF motifs) or the ZNF4 motif alone abolished its interaction with Uhrf1, whereas deletion of the other three ZNF motifs individually or the SCAN domain had no effect (Figure 5D). GST pull down assays using recombinant His-Uhrf1 and GST-Zscan4c fusion proteins produced in E. coli confirmed that the Zscan4-Uhrf1 interaction is direct and that the ZNF4 motif is required (Figure 5E). The SCAN domain mediates protein-protein interactions, including self-association (Edelstein and Collins, 2005). Indeed, deletion of the Zscan4c SCAN domain abolished its ability to interact with itself (Figure 5F), suggesting that Zscan4 selfassociates to form dimers or oligomers.

We then investigated the functional relevance of the Uhrf1-binding and selfassociation abilities of Zscan4. When expressed in mESCs, mutant Zscan4c lacking the ZNF4 motif (ΔZNF4) or the SCAN domain (ΔSCAN) failed to induce Uhrf1 and Dnmt1 degradation and global DNA demethylation, in contrast to the dramatic effects of WT Zscan4c (Figures 6A and 6B). Consequently, these mutant Zscan4c proteins were no longer able to induce telomere elongation, as analyzed by Q-FISH and Flow-FISH (Figures 6C and 6D), and telomere recombination, as measured by T-SCE frequency (Figures 6E and 6F). These results indicate that the abilities of Zscan4 to bind Uhrf1 and self-associate are both critical for its effects on DNA methylation, telomere recombination and telomere length.

Zscan4-Induced Uhrf1 and Dnmt1 Degradation Is Dependent on the E3 Ubiquitin Ligase Activity of Uhrf1

Our finding that MG132 prevents Uhrf1 and Dnmt1 downregulation in 2C-like cells suggests that Zscan4 induces their degradation (Figure 1). We examined the stability of Uhrf1 and Dnmt1 by measuring their turnover rates over time after blocking protein synthesis with cycloheximide. Indeed, WT Zscan4, but not the ΔZNF4 or ΔSCAN mutant, greatly accelerated degradation of both Uhrf1 and Dnmt1 proteins (Figures 7A-C), thus confirming that Zscan4 induces Uhrf1 and Dnmt1 degradation and that its Uhrf1-binding and self-association abilities are both required for the effect.

Uhrf1 has a RING domain with E3 ubiquitin ligase activity and has been shown to ubiquitinate itself and Dnmt1, leading to their degradation (Du et al., 2010; Jenkins et al.,
2005). We therefore asked whether the E3 ligase activity of Uhrf1 is involved in Zscan4-mediated Uhrf1 and Dnmt1 degradation. We first performed in vivo ubiquitination assays by co-expressing Myc-Zscan4c (or the ΔZN4 and ΔSCAN mutants), Flag-Uhrf1 (or the E3 ligase-inactive mutant H730A) and HA-ubiquitin in HEK293 cells. As shown in Figure 7D, WT Zscan4c, but not ΔZN4 or ΔSCAN, greatly enhanced Uhrf1 ubiquitination, and this effect was abolished when the E3 ligase activity of Uhrf1 was eliminated (H730A mutant). These data indicate that Zscan4 enhances Uhrf1-mediated self-ubiquitination and that its abilities to bind Uhrf1 and self-associate are required for the effect. When reexpressed in Uhrf1−/− mESCs, WT Uhrf1 displayed severe downregulation in Zscan4+ cells, whereas the E3 ligase-inactive mutants (H730A and 3C:3A) maintained their levels (Figures 7E and 7F), further supporting that Zscan4 facilitates Uhrf1 self-ubiquitination and degradation. Apparently, Dnmt1 degradation in 2C-like cells also requires Uhrf1, as Dnmt1 downregulation was not observed in Uhrf1-deficient Zscan4+ mESCs, whereas Uhrf1 downregulation was not affected by Dnmt1 deficiency (Figures 7G and 7H). Taken together, our results suggest that Zscan4 induces Uhrf1-dependent degradation of both Uhrf1 itself and Dnmt1.

**DISCUSSION**

Telomere length homeostasis is essential for ESC self-renewal and differentiation (Blasco, 2007; Dan et al., 2014; Huang et al., 2011; Pucci et al., 2013). Zscan4, which is transiently and highly expressed in 2C-like mESCs, plays a critical role in telomere elongation (Zalzman et al., 2010). However, how Zscan4 facilitates telomere elongation is unknown. Based on our data, we propose that global DNA demethylation, which promotes telomere recombination (Gonzalo et al., 2006), mediates the effect of Zscan4 on telomere extension in 2C-like mESCs (Figure 7I). We demonstrate that Zscan4 forms a complex with Uhrf1 and Dnmt1, leading to their degradation. Even though Zscan4 monomers can bind Uhrf1 through the ZNF4 motif, Zscan4 self-association, mediated by its SCAN domain, is required for Uhrf1 and Dnmt1 degradation. One possibility is that Zscan4, via dimerization/oligomerization, brings Uhrf1-Dnmt1 heterodimers to close proximity, thus facilitating cross-ubiquitination (Figure 7I). Alternatively, the SCAN domain may recruit one or more proteins that promote Uhrf1 and Dnmt1 ubiquitination and degradation. Regardless, the transient burst of expression makes Zscan4 highly abundant in 2C-like mESCs, which can explain the drastic effects on Uhrf1 and Dnmt1 levels. Rapid turnover of Uhrf1 and Dnmt1 proteins provides a mechanism that allows their levels to change quickly as mESCs cycle in and out of the transient totipotent state.

Recently, Eckersley-Maslin et al. also reported DNA hypomethylation in 2C-like mESCs and speculated that inhibition of Dnmt1, Dnmt3a and Dnmt3b translation was responsible (Eckersley-Maslin et al., 2016). Our study extends their work in several aspects. First, we demonstrate that Zscan4 is responsible for global DNA demethylation mainly by inducing Uhrf1 and Dnmt1 degradation in 2C-like cells, thus elucidating the underlying mechanism. Second, we show that DNA hypomethylation acts downstream of Zscan4 in regulating telomere length in 2C-like cells, providing functional relevance of DNA demethylation in these cells. Third, our results help clarifying some discrepancies. Eckersley-Maslin et al. showed Dnmt3a downregulation based on IF analysis with a Dnmt3a antibody against the N-
terminal region (Santa Cruz, sc-20703), which would not recognize Dnmt3a2, the predominant isoform in mESCs (Chen et al., 2002). Using an antibody recognizing both Dnmt3a1 and Dnmt3a2, our data indicate that Dnmt3a1, an isoform expressed at low levels in mESCs, is moderately downregulated (consistent with Eckersley-Maslin et al.), whereas Dnmt3a2 and overall Dnmt3a levels are unchanged. Both studies show modest downregulation in Dnmt3b mRNA and protein in 2C-like cells, suggesting inhibition of Dnmt3b transcription. While downregulation of Dnmt3a1 and Dnmt3b may contribute to DNA hypomethylation in 2C-like cells, deficiency in maintenance methylation due to Uhrf1 and Dnmt1 degradation is likely the major cause. In support of this conclusion, we show that co-expression of a Dnmt1-PCNA fusion protein completely prevents global DNA hypomethylation and telomere elongation associated with Zscan4 expression.

2C-like mESCs undergo drastic chromatin remodeling. Global DNA demethylation, which is likely an early event as ESCs transition to the 2C-like state, may contribute to chromatin changes (Akiyama et al., 2015; Ishiuchi et al., 2015). While DNA methylation and repressive histone marks such as H3K9me3 and H4K20me3 usually act cooperatively in maintaining heterochromatin, their roles and crosstalk in telomere regulation are complex and poorly understood. For example, Dnmt1−/− or Dnmt3a3b−/− mESCs show aberrant telomere elongation without affecting H3K9me3 and H4K20me3 density at telomeres (Gonzalo et al., 2006), whereas Dicer−/− mESCs display similar subtelomeric DNA hypomethylation and telomere elongation but increased enrichment of H3K9me3 and K4K20me3 at telomeres (Benetti et al., 2008). Our results indicate that Zscan4 induces DNA demethylation but has little effects on repressive histone marks at telomeres. It is worth noting, however, that Zscan4-overexpressing mESCs likely do not fully recapitulate chromatin changes in 2C-like mESCs.

The mechanisms by which Uhrf1 regulates DNA methylation are complex. On the one hand, Uhrf1 is a positive regulator of DNA methylation by directing Dnmt1 to newly replicated DNA (Bostick et al., 2007; Liu et al., 2013; Nishiyama et al., 2013; Qin et al., 2015; Rothbart et al., 2013; Sharif et al., 2007). On the other hand, Uhrf1, an E3 ubiquitin ligase, can ubiquitinate itself and Dnmt1 and, thus, may function as a negative regulator of DNA methylation (Du et al., 2010; Jenkins et al., 2005). It is unclear how the intricate balance of these seemingly opposing effects is regulated. Our results suggest that molecular and cellular processes that lead to concentration of the Dnmt1-Uhrf1 complex would favor Uhrf1 and Dnmt1 degradation, resulting in passive DNA demethylation.

Our discovery that Zscan4 induces DNA demethylation may be relevant to multiple biological processes. Somatic cell reprogramming to generate induced pluripotent cells (iPSCs) is an inefficient process. DNA methylation is a major barrier for iPSC generation, as DNA demethylation greatly enhances reprogramming efficiency (Costa et al., 2013; Doege et al., 2012; Guo et al., 2013; Huangfu et al., 2008). Interestingly, Zscan4 has also been shown to facilitate somatic cell reprogramming when combined with the Yamanaka factors (Hirata et al., 2012; Jiang et al., 2013), raising the possibility that Zscan4 exerts its effect, at least in part, by inducing DNA demethylation. ESCs and iPSCs can be maintained in a ground or naïve state in the presence of molecules that inhibit ERK and GSK3β signaling (2i inhibitors) (Ying et al., 2008). The 2i inhibitors have been shown to induce global DNA
demethylation by multiple mechanisms, including downregulation of Uhrf1 at the protein level (von Meyenn et al., 2016). It is possible that Zscan4 is involved in Uhrf1 degradation. During preimplantation development, the embryo undergoes global erasure of DNA methylation marks by active and passive demethylation processes. Recently, great progress has been made in elucidating the molecular pathways involved in active demethylation, thanks to the discovery of the Tet family of 5mC dioxygenases (Wu and Zhang, 2014). The mechanisms underlying passive demethylation, due to deficiency in maintenance methylation, remain poorly understood. It will be interesting to determine whether Zscan4, which is highly expressed at the two-cell stage (Falco et al., 2007), plays a role in this process. Notably, two-cell embryos also activate telomere recombination to rapidly extend telomeres (Liu et al., 2007), suggesting that telomere elongation may be linked to Zscan4-mediated DNA demethylation. Finally, cancer cells usually exhibit global DNA hypomethylation and loci-specific hypermethylation. Zscan4 is expressed in some cancer cells (Lee and Gollahon, 2014) and may contribute to DNA hypomethylation. Further work is required to determine the significance of Zscan4 in DNA methylation changes in these biological contexts.

**EXPERIMENTAL PROCEDURES**

Additional experimental procedures are provided in the Supplemental Experimental Procedures.

**Vector Construction**

All plasmid vectors were generated by molecular cloning. The primers and oligonucleotides used are listed in Table S1.

**mESC Culture**

WT (J1) and genetically modified (Flag-Dnmt1 KI/+, Dnmt1+/− and Uhrf1−/−) mESCs were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 15% fetal bovine serum, 0.1 mM nonessential amino acids, 0.1 mM β-mercaptoethanol, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1,000 U/ml leukemia inhibitory factor. The cells were grown on gelatin-coated petri dishes without feeder cells. Normally, culture medium was changed daily, and the cells were passaged every other day.

**Immunofluorescence (IF)**

ESCs grown on gelatin-coated cover slips were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min on ice, permeabilized with 0.1% Triton X-100 in blocking solution (3% goat or donkey serum plus 0.5% BSA in PBS) for 40 min at room temperature, washed three times, and left in blocking solution at room temperature for 1 hr. For 5mC staining, cells were treated with 4N hydrochloric acid (HCl) for 10 min at room temperature after the permeabilization step. Cells were incubated with appropriate primary antibodies overnight at 4°C, followed by appropriate secondary antibodies for 1 hr at room temperature, and then counterstained with Prolong Gold Antifade reagent with 4’,6-diamidino-2-phenylindole (DAPI). Fluorescence was detected and imaged with fluorescence microscopy. The antibodies used are listed in Table S2.
**Co-Immunoprecipitation (Co-IP)**

HEK293 cells or mESCs were co-transfected with epitope-tagged expression plasmids. 48 hr after transfection, cells were harvested with lysis buffer (20 mM Tris-HCl PH 7.9, 25% glycerol, 150mM NaCl, 1.5mM MgCl₂, 0.1% NP-40, 0.2 mM EDTA, 0.5 mM DTT) supplemented with protease inhibitor cocktail (1861279, Thermo Scientific) and phosphatase inhibitor cocktail (78427, Thermo Scientific) and then sonicated for 5 cycles with 5 sec on and 30 sec off at 4°C. After centrifugation at 13,000g for 2 min, the supernatants were used for immunoprecipitation (IP). The cell extracts were incubated with anti-Flag, -Myc or -HA antibody for 2 hr at 4°C, followed by incubation with 25 μl of Protein A/G Ultralink Resin beads (53133, Thermo Scientific) for 1 hr. The beads were washed four times with lysis buffer at 4°C. The cell extracts (input) and IP samples were then subjected to Western blot with anti-Flag, -Myc and/or -HA antibodies for detecting protein-protein interactions. For antibody information, see Table S2.

**In Vivo Ubiquitination Assay**

HEK293 cells were co-transfected with plasmids expressing HA-ubiquitin, Myc-Zscan4c (or the ΔZNF4 mutant or empty Myc vector) and Flag-Uhrf1 (or the H730A mutant). 48 hr after transfection, the cells were treated with 20 μM of MG132 for 2 hr and then harvested. The cell extracts were subjected to IP with anti-Flag antibody, and the IP samples were immunoblotted with anti-ubiquitin antibody. For antibody information, see Table S2.

**Telomere Analyses**

For Q-FISH, cells were incubated with 0.1 μg/ml demecolcine for 4 hr to enrich metaphase cells, then exposed to hypotonic treatment with 75 mM KCl, fixed with methanol:glacial acetic acid (3:1), and spread onto clean slides. Telomere FISH and quantification were performed as described previously (Poon and Lansdorp, 2001). For Flow-FISH, a Telomere PNA Kit/FITC for Flow Cytometry (Dako, K5327) was used following the manufacturer’s protocol. Strand-specific CO-FISH was performed as reported previously (Bailey et al., 2004).

**DNA Methylation Analyses**

Total 5mC levels were measured by dot blot analysis. Briefly, genomic DNA was denatured in 0.4 N NaOH, 10 mM EDTA at 95 °C for 10 minutes and twofold serial dilutions were spotted on positively charged nylon membranes. The membranes were immunooblotted with 5mC antibody, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG. DNA loading was verified by staining with SYTOX Green. Southern blot analysis of DNA methylation at major and minor satellite repeats and the IAP retrotransposons was performed as previously described (Lei et al., 1996; Wang et al., 2009).

**Statistical Analysis**

Statistical comparisons between samples were made using unpaired t-test or one-way ANOVA, and P < 0.05 was considered statistically significant.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank P. Whitney for technical assistance and P. Lansdorp for help with the TFLTELO software. This work is supported by a Rising Star Award (R1108, to T.C.) from the Cancer Prevention and Research Institute of Texas (CPRIT), a grant (1R01AI121403-01A1, to T.C.) from the National Institutes of Health (NIH), and a grant (MOP133449, to C.A.) from Canadian Institute for Health Research (CIHR). J.D. is supported by a scholarship from the Center for Cancer Epigenetics (CCE) at The University of Texas MD Anderson Cancer Center. N.V. is supported by scholarships from CCE and CPRIT (RP140106). T.C. is a CPRIT Scholar in Cancer Research.

References

Akiyama T, Xin L, Oda M, Sharov AA, Amano M, Piao Y, Cadet JS, Dudekula DB, Qian Y, Wang W, et al. Transient bursts of Zscan4 expression are accompanied by the rapid derepression of heterochromatin in mouse embryonic stem cells. DNA Res. 2015; 22:307–318. [PubMed: 26324425]

Amano T, Hirata T, Falco G, Monti M, Sharova LV, Amano M, Sheer S, Hoang HG, Piao Y, Stagg CA, et al. Zscan4 restores the developmental potency of embryonic stem cells. Nat Commun. 2013; 4:1966. [PubMed: 23739662]

Bailey SM, Brenneman MA, Goodwin EH. Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. Nucleic Acids Res. 2004; 32:3743–3751. [PubMed: 15258249]

Benetti R, Gonzalo S, Jaco I, Munoz P, Gonzalez S, Schoeftner S, Murchison E, Andl T, Chen T, Klatt P, et al. A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. Nat Struct Mol Biol. 2008; 15:268–279. [PubMed: 18311151]

Blasco MA. Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet. 2005; 6:611–622. [PubMed: 16136563]

Blasco MA. The epigenetic regulation of mammalian telomeres. Nat Rev Genet. 2007; 8:299–309. [PubMed: 17363977]

Bostick M, Kim JK, Esteve PO, Clark A, Pradhan S, Jacobsen SE. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. Science. 2007; 317:1760–1764. [PubMed: 17673620]

Chen T, Li E. Structure and function of eukaryotic DNA methyltransferases. Curr Top Dev Biol. 2004; 60:55–89. [PubMed: 15094296]

Chen T, Ueda Y, Dodge JE, Wang Z, Li E. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. Mol Cell Biol. 2003; 23:5594–5605. [PubMed: 12897133]

Chen T, Ueda Y, Xie S, Li E. A novel Dnmt3a isoform produced from an alternative promoter localizes to euchromatin and its expression correlates with active de novo methylation. J Biol Chem. 2002; 277:38746–38754. [PubMed: 12138111]

Chuang LS, Ian HI, Koh TW, Ng HH, Xu G, Li BF. Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. Science. 1997; 277:1996–2000. [PubMed: 9302295]

Costa Y, Ding JJ, Theunissen TW, Faiola F, Hore TA, Shliaha PV, Fidalgo M, Saunders A, Lawrence M, Dietmann S, et al. NANOG-dependent function of TET1 and TET2 in establishment of pluripotency. Nature. 2013; 495:370–374. [PubMed: 23395962]

Dan J, Liu Y, Liu N, Chiourea M, Okuka M, Wu T, Ye X, Mou C, Wang L, Wang L, et al. Rif1 maintains telomere length homeostasis of ESCs by mediating heterochromatin silencing. Dev Cell. 2014; 29:7–19. [PubMed: 24735877]

Doege CA, Inoue K, Yamashita T, Rhee DB, Travis S, Fujita R, Guarnieri P, Bhagat G, Vanti WB, Shih A, et al. Early-stage epigenetic modification during somatic cell reprogramming by Parp1 and Tet2. Nature. 2012; 488:652–655. [PubMed: 22902501]
Du Z, Song J, Wang Y, Zhao Y, Guda K, Yang S, Kao HY, Xu Y, Willis J, Markowitz SD, et al. DNMT1 stability is regulated by proteins coordinating deubiquitination and acetylation-driven ubiquitination. Sci Signal. 2010; 3:ra80. [PubMed: 21045206]

Eckersley-Maslin MA, Svensson V, Krueger C, Stubbs TM, Giehr P, Krueger F, Miragaia RJ, Kyriakopoulou C, Berrens RV, Milagre I, et al. MERVL/Zscan4 Network Activation Results in Transient Genome-wide DNA Demethylation of mESCs. Cell Rep. 2016; 17:179–192. [PubMed: 27681430]

Edelstein LC, Collins T. The SCAN domain family of zinc finger transcription factors. Gene. 2005; 359:1–17. [PubMed: 16139965]

Falco G, Lee SL, Stanghellini I, Bassey UC, Hamatani T, Ko MS. Zscan4: a novel gene expressed exclusively in late 2-cell embryos and embryonic stem cells. Dev Biol. 2007; 307:539–550. [PubMed: 17553482]

Fang J, Cheng J, Wang J, Zhang Q, Liu M, Gong R, Wang P, Zhang X, Feng Y, Lan W, et al. Hemimethylated DNA opens a closed conformation of UHRF1 to facilitate its histone recognition. Nat Commun. 2016; 7:11197. [PubMed: 27045799]

Gonzalo S, Jaco I, Fraga MF, Chen T, Li E, Esteller M, Blasco MA. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol. 2006; 8:416–424. [PubMed: 16565708]

Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell. 1985; 43:405–413. [PubMed: 3907856]

Greider CW, Blackburn EH. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature. 1989; 337:331–337. [PubMed: 2463488]

Guo X, Liu Q, Wang G, Zhu S, Gao L, Hong W, Chen Y, Wu M, Liu H, Jiang C, et al. microRNA-29b is a novel mediator of Sox2 function in the regulation of somatic cell reprogramming. Cell Res. 2013; 23:142–156. [PubMed: 23266899]

Hirata T, Amano T, Nakatake Y, Amano M, Piao Y, Hoang HG, Ko MS. Zscan4 transiently reactivates early embryonic genes during the generation of induced pluripotent stem cells. Sci Rep. 2012; 2:208. [PubMed: 22355722]

Huang J, Wang F, Okuka M, Liu N, Ji G, Ye X, Zuo B, Li M, Liang P, Ge WW, et al. Association of telomere length with authentic pluripotency of ES/iPS cells. Cell Res. 2011; 21:779–792. [PubMed: 21283131]

Hung SS, Wong RC, Sharov AA, Nakatake Y, Yu H, Ko MS. Repression of global protein synthesis by Eif1a-like genes that are expressed specifically in the two-cell embryos and the transient Zscan4-positive state of embryonic stem cells. DNA Res. 2013; 20:391–402. [PubMed: 23649898]

Ishii T, Enriquez-Gasca R, Mizutani E, Boskovic A, Ziegler-Birling C, Rodriguez-Terrones D, Wakayama T, Vaquerizas JM, Torres-Padilla ME. Early embryonic-like cells are induced by downregulating replication-dependent chromatin assembly. Nat Struct Mol Biol. 2015; 22:662–671. [PubMed: 26237512]

Jenkins Y, Markovtsov V, Lang W, Sharma P, Pearsall D, Warner J, Franci C, Huang B, Huang J, Yam GC, et al. Critical role of the ubiquitin ligase activity of UHRF1, a nuclear RING finger protein, in tumor cell growth. Mol Biol Cell. 2005; 16:5621–5629. [PubMed: 16195352]

Jiang J, Lv W, Ye X, Wang L, Zhang M, Yang H, Okuka M, Zhou C, Zhang X, Liu L, et al. Zscan4 promotes genomic stability during reprogramming and dramatically improves the quality of iPS cells as demonstrated by tetraploid complementation. Cell Res. 2013; 23:92–106. [PubMed: 23147797]

Lee K, Gollahon LS. Zscan4 interacts directly with human Rap1 in cancer cells regardless of telomerase status. Cancer Biol Ther. 2014; 15:1094–1105. [PubMed: 24840609]

Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R, Li E. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development. 1996; 122:3195–3205. [PubMed: 8898232]

Cell Rep. Author manuscript; available in PMC 2017 September 12.
Liu L, Bailey SM, Okuka M, Munoz P, Li C, Zhou L, Wu C, Czerwiec E, Sandler L, Seyfang A, et al. Telomere lengthening early in development. Nat Cell Biol. 2007; 9:1436–1441. [PubMed: 17982445]

Liu X, Gao Q, Li P, Zhao Q, Zhang J, Li J, Koseki H, Wong J. UHRF1 targets DNMT1 for DNA methylation through cooperative binding of hemi-methylated DNA and methylated H3K9. Nat Commun. 2013; 4:1563. [PubMed: 23463006]

Macfarlan TS, Gifford WD, Agarwal S, Driscoll S, Lettieri K, Wang J, Andrews SE, Franco L, Rosenfeld MG, Ren B, et al. Endogenous retroviruses and neighboring genes are coordinately repressed by LSD1/KDM1A. Genes Dev. 2011; 25:594–607. [PubMed: 21357675]

Macfarlan TS, Gifford WD, Driscoll S, Lettieri K, Rowe HM, Bonanomi D, Firth A, Singer O, Trono D, Pfaff SL. Embryonic stem cell potency fluctuates with endogenous retrovirus activity. Nature. 2012; 487:57–63. [PubMed: 22722858]

Nakai-Futatsugi Y, Niwa H. Zscan4 Is Activated after Telomere Shortening in Mouse Embryonic Stem Cells. Stem Cell Reports. 2016; 6:483–495. [PubMed: 26997646]

Nishiyama A, Yamaguchi L, Sharif J, Johmura Y, Kawamura T, Nakanishi K, Shimamura S, Arita K, Kodama T, Ishikawa F, et al. Uhrf1-dependent H3K23 ubiquitylation couples maintenance DNA methylation and replication. Nature. 2013; 502:249–253. [PubMed: 24013172]

Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999; 99:247–257. [PubMed: 10555141]

Poon SS, Lansdorp PM. Quantitative fluorescence in situ hybridization (Q-FISH). Curr Protoc Cell Biol Chapter. 2001; 18(Unit 18):14.

Qin W, Wolf P, Liu N, Link S, Smets M, La Mastra F, Forne I, Pichler G, Horl D, Fellinger K, et al. DNA methylation requires a DNMT1 ubiquitin interacting motif (UIM) and histone ubiquitination. Cell Res. 2015; 25:911–929. [PubMed: 26065575]

Rothbart SB, Dickson BM, Ong MS, Krajewski K, Houliston S, Kireev DB, Arrowsmith CH, Strahl BD. Multivalent histone engagement by the linked tandem Tudor and PHD domains of UHRF1 is required for the epigenetic inheritance of DNA methylation. Genes Dev. 2013; 27:1288–1298. [PubMed: 23752590]

Sharif J, Muto M, Takebayashi S, Suetake I, Iwamatsu A, Endo TA, Shinga J, Mizutani-Koseki Y, Toyoda T, Okamura K, et al. The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. Nature. 2007; 450:908–912. [PubMed: 17994007]

von Meyenn F, Iurlaro M, Habibi E, Liu NQ, Salehzadeh-Yazdi A, Santos F, Petrinie E, Milagre I, Yu M, Xie Z, et al. Impairment of DNA Methylation Maintenance Is the Main Cause of Global Demethylation in Naive Embryonic Stem Cells. Mol Cell. 2016; 62:848–861. [PubMed: 27237052]

Wang J, Hevi S, Kurash JK, Lei H, Gay F, Bajko J, Su H, Sun W, Chang H, Xu G, et al. The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. Nat Genet. 2009; 41:125–129. [PubMed: 19098913]

Wu H, Zhang Y. Reversing DNA methylation: mechanisms, genomics, and biological functions. Cell. 2014; 156:45–68. [PubMed: 24439369]

Ying QL, Wray J, Nichols J, Battle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. The ground state of embryonic stem cell self-renewal. Nature. 2008; 453:519–523. [PubMed: 18497825]

Zalzman M, Falco G, Sharova LV, Nishiyama A, Thomas M, Lee SL, Stagg CA, Hoang HG, Yang HT, Indig FE, et al. Zscan4 regulates telomere elongation and genomic stability in ES cells. Nature. 2010; 464:858–863. [PubMed: 20336070]
Figure 1. 2C-Like mESCs Show Global DNA Hypomethylation Due to Uhrf1 and Dnmt1 Degradation

(A,B) IF analysis of stable mESC clones transfected with the Zscan4-GFP reporter, which shows that GFP+ cells (indicated by arrowheads) are severely hypomethylated. Representative images (A) and quantification of the data (B) are shown. Scale bar in (A), 10 μm. The total numbers of cells counted in (B) are indicated, and the results are shown as percentages.

(C) Dot blot analysis of DNA from FACS-sorted GFP+ and GFP- populations, which confirms global hypomethylation in GFP+ cells. The same membrane was stained with SYTOX Green to verify equal DNA loading.

(D) Quantification of the data in (C) by measuring the signal intensities. Presented are relative 5mC levels (mean ± s.d.). **** P<0.001.

(E) Southern blot analysis of the major and minor satellite repeats after digestion of genomic DNA with methylation-sensitive restriction enzymes (MaeII for major satellite; HpaII for minor satellite), which shows DNA hypomethylation in GFP+ cells.

(F) Western blot analysis of FACS-sorted GFP+ and GFP- cells, which shows severe downregulation of Uhrf1 and Dnmt1 in the GFP+ population.

(G,H) IF analysis of stable mESC clones transfected with the Zscan4-GFP reporter, which confirms that Uhrf1 and Dnmt1 levels are extremely low in GFP+ cells (G) but largely maintained with MG132 treatment (H) (10 μM MG132, 13 hr for Uhrf1 detection, and 6 hr for Dnmt1 detection).

(I) Quantification of the data in (G,H). The total numbers of cells counted are indicated, and the results are shown as percentages.
(J) Western blot analysis of GFP⁺ and GFP⁻ cells sorted after 12 hr of treatment with MG132 or DMSO, which shows that MG132 prevents Dnmt1 and Uhrf1 degradation in GFP⁺ cells.
See also Figures S1-S3.
Figure 2. Zscan4 Overexpression Leads to Uhrf1 and Dnmt1 Downregulation and DNA Demethylation

(A) Western blot analysis of stable clones expressing Myc-Zscan4c in Flag-Dnmt1 KI/+ mESCs, which show severe downregulation of Uhrf1 and Dnmt1 (examined by both Dnmt1 and Flag antibodies). Mock, stable Flag-Dnmt1 KI/+ mESC clones transfected with empty Myc vector.

(B) Dot blot analysis confirming global DNA hypomethylation in cells overexpressing Myc-Zscan4c.

(C) IF analysis of co-cultured Myc-Zscan4c+ cells (indicated by arrowheads) and Mock (Myc-) cells, which shows severely lower levels of Uhrf1, Flag-Dnmt1, and 5mC in Myc-Zscan4c+ cells. Scale bars, 10 μm.

(D) Western blot analysis showing that Zscan4 shRNA (shZscan4) efficiently depletes Myc-Zscan4c in an overexpression clone and, as a consequence, Uhrf1 and Dnmt1 levels are greatly restored. Untrans, untransfected Flag-Dnmt1 KI/+ mESCs; shControl, control shRNA.

(E) Dot blot analysis showing the restoration of 5mC levels in shZscan4-mediated knockdown clones.

See also Figure S4.
Figure 3. Zscan4 Depletion Prevents Uhrf1 and Dnmt1 Downregulation and DNA Demethylation in 2C-Like mESCs

(A,B) Western blot and IF analyses of Zscan4-GFP reporter mESC lines stably transfected with Zscan4 shRNA (shZscan4) or control shRNA (shControl), which show that shZscan4 efficiently depletes endogenous Zscan4. Two shControl and four shZscan4 clones are shown in (A).

(C-E) DNA methylation analyses by dot blot (C), Southern blot (D), and IF (E) showing that depletion of endogenous Zscan4 prevents DNA hypomethylation in GFP+ (2C-like) cells.

(F,G) IF analysis showing that Zscan4 depletion prevents downregulation of Uhrf1 (F) and Dnmt1 (G) in GFP+ (2C-like) cells (indicated by arrowheads). Scale bars, 10 μm.
Figure 4. Blocking DNA Demethylation Prevents Zscan4-Mediated Telomere Elongation

(A) Western blot analysis of stable mESC clones expressing Myc-Zscan4c alone or both Myc-Zscan4c and Flag-Dnmt1-PCNA, which shows the expression of the transfected proteins. Mock, stable mESC clones transfected with empty Myc vector.

(B) Dot blot analysis showing that hypomethylation associated with Myc-Zscan4c expression is blocked with co-expression of Flag-Dnmt1-PCNA. The same membrane was stained with SYTOX Green to verify equal DNA loading.
Telomere length analyses by Q-FISH (C) and Flow-FISH (D) showing that telomere elongation induced by Myc-Zscan4c is prevented with co-expression of Flag-Dnmt1-PCNA. The relative telomere length (mean ± s.d.) for each sample is indicated. Green lines in (C) indicate median relative telomere length. TFU, telomere fluorescence units.

CO–FISH analysis after labeling leading strand telomeres showing increased TSCE frequency with Zscan4c expression, which is prevented with co-expression of the Dnmt1-PCNA fusion protein. Chromosomes were stained with DAPI (blue), and telomeres were marked with a Cy3-conjugated telomere probe (red). In the absence of recombination between telomeric repeats, only one telomere at each chromosome end is labeled. A T-SCE event gives rise to three telomeric signals per chromosome (indicated by arrowheads). Representative CO-FISH images (E) and quantification (mean ± s.d.) of T-SCE frequency in different groups (F) are shown. See also Figures S5 and S6.
Figure 5. Zscan4 Recruits the Dnmt1-Uhrf1 Complex by Directly Interacting with Uhrf1
(A) Co-IP experiments with Myc- and Flag-tagged proteins expressed in HEK293 cells, which demonstrate that Zscan4c interacts with both Dnmt1 and Uhrf1.
(B) Co-IP experiments with Myc-Zscan4c and Flag-Uhrf1 expressed in wild-type (WT) or Dnmt1⁻/⁻ mESCs, which show that Dnmt1 deficiency does not affect Zscan4c-Uhrf1 interaction.
(C) Co-IP experiments with Flag-Zscan4c and Myc-Dnmt1 expressed in WT or Uhrf1⁻/⁻ mESCs, which show that Uhrf1 is required for Zscan4c-Dnmt1 interaction.
(D) Mutagenesis and co-IP experiments showing that deletion of the Zscan4c ZNF domain (∆ZNF1-4) or the ZNF4 motif (∆ZNF4) abolishes its interaction with Uhrf1.

(E) GST pull down assay showing that GST-Zscan4c, but not GST-∆ZNF1-4 or GST-∆ZNF4, interacts with His-Uhrf1. Shown below is a gel of the purified GST- Zscan4c fusion proteins stained by Coomassie Brilliant Blue.

(F) Co-IP experiments with Myc- and HA-tagged Zscan4c proteins showing that Zscan4c self-associates and that deletion of the SCAN domain abolishes selfassociation. See also Figure S7.
Figure 6. Both Uhrf1-Binding and Self-Association abilities of Zscan4 Are Required for Its Effects on DNA Methylation and Telomere Length

(A,B) Western blot and dot blot analyses of mESC stable clones expressing Myc-Zscan4c or deletion mutants, which demonstrate that the ΔZNF4, ΔZNF1-4, and ΔSCAN mutants fail to induce Uhrf1 and Dnmt1 downregulation (A) and hypomethylation (B). Mock, mESC stable clones transfected with empty Myc vector.
(C,D) Telomere length analyses by Q-FISH (C) and Flow-FISH (D) showing that WT Zscan4c, but not the ΔZNF4 and ΔSCAN mutants, induces telomere elongation. The relative telomere length (mean ± s.d.) for each sample is indicated.

(E,F) CO-FISH analysis showing that WT Zscan4c, but not the ΔZNF4 and ΔSCAN mutants, leads to increased T-SCE events (indicated by arrowheads) compared to Mock control cells. Representative CO–FISH images (E) and quantification (mean + s.d.) of TSCE frequencies (F) in different groups are shown.
Figure 7. Zscan4 Enhances Uhrf1 Self-Ubiquitination and Degradation

(A) Western blot analysis of Dnmt1 and Uhrf1 in mESC stable clones expressing Myc-Zscan4c or mutants (ΔZNF4 or ΔSCAN) at different time points after blocking protein synthesis with 100 μg/ml cycloheximide (CHX) to determine Dnmt1 and Uhrf1 stability.

(B) Quantification of data in (A). The intensities of the Uhrf1 and Dnmt1 bands were measured and normalized to those of β-actin. Shown are changes in Uhrf1 and Dnmt1 levels over time relative to their levels before CHX treatment.
(C) The half-lives of Uhrf1 and Dnmt1 proteins (mean ± s.d.) in mESCs expressing WT or mutant Zscan4c. ** p<0.01; *** p<0.001.

(D) Ubiquitination assay showing that Myc-Zscan4c, but not the ΔSCAN or ΔZNF4 mutant, enhances ubiquitination of Flag-tagged WT Uhrf1, but not the E3 ligase-inactive mutant (H730A).

(E,F) IF analysis of Uhrf1−/− mESC stable clones expressing Flag-tagged WT Uhrf1 or the E3 ligase-inactive mutants, which shows downregulation of Flag-Uhrf1, but not the E3 ligase-inactive mutants, in Zscan4+/2C-like cells. Representative images (E) and quantification of the data (F) are shown.

(G,H) IF analysis of Uhrf1−/− mESCs or Dnmt1−/− mESCs (transfected with the Zscan4-GFP reporter) showing that Uhrf1 deficiency prevents Dnmt1 downregulation and Dnmt1 deficiency has no effect on Uhrf1 downregulation in Zscan4+/2C-like cells. Representative images (G) and quantification of the data (H) are shown.

(I) Proposed mechanism by which Zscan4 induces global DNA demethylation and telomere elongation. As mESCs proliferate, they undergo telomere shortening, which ultimately triggers the transition to the 2C-like totipotent state, resulting in a burst of Zscan4 expression. Zscan4, via its ZNF4 motif, recruits the Uhrf1-Dnmt1 complex and, through self-association mediated by its SCAN domain, brings two or more Uhrf1-Dnmt1 heterodimers to close proximity, facilitating Uhrf1-mediated ubiquitination of Uhrf1 itself and Dnmt1, likely through cross actions between Uhrf1 molecules. Subsequent Uhrf1 and Dnmt1 degradation leads to global DNA demethylation, which facilitates telomere recombination and elongation. After telomere lengths are recovered, the cells transition back to the pluripotent state.