Zscan4 Is Activated after Telomere Shortening in Mouse Embryonic Stem Cells

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SUMMARY

ZSCAN4 is a DNA-binding protein that functions for telomere elongation and genomic stability. In vivo, it is specifically expressed at the two-cell stage during mouse development. In vitro, it is transiently expressed in mouse embryonic stem cells (ESCs), only in 5% of the population at one time. Here we attempted to elucidate when, under what circumstances, Zscan4 is activated in ESCs. Using live cell imaging, we monitored the activity of Zscan4 together with the pluripotency marker Rex1. The lengths of the cell cycles in ESCs were diverse. Longer cell cycles were accompanied by shorter telomeres and higher activation of Zscan4. Since activation of Zscan4 is involved in telomere elongation, we speculate that the extended cell cycles accompanied by Zscan4 activation reflect the time for telomere recovery. Rex1 and Zscan4 did not show any correlation. Taken together, we propose that Zscan4 is activated to recover shortened telomeres during extended cell cycles, irrespective of the pluripotent status.

INTRODUCTION

Zinc finger and SCAN domain containing 4 (ZSCAN4) is a DNA-binding protein that is specifically expressed in two-cell stage embryos during mouse development (Falco et al., 2007). In vitro, interestingly, Zscan4 is transiently expressed in a minor population of embryonic stem cells (ESCs) at one time (Carter et al., 2008) but is eventually expressed in all (Zalzman et al., 2010). It functions for telomere elongation and genomic stability (Zalzman et al., 2010) and thus is considered as a rejuvenation factor.

ESCs are a heterogeneous population. If cultured in conventional serum-containing medium supplemented with leukemia inhibitory factor (LIF), they remain undifferentiated but closer studies show they are actually a mixture of cells with higher and lower potential of differentiation (reviewed in Nakai-Futatsugi and Niwa, 2013). Recently even a minor population of two-cell-stage-like ESCs that are not only pluripotent but also capable of differentiating into extra-embryonic lineages was found in the heterogeneous ESC population (Maclaran et al., 2012). The heterogeneity of ESCs is accompanied by fluctuation of the expression of pluripotency-associated genes such as Rex1 (also known as Zfp42) (Toyooka et al., 2008), Nanog (Chambers et al., 2007; Singh et al., 2007), Klf4 (Niwa et al., 2009), Tbx3 (Niwa et al., 2009), Stella (Hayashi et al., 2008), and so on. However, among the pluripotency-associated genes, Oct3/4 (also known as Pou5f1), whose expression does not fluctuate, is an exception. It is the master gene of pluripotency (Nichols et al., 1998). A constant expression level of Oct3/4 is crucial for the maintenance of pluripotency, as a slight increase leads to differentiation into primitive endoderm and mesoderm while a slight decrease leads to differentiation into trophoderm (Niwa et al., 2000). The expression level of Oct3/4 is maintained at a constant level downstream of a robust transcription factor network in mouse ESCs (Niwa et al., 2009). Rex1, although not essential for ESC self-renewal and pluripotency (Masui et al., 2008), decreases in its mRNA-expression level when the master gene Oct3/4 is either hyper-expressed or hypo-expressed (Niwa et al., 2000). Thus we consider the promoter activity of Rex1, which is high only when the expression of Oct3/4 is maintained at an optimal range, as a good indicator of pluripotency.

To elucidate whether the expression pattern of Zscan4 has any correlation with ESC proliferation, we monitored Zscan4 activity at single cell level. Also to see whether the rejuvenation factor Zscan4 correlates with the fluctuating wave of ESC pluripotency (Figure S1), we monitored Zscan4 and the pluripotency indicator Rex1 simultaneously under live cell imaging. Unexpectedly, we did not see any correlation between the two factors. Instead, we found Zscan4 is activated when the cell-cycle lengths become long, irrespective of the pluripotent status, presumably sensing shortened telomeres.

RESULTS

Cell-Cycle Length of Mouse ESCs Is Diverse

First, we analyzed the proliferation profile of ESCs at the single cell level. ESCs were stably transfected with Fucci vector
(Sakaue-Sawano et al., 2008), which expresses fluorescence Kusabira orange at the G1 phase and fluorescence Azami green at the S/G2/M-phase. They were monitored under the microscope for up to 5 days in conventional medium that contains fetal calf serum (FCS) supplemented with leukemia inhibitory factor (LIF) (FCS/LIF medium). Images were taken every 15 min. After the images were taken, each cell was tracked manually and the data were converted into lineage trees using a handmade program (source code provided in Data S1). Figure 1A shows examples of the lineage trees, in which each vertical line shows the fate of each cell, plotted for every time point with the intensities of Kusabira orange and Azami green converted into 256 intensity scale of red and green, respectively. Horizontal lines indicate cell division. Cells were sequentially numbered in the order they emerged (small black numbers). The timescale is on the left of the lineage tree. Green numbers indicate the cell-cycle length (hr).

Although previous studies have suggested the cell-cycle length of mouse ESCs should be around 10–14 hr (Pauklin et al., 2011), under our conditions, the length of the cell cycle was more diverse than expected; it varied from less than 10 hr to more than 20 hr (Figures 1A, 2C, and S5, green numbers; see also Figure 1B). Interestingly, the cell cycles of the sister cells were similar (Figures 1A, 2C, and S5, compare green numbers between sisters), probably because the cell components including the cell-cycle determinants were divided evenly between the daughter cells. When the difference in the cell-cycle length between mother and daughter, and between sisters were quantified, sister cells showed a significantly smaller difference (Figure 1C).

The G1 phase in ESCs is characteristically short. Typically the G1 phase occupies only 20% of the total cell-cycle length in ESCs, while in somatic cells it is more than 50% (White and Dalton, 2005). Our Fucci-based live imaging confirmed that cells at longer cell cycles still had the typical ESC-like...
ratio of the G1 phase (Figure 1D, right panel). At the same time, this meant that not only the G1 phase but also the actual duration of the S/G2/M phase was elongated in ESCs when the cell cycles became longer (Figure 1D, left panel).

**Monitoring Zscan4 Promoter Activity**

We monitored the promoter activities of Zscan4 and the pluripotency marker Rex1 under our live imaging system. For monitoring of Rex1 promoter activity, chemiluminescence Luciferase2 was knocked in downstream of the Rex1 promoter (Figure S2A). We used Luciferase2, which has a short half-life, for quasi real-time imaging of Rex1 promoter activity (Figure S2). With this probe, the fluctuation of Rex1 promoter activity was clearly visualized (Figure S2E) and the cycle of fluctuation was estimated to be approximately 7 days (Figure S3A). Rex1 promoter activity showed negative correlation with the cell-cycle length (Figure S3B; also discussed in Figure 7A).

For monitoring of Zscan4 promoter activity, ESCs were stably transfected with a set of transgenes that express enhanced green fluorescent protein (EGFP) downstream of Zscan4 promoter (Figure 2A). Images were taken every 1 hr. Figure 2B shows an example of the live imaging data starting from two cells (#1 and #2). Marks of manual tracking are shown by pink circles. Figure 2C shows the lineage trees of cells #1 and #2 in Figure 2B. Each vertical line shows the fate of each cell plotted for every time point with the intensities of Luciferase2 (for Rex1) and GFP (for Zscan4) converted into a 256 intensity scale of gray and green, respectively. As shown in Figure 2C, cells with strong GFP
intensities were prone to die (red circles), which was distinguished from autofluorescence of dying cells as it was not detected through an red fluorescent protein filter. This was consistent with our previous study using a Zscan4-mCherry reporter showing that cells with hyperactive Zscan4 had higher incidence of cell death (Fujii et al., 2015). With our previous version of the Zscan4 probe that coded fluorescence mCherry directly downstream of the Zscan4 promoter, most likely only the hyperactive Zscan4 that led to cell death was detectable. Thus, in order to elucidate the function of Zscan4, we thought that detecting much weaker Zscan4 activity might be required. trans-Activator systems are generally used for signal amplification (Iyer et al., 2001) and our preliminary study showed trans-activation by Gal4-UAS amplifies the signal by 10–100 times (H.N., unpublished data). So we applied the Gal4-UAS-trans-activator system to amplify small Zscan4 signals into large expression levels of the EGFP reporter (Figure 2A). The signal amplification by the Gal4-UAS-trans-activator system was indeed efficient as the population with a weak Zscan4-GFP signal that was not detected in previous studies emerged by fluorescence-activated cell sorting (FACS) analysis (Figure S4A). This enabled the visualization of very weak signals as shown in Figure 2C (red arrows).

While Luciferase2 was efficient for monitoring the kinetics of the Rex1 promoter activity due to its short half-life (Figure S2), the intensity of EGFP itself does not directly indicate promoter activity of Zscan4 because the half-life of EGFP is rather long (Figure S2B) so may not degrade even after the promoter is “off” (Figure 3A, “not active”). To interpret the kinetics of the Zscan4 promoter activity from the EGFP intensity, we used the increment of the EGFP signal during one time point (Figure 3A, delta; see also Figure S4B) as a variable that represents the promoter activity at longer cell cycles (Figures 4A and 4B) and the activation of Zscan4 at longer cell cycles may indicate activation of Zscan4 in response to telomere shortening, which extends the cell-cycle length during repair of the telomere, presumably at the G2/M phase.

Zscan4 Activity Is Followed by Shortening of the Cell-Cycle Length in the Next Generation

If Zscan4 is activated for repair by sensing shortened telomeres, the upregulation of Zscan4 should be followed by shortening of the cell cycle after the recovery of the telomere. Thus we analyzed the correlation between the Zscan4 promoter activity and the change in the cell-cycle length in the next generation. For this we quantified the total activity of Zscan4 during one cell cycle, and plotted it against the difference between the length of that cell cycle and the next cell cycle (which becomes negative when the cell cycle shortens). As shown in Figure 4C, when the cell cycle was shortened, it was preceded by a significantly high activity of Zscan4. The same tendency was also shown in 2i/LIF culture conditions (Figure 4D).

Telomere Shortening Accompanies Cell-Cycle Elongation

As shown in Figure 2C (see also Figures 1A and S5), the cell-cycle length of ESCs fluctuates. It is noteworthy that the cell cycles not only elongate but also shrink. As Zscan4 was active at longer cell cycles (Figures 4A and 4B) and the activation of Zscan4 led to shrinkage of the length of the next cell cycle (Figures 4C and 4D), next we attempted to measure the telomere length in cohorts of ESCs that had short, medium, or longer cell cycles. ESCs that were labeled with carboxyfluorescein succinimidyl ester (CISE) dye were cultured for 48 hr and collected according to the dilution of the dye (Figure S5, upper panel). We speculate that when
the telomere becomes short, time for telomere recovery presumably at the G2/M phase is required, and as a consequence the cell-cycle length becomes long. A standard method for telomere length measurement that applies quantitative fluorescent in situ hybridization (qFISH) (Lansdorp et al., 1996) only allows measurement of cells at the M phase, which may count only the cells after recovery of the telomeres, especially for longer cell cycles. So we applied a method using flow cytometry (flow-FISH) (Rufer et al., 1998; Baerlocher et al., 2006) and a method using quantitative PCR (qPCR) for telomere measurement (Cällcott and Womack, 2006), which are both advantageous as a large number of unbiased cells, not only at the M phase, can be measured. Of course telomere shortening is not the only cause of cell-cycle elongation (this is discussed later in Figure 7). Thus ESCs that had longer cell cycles during the last 48 hr should be a mixture of several conditions. Nevertheless, the cohort of ESCs that had longer cell cycles had significantly short telomeres (Figure 5, see also Figure S6A). This again supports the idea that the activation of \textit{Zscan4} at longer cell cycles might be a consequence of sensing telomere shortening.

ESCs with Constitutive Expression of \textit{Zscan4} Have Stable Cell-Cycle Length

We generated ESC lines that stably express exogenous \textit{Zscan4}c (Z4ex-ESC) (Figure S7, Z4ex). When the telomere length was measured by flow-FISH (Figure 6A), qFISH (Figure S6B), and qPCR (Figure S6C) Z4ex-ESCs had longer telomeres, consistent with the pioneer study (Zalzman et al., 2016).
demonstrating the function of Zscan4 in telomere elongation. With these Z4ex-ESCs, we analyzed whether the expression of Zscan4 affects the cell-cycle length. Examples of the lineage trees of Z4ex-ESCs are shown in Figure 6B. In Z4ex-ESCs, the majority of the cell-cycle length remained within 15 hr (Figure 6B; see also Figure 6D, Z4ex), which was relatively short compared with wild-type ESCs (Figure 1B). Probably in Z4ex-ESCs with sufficient telomere length (Figures 6A, S6B, and S6C), time for telomere recovery is not required, and thus the cell-cycle lengths remain short.

**Zscan4 Is Required for Survival of ESCs with Longer Cell Cycles**

As the activation of Zscan4 was followed by shortening of the cell-cycle length (Figures 4C and 4D), next we analyzed whether the downregulation of Zscan4 affects the recovery from elongated cell cycle. We knocked down Zscan4 by shRNA (Z4sh-ESC) (Figure S7, Z4sh). Although we used the same shRNA sequence as in a previous study (Zalzman et al., 2010), our knockdown was less effective (83% and 96% of reduction, respectively). This was probably because, while we used inducible shRNA, Zalzman et al. (2010) stably expressed the shRNA and overexpressed exogenous Zscan4 during the establishment of knockdown, which should give more efficient knockdown by continuous expression of shRNA (Figure S7).

Thus unlike the Zscan4 knocked down ESCs of Zalzman et al. (2010) that ceased to proliferate after eight passages, our Z4sh-ESCs slowly proliferated without crisis for at least 19 passages. Figure 6C shows examples of the lineage trees of Z4sh-ESCs. Z4sh-ESCs showed higher incidence of cell death (Figure 6C). The distribution of overall cell cycle length in Z4sh-ESCs did not alter much from wild-type ESCs (Figure 6D compared with Figure 1B). This suggests that the majority of the cells with presumably sufficient length of telomeres were not affected by the downregulation of Zscan4, which is reasonable considering the transient activation of Zscan4 in ESCs. Our results so far suggest that Zscan4 is activated by sensing shortened telomeres. Probably during the repair of the telomeres, the cell cycle lengthens followed by shortening of the cell cycle after recovery. If this is the case, the question is whether Z4sh-ESCs could survive after elongated cell cycles without sufficient activation of Zscan4. The gray portion of the histograms in Figure 6D indicates the number of the cells whose daughter cells could not proliferate. Z4sh-ESCs became less proliferative (died or did not divide for more than 30 hr) after longer cell cycles. For quantification, the ratio of the surviving cells (Figures 6D and 488 Stem Cell Reports | Vol. 6 | 483–495 | April 12, 2016 | ©2016 The Authors

**Figure 4. Zscan4 Activity Correlates with the Cell-Cycle Length**

(A and B) Zscan4 is activated when the cell-cycle length is long. Zscan4 promoter activity represented by the delta of GFP intensity in the lineage trees was analyzed against the cell-cycle length in FCS/LIF culture conditions (A; n = 386 cell divisions in eight lineages from two independent experiments) or in 2i/LIF culture conditions (B; n = 283 cell divisions in four lineages from two independent experiments). Zscan4 activity became significantly high when the cell-cycle length became long.

(C and D) Expression of Zscan4 leads to shortening of the cell cycle at the next cell division. Total Zscan4 activity during a given cell cycle was analyzed against the difference between the lengths of that cell cycle and the next cell cycle in FCS/LIF culture conditions (C; n = 276 cell divisions in eight lineages from two independent experiments) or in 2i/LIF culture conditions (D; n = 255 cell divisions in four lineages from two independent experiments). Negative x axis means the cell cycle shortened. Bar graph represents the average with error bars of the SD. Student’s t test was used for statistical analysis. *p < 0.05, **p < 0.005, ***p < 0.001.
1B, black portion versus total) was calculated (Figure 6E), which indicated that knockdown of Zscan4 significantly reduced the number of the cells that could survive or recover from longer cell cycles. By qPCR, which is less sensitive for telomere measurement compared with flow-FISH or qFISH (Gutierrez-Rodrigues et al., 2014), the average telomere length in Z4sh-ESCs was comparable with wild-type ESCs (Figure S6C). This could be because cells with extremely short telomeres, which are a minor population that are supposed to be rejuvenated by the transient activation of Zscan4, are prone to die in Z4sh-ESCs without the benefit of telomere elongation (Figure 6E) and thus were excluded from the count, while the majority of the population, in which Zscan4 is not activated anyway, was dominantly counted for the assay. Indeed when the lengths of the telomeres in individual cells were measured by flow-FISH, the population with normal telomere length was not so much affected by the knockdown of Zscan4. Instead, there was an increase in the population with extremely short telomeres in the knocked down cells (Figure 6A, 1.2% and 4.2% in Wt and Z4sh, respectively), which was reflected in the average telomere length. Taken together, we speculate that Zscan4 is activated in response to telomere shortening, which may lead to genomic instability, and without sufficient expression of Zscan4, ESCs cannot divide.

In summary, we interpret correlation of Zscan4 activity with cell-cycle length as follows: (1) the telomere becomes short after each cell division due to end replication problems; (2) then Zscan4 becomes active sensing shortened telomeres; (3) activation of Zscan4 elongates the telomeres, and meanwhile the cell cycle lengthens; (4) after the repair of the telomere, the cell cycle shortens and Zscan4 becomes silent in the next cell cycle (Figure 7B).

DISCUSSION

In this study, we showed that Zscan4 is activated independent of the pluripotent status represented by Rex1 activity. On the other hand, increasing evidence from recent studies suggests involvement of the maintenance of telomere length in pluripotency (Hoffmeyer et al., 2012; Huang et al., 2011; Pucci et al., 2013; Wong et al., 2010). It is also shown that exogenous expression of Zscan4 gives more stable pluripotency during embryogenesis (Amano et al., 2013) and also enhances the efficiency of iPSC generation (Hirata et al., 2012). If Zscan4 is irrelevant to pluripotency, under what mechanisms can these be explained? As described by Zalzman et al. (2010), Zscan4 contributes not only to telomere elongation but also to genomic stability. We interpret the contribution of Zscan4 to more stable chimeric contribution (Amano et al., 2013) or higher efficiency of iPSC generation (Hirata et al., 2012) as the consequence of more stable proliferation of pluripotent ESCs owing to the activation of Zscan4, as shown by the short and stable cell cycles of Z4ex-ESCs in our experiments. Reversely, the low chimeric contribution by telomerase-deficient ESCs (Huang et al., 2011) may be a consequence of higher incidence of cell death in the short telomere ESCs as shown in our Z4sh-ESCs.

If the function of Zscan4 is to elongate the telomeres, why is it co-expressed with telomerase? At the time of cell division, cells are exposed not only to telomere shortening...
attributed to the end replication problem but also to DNA replication stress by which a DNA damage response is activated (Mazouzi et al., 2014). Also, telomere shortening beyond the threshold can activate a DNA damage response (Blackburn, 2001). It has been shown that telomere recovery and DNA repair share common mechanisms (Maser and DePinho, 2004; Doksani and de Lange, 2014). Zscan4 may contribute to the repair of largely damaged telomeres caused by DNA replication stress, which may be the case homologous recombination is more efficient than telomerase-mediated telomere synthesis. This may also explain the characteristically transient and population restricted activation of Zscan4. Indeed, artificial DNA damage induced by reagents such as zeocin, cisplatin (Storm et al., 2014), and doxorubicin (Y.N.-F. and H.N., unpublished data) led to strong activation of Zscan4. If Zscan4 is upregulated in response to DNA damage, this may also explain the expression of Zscan4 at longer cell cycles as generally DNA damage response leads to cell-cycle elongation to repair the damaged DNA after corresponding checkpoints (Harper and Elledge, 2007). In this study, we showed that longer cell cycles in ESCs were accompanied by a

Figure 6. Expression Levels of Zscan4 Affects the Cell-Cycle Length
(A) Telomere lengths of Z4ex-, wild-type or Z4sh-ESCs were measured by flow-FISH using an FITC-conjugated telomere probe. The average telomere intensity (×1,000) per cell ±SD is indicated. n > 7,000 cells. Two technical replicates showed the same tendency. Note that cells with longer telomeres were increased in Z4ex-ESCs compared with wild-type ESCs (11.4% vs 2.3%) and cells with shorter telomeres were increased in Z4sh-ESCs compared with wild-type ESCs (4.2% vs 1.2%). See also Figures S6B and S6C.

(B and C) Examples of the lineage trees of Z4ex-ESCs (B) and Z4sh-ESCs (C) with Rex1-Luciferase and Zscan4-Gal4-UAS-EGFP probes. The green scale on the right side of each lineage indicates the intensities of the Zscan4-Gal4-UAS-EGFP. Note that Z4ex-ESCs showed stable cell cycles without strong Zscan4 activities (B), while Z4sh-ESCs showed a longer cell cycle with high basal EGFP expression (C).

(D) Histograms of the cell-cycle length of Z4ex- and Z4sh-ESCs (n = 194 and 289 cell divisions in three and 11 lineages from two independent experiments, respectively). Z4ex-ESCs increased in cells with shorter cell cycles. Black, the cells whose daughter cells divided within 30 hr; gray, the cells whose daughter cells died or did not divide within 30 hr.

(E) Equivalent of the histograms of the wild-type and Z4sh-ESCs from Figures 1B and (D), respectively, highlighting the difference in the survival rate of the daughter cells, i.e., black portion divided by black + gray. Student’s t test was used for statistical analysis. *p < 0.01, **p < 0.005. See also Figure S7.
Figure 7. *Rex1* and Zscan4 Correlate with the Cell-Cycle Length Under Different Mechanisms

(A) ESCs are continuously exposed to differentiation factors. For example, a very general growth factor, fibroblast growth factor (FGF), is always around ESCs as it is secreted by ESCs themselves. Downstream of FGF, MAPK is activated, which suppresses pluripotent factors such as *Tbx3* and *Nanog* (Niwa et al., 2009). But ESCs form a robust transcription factor (TF) network that gradually adjusts the pluripotent status when the balance among the TFs becomes chaotic (Niwa et al., 2009). Since the battle between differentiation and pluripotency occurs at the transcription level, the conditions at the G1 phase should be the platform (Pauklin and Vallier, 2013). Thus, when the G1 phase remains in a typical ESC-like proportion (Figure 1D), and both the cell-cycle length (Figures 1A, 2C, and S5) and *Rex1* expression (Figure S3A) are reversible. In conventional culture (i.e., in serum-containing medium supplemented with LIF), ESCs are probably fluctuating between the two statuses illustrated on the left and right in (A). At longer cell cycles, the chances of prolonged S/G2/M phase, and our previous study showed that activation of *Zscan4* induces arrest at the G2/M phase (Fujii et al., 2015), which is the phase for DNA repair. Also a recent study showed that activation of *Zscan4* induces heterochromatin decondensation, which permits DNA repair (Akiyama et al., 2015). If *Zscan4* is activated in response to DNA damage, this could also underlie the cell death observed in ESCs with hyperactive *Zscan4* that was shown in our live imaging, as these ESCs may have unbearable levels of DNA damage, by which they were eliminated.

Our previous study identified *Dax1* as a suppressor of *Zscan4* (Fujii et al., 2015). In *Dax1*-null ESCs, *Zscan4* was hyperactive and had a higher incidence of cell death, which was suppressed by the restoration of *Dax1*. This clearly indicates the significance of *Zscan4* suppression in ESC survival, which suggests that *Zscan4* can be a cause of cell death. Probably in *Dax1*-null ESCs, *Zscan4* was more sensitive to telomere shortening or DNA damage due to the lack of suppression by *Dax1*, and thus became hyperactive. Generally, too strong DNA damage response results in apoptosis (Harper and Elledge, 2007). Similarly, we speculate that hyperactive *Zscan4* activates signal cascades leading to cell death. In other words, at normal expression levels, *Zscan4* functions for telomere elongation and genomic stability, however when it is hyper-expressed, *Zscan4* can be a cause of cell death.

*Zscan4* is sometimes considered as a marker of highly pluripotent status, merely due to its expression at the two-cell stage during development (Cerulo et al., 2014). However, based on our observation, we would like to propose that the expression of *Zscan4* is activated in response to telomere shortening and maybe to DNA damage, independent of the expression of the pluripotency-associated transcription factors. Indeed, a previous study has shown that *Zscan4* can respond to artificial telomere shortening without affecting the expression of *Oct3/4* (Huang et al., 2011). Thus it should be more reasonable to consider that *Zscan4* is activated merely for the physical maintenance of the genome and does not necessarily represent the two-cell-stage-like status of ESCs, the status in which ESCs have the potential to develop into both embryonic and extra-embryonic lineages (Macfarlan et al., 2012). Then if the function of *Zscan4* is restricted to telomere elongation and genomic stability, why is *Zscan4* specifically expressed at the two-cell stage in vivo? One bold idea could be that two-cell-stage embryos have a special invasion by differentiation cues can be higher, resulting in negative correlation of *Rex1* and the cell-cycle length (Figure S3B).
mechanism to recover from telomere shortening after meiosis, otherwise the telomeres will be short generation after generation. And maybe for this special mission required for just one cell division, Zscan4 is expressed. In the inner cell mass of the blastocyst, from where ESCs derive, this mechanism might be silent or just invisible, and may become visible in long-term culture.

By retrospective analysis of the lineage tree, we were able to elucidate the expression pattern of Zscan4 in terms of the correlation with the cell-cycle length. Zscan4 was activated regardless of the pluripotent status probably sensing shortened telomeres and genomic instability. Maybe the physiological regulation of pluripotency and physical maintenance of the genome should be considered separately. If live imaging of telomere shortening and/or manipulation of the telomere length was possible, this should give more direct evidence, but for now it is technically difficult and will be our next challenge. This study visualized the fluctuation of the cell-cycle length and showed one of the mechanisms to maintain self-renewability in ESCs opposing naturally occurring stem cell aging.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

ESB5 ESCs (derived from male E14tg2a ESCs) were cultured on a gelatin-coated culture dish in Glasgow minimal essential medium supplemented with 10% FCS, 1× sodium pyruvate, 1× non-essential amino acids, 0.1 mM 2-mercaptoethanol, and 1,000 U/ml of LIF. For 2i/LIF culture, NDiiH227 medium (Stem Cells) was supplemented with 1,000 U/ml LIF, 3 μM GSK3β-inhibitor CHIR99021 (Stemgent), and 1 μM MEK inhibitor PD0325901 (Stemgent).

**Generation of Rex1p-Luc/Zscan4p-Gal4-4US-EGFP ESC**

Luciferase2 was inserted downstream of the Rex1 promoter in one of the alleles of a Cre-loxP mediated cassette exchange system based on the system we previously reported modified for manipulation of the Rex1 allele (Masui et al., 2005). Briefly, host cells were generated by electroporation of EBS ESCs using Gene Pulser (Bio-Rad) with a linearized vector possessing a 5′-activating domain of Rex1p

The resulting vector was transfected into EBS or Rex1p-Luc ESCs with a PiggyBac vector coding the Zscan4p promoter (amplified from the EBS genomic DNA) and the trans-activating domain of Gal4 (Figure 2A), together with the PiggyBac vectors pPB-UAS-hCMV promoter-EGFP and pPB-H2BmCherry-IRESpac, followed by selection with puromycin to obtain Rex1p-Luc ESCs stably transfected with Zscan4p-Gal4TD, UAS-EGFP, and H2B-mCherry.

**Generation of Z4ex-ESC and Z4sh-ESC**

For generation of Z4ex-ESCs, EBS or Rex1p-Luc ESCs were transfected with a PiggyBac vector coding Zscan4p5 (amplified from cDNA pool derived from wild-type mouse ESCs) downstream of the Tet-responsive element with a minimal CMV promoter (hCMV*1) (Figure S6A), together with the PiggyBac vectors pPB-CAG-rtTA-IRESpno and pPB-H2BmCherry-IRESpac, followed by selection with G418 and puromycin to obtain ESCs stably transfected with Tet-inducible Zscan4p, rtTA, and H2B-mCherry.

For generation of Z4sh-ESCs, shRNA was designed based on the 19-nucleotide shRNA sequence previously identified (Zalzman et al., 2010): 5′-<ATT GTG AGA CC>AAA AAA [CAG AAG CCT GGC ATT CCC TTG AAG CTT [AGG GAA TGC CAG GCT TGT C]<GGT CTC ACA GGS–3′, that is 5′-linker sense>AAA AAA[19-nucleotide sense] a hairpin loop (19-nucleotide antisense)-linker antisense>

After annealing with complementary sequence, the shRNA was cloned into the mir155 region of a PiggyBac vector that was modified from BLOCK-it mir155 expression vector (Invitrogen) (Adachi et al., 2013), which now has a Tet-responsive element with a minimal CMV promoter (Figure S6A). The resulting vector was transfected into EBS or Rex1p-Luc ESCs together with the PiggyBac vectors pPB-CAG-rtTA-IRESpno and pPB-H2BmCherry-IRESpac, followed by selection with G418 and puromycin to obtain ESCs stably transfected with Tet-inducible Zscan4p-shRNA, rtTA, and H2B-mCherry.

**Live Imaging**

ESCs were seeded 1,000 cells per well on a thin plastic-bottom eight-well chamber (Ibidi) coated with LamininS111 (1 μg/cm²; Nippi) in FCS/LIF medium. 2i/LIF medium was also used for some experiments. Cells were monitored on a stage incubator in a humid atmosphere and 5% CO₂ at 37°C (Tokai Hit) under an inverted microscope (IX81; Olympus) equipped with MetaMorph imaging software (Molecular Devices).

For simultaneous monitoring of chemiluminescence and fluorescence, the IX81 microscope was modified to shut out all the lights from the mechanics of the microscope (Olympus), tightly covered with light-shielding tent (Hamamatsu Photonics), and equipped with a CCD camera (ImageEM; Hamamatsu Photonics) and an LED illuminator (Olympus). Chemiluminescence was induced by addition of 1 mM Luciferin (Wako) to the medium and was detected by opening the shutter for 5 min with an electron-multiplying (EM) gain of 255 and the CCD resolution set at binning 1 or 2. Fluorescence was detected by exposure for 300–500 ms with EM gain of 100. Images were taken every 1 hr with a 20× objective lens.

**Fucci-Based Cell-Cycle Analysis**

Fucci vectors (provided by Dr. Atushi Miyawaki) (Sakaue-Sawano et al., 2008) were modified by fusing the two elements, Fucci-G1-Kusabira_orange and Fucci-S/G2/M-Atami_green with P2A. The resulting Fucci-G1-KO-P2A-S/G2/M-AG sequence was inserted into a PiggyBac vector cassette under the control of CAG
promoter and was transfected together with pPB-H2BCFP-IRESpac, followed by selection with puromycin to obtain stably transfected ESCs. Cells were monitored under the IX81 microscope equipped with a stage incubator, a confocal spinning disk (CSU-X1; Yokogawa), a CCD camera (iXon; Andor), and a laser illuminator (LMMS; Spectral) with wavelength 448, 488, and 561 nm, an exposure time of 500 ms, and EM gain of 300. Time-lapse images were taken every 15 min or 30 min with a 20× objective lens.

**Lineage Tracking**

Each cell was tracked manually by drawing a region surrounding the nuclear marker H2B-mCherry (Figure 2B). The average intensities of the chemiluminescence and fluorescence were measured using MetaMorph imaging software. The intensity data were manually converted to a Microsoft Excel sheet in the format of the lineage. Then the data were used as input for a VBA macro (input data and source code are provided in Data S1) that was programmed to automatically draw a lineage tree with the intensity color scale (Figures S2E and 3A) in Microsoft PowerPoint.

**qPCR for mRNA Expression Level**

First-strand DNA was synthesized from total RNA prepared by a QuickGene RNA cultured cell HC kit (KURABO) in 20 μl of the reaction mixture containing oligo-dT primers using a ReverTra Ace first-strand synthesis kit (Toyobo). Real-time PCR was performed with THUNDERBIRD SYBR qPCR Mix (Toyobo) using a CFX384 Real-Time System (Bio-Rad). The expression level of Gapdh was used as an internal control. The average of triplicate reactions was calculated. Sequences of primer pairs are as follows:

| Gene            | Forward primer | Reverse primer |
|-----------------|----------------|----------------|
| Zscan4lowendo   | 5'-GAGATTCTAGGAGATCGACTGATGATG-3' | 3'-GCTGTTTGTTCAAATTGATGACCTC-5' |
| Zscan4highendo  | 5'-ATTCCTCATAGGTCTTGAC-3'   | 3'-CTGACTTGAGGATGAAAC-5'   |
| Gapdh           | 5'-ACCACACGTCCATGCCATAC-3'   | 3'-TCCACACCCCTGTTGCTGTA-5'  |

**CFSE Dilution Assay**

Cells were labeled with 20 μM CFSE dye (Wako) for 15 min at 37°C, followed by centrifugation and two washes in PBS, and cultured for 48 hr. After 48 hr, the cells were trypsinized, centrifuged, and washed twice, then suspended in Hanks buffer with 1% BSA and subjected to FACS sorting by AriaIII (BD Biosciences). Cells labeled just before sorting and unlabeled cells were used as positive and negative controls, respectively.

**Telomere Length Measurement by Flow-FISH**

A telomere PNA kit/fluorescein isothiocyanates (FITC) for flow cytometry (Dako, K5327) was used following the manufacturer's protocol. Briefly, 10^6 cells were hybridized with an FITC-conjugated telomere PNA probe at 82°C for 5 min and incubated at room temperature overnight, followed by DNA staining with propidium iodide. For cells that were collected after the CFSE dilution assay, 5 × 10^5 cells were hybridized with 150 ng/ml Cy3-conjugated telomere PNA probe (TelG-Cy3, Panagene) instead of the FITC telomere PNA probe provided in the kit, because carry over of CFSE dye with wavelength close to that of FITC was still detected after in situ hybridization. The intensities of telomere-bound fluorescence (either FITC or Cy3) were analyzed by FACS with AriaIII (BD Biosciences).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.02.010.

**AUTHOR CONTRIBUTIONS**

Y.N.-F. designed and performed the experiments, analyzed the data, and wrote the manuscript. H.N. generated the plasmids and gave scientific guidance.

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