Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Nature. Author manuscript; available in PMC 2010 October 08.

Published in final edited form as:

Nature. 2010 April 8; 464(7290): 858–863. doi:10.1038/nature08882.

Zscan4 regulates telomere elongation and genomic stability in ES cells

Michal Zalzman[1], **Geppino Falco**[1,*], **Lioudmila V. Sharova**[1], **Akira Nishiyama**[1], **Marshall Thomas**[1], **Sung-Lim Lee**[1,†], **Carole A. Stagg**[1], **Hien G. Hoang**[1], **Hsih-Te Yang**[1], **Fred E. Indig**[2], **Robert P. Wersto**[2], and **Minoru S. H. Ko**[1]

[1]Developmental Genomics and Aging Section, Laboratory of Genetics, National Institute on Aging, NIH, Baltimore, MD 21224, USA

[2]Research Resources Branch, National Institute on Aging, NIH, Baltimore, MD 21224, USA

Abstract

Exceptional genomic stability is one of the hallmarks of mouse embryonic stem (ES) cells. However, the genes contributing to this stability remain obscure. We previously identified Zscan4 as a specific marker for 2-cell embryo and ES cells. Here we show that Zscan4 is involved in telomere maintenance and long-term-genomic stability in ES cells. Only 5% of ES cells express Zscan4 at a given time, but nearly all ES cells activate Zscan4 at least once within nine passages. The transient Zscan4-positive state is associated with rapid telomere extension by telomere recombination and upregulation of meiosis-specific homologous recombination genes, which encode proteins that are colocalized with ZSCAN4 on telomeres. Furthermore, Zscan4 knockdown shortens telomeres, increases karyotype abnormalities and spontaneous sister chromatid exchange, and slows down cell proliferation until reaching crisis by eight passages. Together, our data reveal a unique mode of genome maintenance in ES cells.

Mouse embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocysts1,2 and have gene expression patterns similar to ICM cells3. The defining features of ES cells are pluripotency and self-renewal, both of which have been the focus of intensive research for many years4. Another important hallmark of mouse ES cells is their ability to defy cellular senescence and to undergo more than 250 doublings without undergoing crisis or transformation5. Although the fraction of euploid cells tends to decrease in long-term culture6, the genomic integrity of mouse ES cells is more strictly maintained than in any other cultured cells. For example, ES cells maintain their ability to

Users may view, print, copy, download and text and data- mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

Correspondence and requests for materials should be addressed to M.S.H.K. (kom@mail.nih.gov).

*Present address: Istituto di Ricerche Genetiche Gaetano Salvatore Biogem s.c.ar.l., Ariano Irpino 83031, Italy

†Present address: College of Veterinary Medicine, Gyeongsang National University, Jinju, Gyeongnam 660701, Republic of Korea

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Author contributions M.Z., G.F., L.V.S., A.N., M.T., S.-L.L., C.A.S., H.G.H., H.-T.Y., F.E.I., R.P.W. designed and performed experiments. M.S.H.K. conceived and supervised the project. M.Z. and M.S.H.K. wrote the manuscript. All authors participated in the discussion.

Author Information Reprints and permissions information is available at www.nature.com/reprints.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

form chimeric animals with germline competency even after many passages[7,8]. The mutation frequency in ES cells is also much lower (> 100-fold) than in mouse embryonic fibroblast cells and other somatic cells[9]. The unique features of mouse ES cells are further marked by lower frequency of chromosomal abnormalities compared to embryonal carcinoma (EC) cells, which otherwise share characteristics with ES cells[10], or some human ES cells[11]. However, the mechanism by which mouse ES cells maintain genomic stability is currently poorly understood.

A Zscan4 (zinc finger and SCAN domain containing 4) gene cluster includes 6 transcribed paralogous genes (Zscan4a - Zscan4f) which share high sequence similarities and are thus collectively called Zscan4[12]. A sharp expression peak of Zscan4 marks the late 2-cell stage of mouse embryos and is essential for embryo implantation and blastocyst outgrowth in tissue culture[12]. Zscan4d is transcribed predominantly in 2-cell embryos, whereas Zscan4c is transcribed predominantly in ES cells[12,13] and is associated with self-renewal[14]. Both Zscan4c and Zscan4d encode a SCAN domain, predicted to mediate protein-protein interactions, and four DNA binding Zinc-finger domains[15]. Here we found Zscan4 to be essential for long-term culture of ES cells and maintenance of karyotype integrity associated with regulated telomere recombination in normal undifferentiated ES cells.

Zscan4 expression dynamics in ES cells

Zscan4 expression is heterogeneous in mouse ES cell colonies[12,13]. A plasmid containing a GFP-Emerald reporter under the Zscan4c promoter was stably introduced into ES cells (pZscan4-Emerald cells), which allowed us to demonstrate a small fraction of ES cells as Zscan4 positive (Zscan4+), recapitulating our observations by RNA in situ hybridization (Fig. 1a). Fluorescence Activated Cell Sorting (FACS) analysis indicated that approximately 5% of the ES cells were Emerald positive (Em+; Fig. 1b), although the number varied slightly (2–7%) between cultures. Cells were then FACS-sorted into Em+ cells and Em– cells and subsequently replated separately. Both Em+ and Em– cells were able to establish viable, undifferentiated colonies (Fig. 1c). However, by 24 hours in culture, 3.3% of Em– cells became Em+ (Fig. 1c, d), whereas 54% of the Em+ cells became Em– as shown by flow cytometry analysis (Fig. 1c, d). qRT-PCR analysis of sorted cells confirmed a correlation between the expression of Emerald and Zscan4 (Supplementary Fig. 1). These results indicate that Zscan4+ state is transient and reversible, and constitutes ~5% of ES cells in culture.

To trace the fate of Zscan4+ cells, we stably transfected a plasmid carrying CreERT2 [16] under the control of the Zscan4c promoter (pZscan4-CreERT2 cells). In this system, Cre-recombinase translocates from the cytoplasm into the nucleus only in the presence of tamoxifen[16] and excises a neomycin cassette from the LacZ-open reading frame (ORF)[17], leading to heritable constitutive LacZ expression (Supplementary Fig. 2a). As expected, after a short exposure to tamoxifen, cells positive for Zscan4 RNA by fluorescence in situ hybridization were colabeled with nuclear-localized Cre-recombinase protein by immunostaining (Supplementary Fig. 2b). To identify the total population of cells descended from Zscan4+ cells, pZscan4-CreERT2 ES cells were maintained continuously in the presence of tamoxifen for 9 passages (27 days) in undifferentiated conditions.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Immunostaining analysis of the cells revealed that POU5F1 (OCT4, OCT3/4) was costained with LacZ, indicating that they were still undifferentiated (Supplementary Fig. 2c). Analyses of LacZ activity by X-gal staining (Fig. 1e) and by flow cytometry following CMFDG staining (a green fluorescence substrate) (Fig. 1f, Supplementary Fig. 3), however, showed that the number of cells marked with LacZ steadily increased, and the majority of ES cells became LacZ-positive (LacZ+) by passage 9. The steady increase of LacZ+ cells over multiple passages was not likely caused by a growth advantage of LacZ+ cells, as the early withdrawal of tamoxifen at passage 4 mostly halted the further increase of LacZ+ cells (Fig. 1f; Tam+: P1–P3). These data indicate that, although 5% of ES cells are marked by Zscan4+, most of the ES cells in culture are descendants of cells which activated Zscan4 at least once within 9 passages. Based on these data, we estimated that every day, 3% of Zscan4-ES cells turned into Zscan4+ cells, whereas 47% of Zscan4+ cells turned into Zscan4− cells, leading to the equilibrated state of 5% Zscan4+ cells in ES cell culture at any given time (Supplementary Fig. 4).

To further exclude the possibility that Zscan4 expression marks a certain cell lineage commitment of ES cells, we first exposed pZscan4-CreERT2 cells to a pulse of tamoxifen, followed by an embryoid body (EB) formation and attachment assay18. Subsequent to the EB differentiation, both LacZ+ cells (i.e., descendants of Zscan4+ cells) and LacZ− cells (i.e., descendants of Zscan4− cells) were able to contribute to a variety of cell types including lineages from all three embryonic germ layers, as judged by both cell morphology and immunostaining for specific lineage markers (Supplementary Fig. 5a, b). Zscan4+ cells were also able to form chimeras, as demonstrated by blastocyst injection of Em+ cells (Supplementary Fig. 5c).

Zscan4 knockdown leads to culture crisis

To directly test whether the intermittent expression of Zscan4 is dispensable for ES cells, we stably transfected a Zscan4-shRNA knockdown vector targeting a common 3’-untranslated region of Zscan4c and Zscan4d into tet-Zscan4c ES cells19, in which an exogenous copy of Zscan4c is repressed in the presence of doxycycline (Dox+) but is induced in absence of Dox (i.e., Dox− condition). For brevity, the resultant Zscan4-knockdown/rescue cells (tet-Zscan4c-KR cells) are called Zscan4-knockdown cells in the Dox+ condition, or Zscan4-rescue cells in the Dox− condition (see Supplementary methods and Supplementary Fig. 6). Two independent tet-Zscan4c-KR cell lines were used for the following studies. qRT-PCR and Western blot analyses confirmed Zscan4 knockdown (Fig. 2a, Supplementary Fig. 7a, b), whereas the control shRNA did not affect equivalent cells. As expected from the transient expression of Zscan4, Zscan4-knockdown cells showed typical ES cell morphology upon clonal isolation (Supplementary Fig. 7c). However, the population doublings slowed with passages (Fig. 2b), and flat cells accumulated in culture. Most of the cells ceased to proliferate during passage 7 and died abruptly 1–2 days after plating passage 8 (i.e., approximately 31 cell doublings after transfection), leaving a very low number of surviving but slowly proliferating small colonies (Fig. 2b). The presence or absence of Dox alone did not affect proliferation rate, as all the control cells in Dox+ and Dox− conditions showed normal proliferation curves (Fig. 2b, Supplementary Fig. 8a). Apoptosis and viability tests at the same passages (Fig. 2c, Supplementary Fig. 8b) showed that the level of apoptosis in

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Zscan4-knockdown cells increased by passage 7 (P+7) and reached 47.5±1.2% (mean ±s.e.m.) at P+8, leading to the crisis observed a day later. Rescue of Zscan4 was able to recover, to some extent, cell proliferation (Fig. 2b, Supplementary Fig. 8a) and apoptosis (Fig. 2c, Supplementary Fig. 8b), and consequently prevented tissue culture crisis and massive cell death in P+8, suggesting that Zscan4 is essential for the maintenance of ES cells in culture.

To investigate the events associated with the culture crisis, the karyotype of Zscan4-knockdown and control cells was analyzed at passage 3, the earliest passage after clonal isolation and expansion (Fig. 2d, Supplementary Fig. 9). Only 65% of the Zscan4-knockdown cells presented a normal karyotype, compared to 88% of control cells. This is still considered acceptable for cultured ES cells[7,8], as the abnormalities found in these cells seemed random at this stage; however, the Zscan4-rescue cells showed distinctly better karyotype (77.5% normal), although a slight increase of chromosome fusion was noted (Fig. 2d). Moreover, karyotype abnormalities observed included random chromosome fusions and deletions, suggesting that genomic instability was not due to a specific clonal defect but caused by Zscan4 knockdown. When cells were karyotyped at passage 7, only 22.7% of the Zscan4-knockdown cells showed normal karyotype. Remarkably, the karyotype of Zscan4-rescue cells was significantly better, as 60% of the cells were normal and no deletions were observed.

The compromised karyotype in Zscan4-knockdown cells prompted us to examine its effect on telomeres. Telomere length ratios were compared to a single-copy gene by qPCR, as previously described[20,21]. Average telomere length decreased significantly by 30±5% (mean±s.e.m.) in Zscan4-knockdown cells at passage 6 (Supplementary Fig. 10). By contrast, telomeres of control cells remained intact through passages, and telomere length was rescued by Zscan4c overexpression (Supplementary Fig. 10), suggesting that knockdown of Zscan4 causes the telomere shortening. Furthermore, telomere length distribution analyzed by quantitative fluorescence in situ hybridization (Q-FISH)[22] demonstrated that telomeres in the Zscan4-knockdown cells at passage 6 (Fig. 3a, Supplementary Fig. 10) were significantly shorter than those in the control cells ($p<0.001$) (Fig. 3b).

Zscan4 extends telomeres in ES cells

Surprisingly, Zscan4 induction led to elongated telomeres in Zscan4-rescue cells (Fig. 3a, Supplementary Fig. 10). The telomere length distribution in Zscan4-rescue cells also indicated that the average increase of telomere length was due to an overall shift and not due to a few abnormally long telomeres. The effects of Zscan4 overexpression on telomeres were also examined in the parental tet-Zscan4c cells before and after gene induction (Fig. 3b, Supplementary Fig. 11a, b). Consistent with the results of Zscan4 rescue (Fig. 3a), telomere Q-FISH showed a significant increase (1.8-fold) in average telomere length from 39.4±0.13 kb (mean±s.e.m.) in the non-induced tet-Zscan4c cells to 65.9±0.2 kb (mean ±s.e.m.) in Zscan4-overexpressing cells (Fig. 3b). This finding was further confirmed by telomere qPCR data (Fig. 3d). As expected, telomere extension was not caused by Dox itself, as controls showed no significant difference between Dox+ and Dox− conditions.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Furthermore, telomere qPCR analysis correlated telomere extension with the transient Zscan4+ state in FACS-sorted pZscan4-Emerald cells: the relative telomere length in Em+ cells was 2.4-fold longer than in control tet-Empty cells, whereas that of Em− cells was comparable to controls (Supplementary Fig. 11b). The telomere lengthening by Zscan4 was not associated with elevated telomerase activity, as shown by Telomeric Repeat Amplification Protocol (TRAP) assay (Supplementary Fig. 12a), nor abolished by eliminating telomerase activity with *Tert* knockdown (Supplementary Fig. 12b–e), suggesting a telomerase-independent mechanism for the Zscan4 activity.

Recent findings have established that preimplantation embryos can activate rapid telomere extension (within one cell cycle) through telomere recombination or telomere sister chromatid exchange (T-SCE)23. As Zscan4 is a common marker for ES cells and the 2-cell embryos, we investigated whether Zscan4 activated the T-SCE apparatus by a telomere chromosome orientation FISH (CO-FISH) assay24 (Fig. 4a). As expected, the frequency of T-SCE in all control cells was low and showed no significant difference between Dox+ and Dox− conditions (Fig. 4b). Similar frequencies were observed for non-induced tet-Zscan4c cells. By contrast, Zscan4c induction in tet-Zscan4c cells for 3 days resulted in >10-fold more T-SCE events in 76% of the nuclei tested (Fig. 4b). Therefore, we infer that transient expression of Zscan4c promoted telomere recombination, leading to telomere elongation. However, additional mechanisms for telomere elongation by Zscan4 cannot be excluded.

Zscan4 inhibits spontaneous SCE

Given that T-SCE is often coupled with general instability and higher incidence of genomic sister chromatid exchange (SCE)25, we tested for SCE following Zscan4 overexpression in tet-Zscan4c cells or knockdown/rescue in tet-Zscan4c-KR cells. Consistent with previous reports on ES cells26,27 , the basal spontaneous SCE rate in all control cells was relatively low; however, Zscan4c overexpression further reduced SCE events 4-fold compared to non-induced cells and 6-fold compared to tet-Empty controls (Fig. 5a, b). Conversely, a significantly higher frequency of SCEs was found in Zscan4 knockdown cells (Supplementary Fig. 13): 29±0.7% (mean±s.e.m.) (Fig. 4c) of the cells had 11±4.2 SCE/ nucleus (mean±s.e.m.) (Fig. 4d). When Zscan4 expression was rescued, the number of spontaneous SCEs was dramatically lower: only 13% of the cells were SCE-positive (Fig. 4c) with smaller number (2.9±2.1) of SCE/nucleus (mean±s.e.m.) (Fig. 4d), which was comparable to controls. Therefore, our findings suggest that Zscan4 expression reduces the incidence of non-telomeric SCE in ES cells, whereas knockdown increases it.

ZSCAN4 is localized on telomeres

ZSCAN4 protein contains a SCAN domain as well as a DNA binding domain, suggesting its role in recruiting other proteins to chromatin15. To test whether ZSCAN4 protein functions in telomere recombination, we performed a colocalization study in Zscan4-overexpressing tet-Zscan4c cells (Dox−) by ZSCAN4 immunostaining along with telomere FISH (T-FISH). Confocal microscope analysis revealed that 84±0.6% (mean±s.e.m.; n=6) of ZSCAN4 foci were localized on telomeres (Fig. 5a, top panel; Supplementary Fig. 14), and on average, 50±0.6% (mean±s.e.m.; n=6) of telomeres were ZSCAN4 positive (Supplementary Fig. 14).

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Additionally, preliminary microarray analysis indicated that meiosis-specific homologous recombination genes were induced by Zscan4c overexpression in ES cells. Analyses by qRT-PCR and Western blot validated the results for the enzyme Spo11[28,29], which facilitates the double strand DNA breaks (DSBs) required for meiotic recombination, the recombinase Dmc1[30], essential for meiotic recombination, and the cohesin Smc1β required for meiotic sister chromatid exchange and cohesion[31] (Fig. 5b). Immunostaining colabeled with T-FISH showed SPO11 was localized on telomeres (Fig. 5a, middle panel) as well as ZSCAN4 foci (Fig. 5a, top panel) and DMC1 foci (Supplementary Fig. 15a, bottom panel). Colocalization of SPO11 and ZSCAN4 foci was also evident in the G2 phase (Fig. 5a, bottom panel). Consistent with the fact that DSBs are enclosed in γ-H2AX foci to mediate homologous recombination during meiotic recombination[29], immunostaining revealed γ-H2AX foci forming on telomeres after Zscan4c overexpression (Supplementary Fig. 15a, top panel). However, this marker was no longer present during M phase, which may suggest that DSBs are recovered prior to M phase (Supplementary Fig. 15a, top panel). Furthermore, in Em+ cells the RecA homolog DMC1[30] was colocalized with γ-H2AX foci (Supplementary Fig. 15b, top panel) and ZSCAN4 foci was colocalized with TRF1[32] foci (Supplementary Fig. 15b, bottom panel; Supplementary Fig. 15c for controls). Taken together, our results suggest that Zscan4 may facilitate telomere recombinations by inducing the meiosis-specific homologous recombination mediators that are subsequently colocalized with ZSCAN4 at telomeres. However, further experiments will be required to determine if ZSCAN4 acts as a transcription factor on these genes and/or directly recruits these proteins to telomeres.

Discussion

Here we have demonstrated that Zscan4 plays a key role in the critical feature of ES cells, i.e., defying cellular senescence and maintaining normal karyotype for many cell divisions in culture (Fig. 5d). Zscan4 is expressed in about 5% of ES cells at any given time and marks a transient state of ES cells, whereas other heterogeneously expressed genes in ES cells[13,33] often mark specific cell lineages[34] [35]. Yet, nearly all the ES cells in culture express Zscan4 at least once in 9 passages of continuous culture. Although additional experiments will be required to find the signals leading to Zscan4 activation, its unique mode of expression suggests a highly regulated mechanism and seems to be consistent with our observation that the knockdown of Zscan4 does not affect the cells immediately, but eventually leads to karyotype deterioration, reduction of cell proliferation, and apoptosis by 7 – 8 passages. Therefore, without intermittent activation of Zscan4, ES cells lose their ability to proliferate indefinitely.

Karyotype deterioration in Zscan4-knockdown ES cells seems to be associated with telomere shortening as well as an increase in spontaneous non-telomeric SCE. As Zscan4 functions as an activator of spontaneous T-SCE and telomere elongation in undifferentiated ES cells, regulation of telomeres by Zscan4 in mouse ES cells is distinct from previously reported mechanisms. First, Zscan4+ cells undergo rapid telomere extension, most likely by telomere recombination or T-SCE. ZSCAN4 protein seems to function by inducing meiosis-specific homologous recombination mediators and forming foci with them on telomeres (Fig. 5a), suggesting ES cells are able to utilize a novel mechanism for T-SCE. Second,

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

unlike cells usually associated with T-SCE, such as survivors of telomerase knockout *Terc*$^{-/-}$ ES cells25,36,37 and tumor cells lacking telomerase reactivation38–41, we have shown that telomerase is active in undifferentiated ES cells expressing Zscan4. We have also shown that Zscan4-mediated telomere extension does not require telomerase; however, it remains to investigate if Zscan4-mediated telomere extension can compensate the telomere loss in *Terc*$^{-/-}$ ES cells, which cease to proliferate after >450 population doublings25,36,37. Third, most genes previously identified for telomere regulation are inhibitors of T-SCE, as downregulation of these genes increases T-SCE and/or telomere length 40, such as DNA methyltransferase DNMT1 and DNMT342 and Werner syndrome protein (WRN)43. An exception is the Rtel gene, as Rtel$^{-/-}$ ES cells show telomere shortening after induction of differentiation44, but unlike Rtel, Zscan4 exhibits this phenotype in undifferentiated ES cells. Fourth, unlike T-SCE that usually results from general chromosomal instability and is accompanied by increased SCE in non-telomeric sequences25, T-SCE mediated by Zscan4 is not associated with an increase of general SCE, and normal karyotype remains stable with lower spontaneous SCE rate (Fig. 5a, b). Overall, this work provides a first link to a novel mechanism employed by ES cells to sustain long-term genomic stability and telomere maintenance.

The expression of Zscan4 varies among different pluripotent stem cells, which may correlate to their differences in genomic stability. For example, consistent with the inferior ability of EC cells to maintain genomic integrity10, the expression of Zscan4 is much lower in EC cells than in ES cells45. It is interesting to note that the expression level of Zscan4 in induced pluripotent stem (iPS) cells46 is comparable to ES cells45, suggesting that iPS cells may have regained the ability to undergo ES-like genome maintenance. By selecting cells able to activate Zscan4, it may be possible to enrich cultures with cells more suitable for future therapeutic purposes. Moreover, if Zscan4 expression can be induced and controlled, it may provide means to increase genomic stability in other cell types, such as stem cells or cancer cells.

METHODS SUMMARY

ES cell lines used for this study: R26R317and MC147. Zscan4c promoter used for pZscan4-Emerald and pZscan4-CreERT2 was cloned from BAC RP23-63I1. For ROSA/tet-off targeting vector and generation of tet-Zscan4c cells and tet-Empty cells, pZhcSfi plasmid48 was modified with puromycin resistance and 6xHis-FLAG tag to flank Zscan4c. Zscan4 shRNA was cloned into GeneSilencer pGSU6 shRNA Vector (Gelantis). Telomere Q-FISH was performed by PNA-FISH Kit/Cy3 (DAKO), and calculated by TFL-TELO software22. CO-FISH24,49, Telomere qPCR21 and SCE50 assays were performed as previously described. **Full methods** are available in Supplementary materials and methods.

METHODS

ES cell culture

MC1 ES cells, derived from 129S6/SvEvTac47, were purchased from the Transgenic Core Laboratory of the Johns Hopkins University School of Medicine, Baltimore, MD, USA. R26R317 ES cells, a gift from Dr. Philippe Soriano, were used as parental line to generate

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

pZscan4-CreERT2 cells for lineage-tracing experiments. In general, all ES cell lines were cultured for 2 passages on gelatin-coated feeder-free plates and subsequently maintained in gelatin-coated 6-well plates in complete ES medium: DMEM (Gibco); 15% FBS (Atlanta Biologicals); 1000 U/ml leukemia inhibitory factor (LIF) (ESGRO, Chemicon); 1 mM sodium pyruvate; 0.1 mM non-essential amino acids (NEAA), 2 mM GlutaMAX, 0.1 mM beta-mercaptoethanol, and penicillin/streptomycin (50 U/50 μg/ml). For all cell lines, medium was changed daily and cells were split every 2 to 3 days routinely.

pZscan4-Emerald vector construction

A putative Zscan4 promoter that covers between the Zscan4c start codon (methionine) and the 2570 bp upstream site from the start codon was amplified from BAC RP23-63I1 with high fidelity TITANIUM Taq polymerase (Clontech) using primers (Forward primer, *Mlu*I_site_GGCAACCTTACTACTTCTATC; Reverse primer, AGCATCAACCACTTTGGTAC). Subsequently, the PCR product was cloned into the pCDNA6.2/C-EmGFP TOPO vector (Invitrogen). Sequence-verified plasmid DNA was linearized by *Mlu*I digestion.

pZscan4-CreERT2 vector construction

An open reading frame (ORF) of pCreERT216 vector was cloned into *Eco*RI site of pBluescriptIISK(+) plasmid vector. Subsequently, the aforementioned PCR fragment of Zscan4c promoter was cloned into the vector between *Eco*RV and *Eco*RI sites, located at the 5' end of the CreERT2-ORF by blunt-end ligation. A *Sal*I-*Not*I DNA fragment containing Zscan4c promoter-CreERT2 was cut out from the vector and then cloned into pEF6/V5-His-Topo plasmid vector between *Hind*III and *Pme*I sites by blunt-end ligation.

Generation of pZscan4-Emerald and pZscan4-CreERT2 cells

MC1 ES cells (for pZscan4-Emerald transfection) and R26R3 ES cells (for pZscan4-CreERT2 transfection) were grown on gelatin in 6-well plates. $5{\times}10^5$ cells in suspension were transfected with 1 μg of linearized pZscan4-Emerald vector or pZscan4-CreERT2 vector using Effectene (QIAGEN) according to manufacturer's instructions, and plated in gelatin-coated 100 mm dishes. Cells were selected with 5 μg/ml blasticidin. ES cell colonies were picked on the 8th day, expanded, and frozen for further analysis.

Sorting pZscan4-Emerald ES cells

Cells were fed at least 2 hours before harvesting by Accutase (Chemicon), and resuspended in IMDM containing 25 mM HEPES buffer (Chemicon) with 1% fetal bovine serum and 1000 U/ml LIF. The cells were then FACS-sorted according to the fluorescent intensity of Emerald into IMDM containing 35% serum, 1 mM sodium pyruvate, 2 mM GlutaMAX, 100 μM β-mercaptoethanol, 100 U/ml Penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids, and 1000 U/ml LIF. For qRT-PCR analysis of FACS-sorted cells, total RNAs were collected immediately after sorting by TRIzol (Invitrogen) according to the manufacturer's instructions.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

LacZ analysis of pZscan4-CreERT2 cells

To trace the fate of Zscan4+ cells, pZscan4-CreERT2 ES cells were grown in the standard ES medium in the presence or absence of 100 nM tamoxifen for up to 9 passages. As a control, pZscan4-CreERT2 cells were also cultured in the presence of tamoxifen for 3 passages, followed by 6 additional passages without tamoxifen. To visualize the LacZ+ cells, cells in biological triplicate at passages 1– 9 were stained for beta-galactosidase (LacZ) using a commercial kit (Chemicon) according to manufacturer's instructions. For flow cytometry analysis, LacZ was also detected by the green fluorescence substrate CMFDG using the commercial kit DetectaGene™ (Invitrogen). Suspended cells were then analyzed by the Guava EasyCyte Mini flow cytometry system with the CytoSoft4.1 software (Guava Technologies).

Embryoid body (EB) formation assay of pZscan4-CreERT2 cells

To examine the difference between Zscan4+ and Zscan4– cells for their ability to differentiate into multiple cell lineages, EB formation assay18 was carried out after culturing pZscan4-CreERT2 ES cells on gelatin for 3 days in the complete ES medium containing 100 nM tamoxifen. Cells were then harvested and $4{\times}10^6$ ES were plated on 100 mm bacteriological Ultra-Low Culture Dish (Corning) in LIF-free medium without tamoxifen to form floating EB. On the 7th day floating EB were collected and plated in gelatin-coated 6-well plates in LIF-free medium without tamoxifen to allow attachment. On the 11th day, areas of beating muscles were scored and subsequently cells were fixed in 4% PFA for further analyses by LacZ staining and immunohistochemistry.

Mouse chimera assay of pZscan4-Emerald cells

Eight-week-old C57BL/6J mice were superovulated by injecting 5 IU pregnant mare serum gonadotropin (PMS; Sigma, St Louis, MO, USA) and 5 IU human chorionic gonadotropin (HCG; Sigma) after 46–47 h51. Fertilized eggs were harvested from mated superovulated mice and cultured in synthetic oviductal medium enriched with potassium (KSOMaa MR-121-D) at 37°C in an atmosphere of 5% CO_2. Emerald-positive cells were collected by FACS-sorting pZscan4-Emerald cells and subsequently microinjected into 3.5 d.p.c. blastocysts. 15 blastocysts were transferred into the uteri of 2.5 d.p.c. pseudopregnant ICR female mice. Embryos were collected at day 10.5 d.p.c. and genotyped for chimerism by PCR.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

1 μg of total RNAs, isolated from cells by TRIzol (Invitrogen) in biological triplicate, were reverse transcribed by Superscript III according to the manufacturer's instructions. qPCR analyses were performed using 10 ng of cDNAs per well in triplicate with the SYBR green master mix (Applied Biosystems) according to the manufacturer's instructions. Primers used: Zscan4 (recognize all Zscan4 paralogs; forward, 5'-GAGATTCATGGAGAGTCTGACTGATGAGTG-3'; reverse, 5'-GCTGTTGTTTCAAAAGCTTGATGACTTC-3'); Zscan4c (forward, 5'-CCGGAGAAAGCAGTGAGGTGGA-3'; reverse, 5'-CGAAAATGCTAACAGTTGAT-3'); Zscan4d (forward, 5'-

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

GTCCTGACAGAGGCCTGCC-3'; reverse, 5'-GAGATGTCTGAAGAGGCAAT-3'); Spo11 (forward, 5'-GCTGGACAGCATCCTGAAGAGG-3'; reverse, 5'-GGGTAAGTACACTCTGGACA-3'); Dmc1 (forward, 5'-CAGATCCAGGAGCAACTATGA-3'; reverse, 5'-CGATCCTCAGTTCTCCTCTT-3'); and Smc1β (forward, 5'- GCATCCATCCAAATAGACTACAGC-3'; reverse, 5'-CCTGTGCTCGCAAGTTTGGA-3'). Reactions were run on 7900HT or 7500 system (Applied Biosystems). Fold induction was calculated and normalized by the Ct method52.

Generation of tet-Zscan4c and tet-Empty cells

The detailed procedures for making tet-Zscan4c and tet-Empty ES cells were described previously19. In brief, "MC1 Rosa26 knock-in parental ES cell clone" was made by targeting ROSA26 locus17 of MC1 ES cells47 by pMWRosaTcH vector48 (a gift from Dr. Hitoshi Niwa). The ORF of Zscan4c was amplified by PCR and subcloned into the pZhcSfi plasmid48 (a gift from Dr. Hitoshi Niwa) after replacing the Zeocin-resistance gene by puromycin-resistance gene19. A 6xHis-FLAG epitope tag sequence was inserted into the 5' end of LoxPV site to make ZSCAN4C fused to the epitope tag at the C-terminus19. The resultant Zscan4c plasmid was cotransfected with pCAGGS-Cre plasmid into the MC1 Rosa26 knock-in parental ES cells using Effectene (QIAGEN) according to manufacturer's instructions. After the puromycin selection in the presence of Doxycycline (0.2μg/ml), ES cell clones were isolated and thereafter named tet-Zscan4c cells. Modified pZhcSfi vector without ORF was used to establish tet-Empty control cells.

Telomerase knockdown in tet-Zscan4c cells

The tet-Zscan4c cells were transiently transfected with a SMART pool siRNA (Dharmacon) against mouse Tert gene (the catalytic protein unit of the enzyme telomerase). Non-targeting control (NTC) siRNA pool (Dharmacon) was used as a control for off-target effect. The effects of Zscan4 overexpression in these cells were examined by subsequent induction of Zscan4 in the Dox− condition for 3 days. Tert knockdown was confirmed by qRT-PCR analysis and TRAP assay.

Generation of tet-Zscan4c-KR cells

After testing four different shRNA designs against the sequence common to 3'-UTRs of both Zscan4c and Zscan4d, the following shRNA sequence (linker, 19-mer sense oligos, a hairpin loop, and an anti-sense of the 19-mer sense oligos, and an antisense of the linker; see Supplementary Fig. 6) was selected and used: forward, 5'-CATAACCTGAAAAAACAGAAGCCTGGCATTCCCTAAGCTTAGGGAA TGCCAGGCTTCTGCGCGTCCTTTCCACAAGA TATATA-3'; reverse, 5'-CATAACCTGAAAAAACAGAAGCCTGGCATTCCCTTTCGAAAG GGAATGCC AGGCTTCTGCGCGTCCTTTCCACAAGATATATA-3'. The shRNA was cloned into GeneSilencer U6 PCR kit (Gelantis) according to the manufacturer's instructions. The resultant vector was stably transfected into the tet-Zscan4c cells by the Effectene (QIAGEN) according to manufacturer's instructions. The cells were named tet-Zscan4c-knockdown/rescue (KR) cells, as the endogenous Zscan4 was knocked down in the Dox+ condition and the exogenous Zscan4c provided a rescue in the Dox− condition. As a negative control, an

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

shRNA against Luciferase provided by the kit was stably transfected into tet-Zscan4c cells and named shCont cells.

Telomere Quantitative Fluorescence In Situ Hybridization (Q-FISH)

All the cells were maintained in the complete ES medium containing Dox, except for Zscan4-overexpression condition achieved by culturing cells in the Dox− condition for 3 days. Medium was replaced every day. On the 3rd day medium was supplemented with 0.1 μg/ml colcemid (Invitrogen), followed by 4 hours incubation to arrest the cells in metaphase. After adding hypotonic 0.075 M KCl buffer, cells were fixed in cold methanol/acetic acid (3:1) and metaphase spreads were prepared. Telomere FISH was performed by Telomere peptide nucleic acid (PNA) FISH Kit/Cy3 (DakoCytomation) according to the manufacturer's instructions. Chromosomes were stained with 0.5 μg/ml DAPI. For quantitative measurement of telomere length, digital images of chromosomes and telomeres were captured by Zeiss microscope with Cy3–DAPI filter sets, followed by the quantitation of telomere size and fluorescence intensity by TFL-TELO software 22.

Telomere Chromosome Orientation FISH (CO-FISH)

CO-FISH analysis was done as described 24,49 with several minor modifications. Briefly, ES cells were grown in either Dox+ or Dox− condition for 3 days. Medium was changed every day. On the 3rd day 5'-bromo-2'-deoxyuridine (BrdU) was added for 12 hours to allow BrdU incorporation for one cell cycle. Colcemid (0.1 μg/ml) was added for the final 4 h. Metaphase spreads were prepared. Slides were stained with 0.5 μg/ml Hoechst 33258 (Sigma), washed in 2× SSC for 20 min at room temperature, mounted with McIlvaine's buffer (at pH 8.0), and exposed to 365-nm UV light (Stratalinker 1800 UV irradiator) for 30 min. The BrdU-substituted DNA was digested with 3 units/μl Exonuclease III (Promega) for 10 min at room temperature. The leading strand telomeres were revealed by 3'-Cy3-conjugated $(TTAGGG)_7$ (IDA) without denaturation step and incubated overnight at 37°C. Chromosomes were counterstained with 1 μg/ml DAPI (Vector Laboratories).

Telomere measurement by quantitative real-time PCR

Genomic DNA was extracted from 10^6 cells and quantified by Nanodrop. Average telomere length ratio was measured from total genomic DNA using a real-time PCR assay, as previously described 21. PCR reactions were performed on the Prism 7500 Sequence Detection System (Applied Biosystems) using telomeric primers, control single-copy gene Rplp0, and PCR settings as previously described 21. A standard curve was made for reference gene by serial dilutions of known amounts of DNA from 100 ng to 3.125 ng. The telomere signal was normalized to Rplp0 to generate a T/S ratio indicative of relative telomere length.

Immunohistochemistry with Telomere FISH

High-quality metaphase spreads were prepared as described for Q-FISH method. Slides were unmasked for 1 hour 30 min at 90°C, dehydrated, and incubated for 5 min at 83°C with Telomere Probe Alexa488. Slides were allowed to anneal at room temperature for 1 hour. Slides were incubated at 4° C overnight with primary antibody in block solution. Primary

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

antibodies used: Rabbit anti Zscan4 (1:5000), Mouse anti H2AX (1:1000), Goat anti SPO11 (1:400), Goat anti-DMC1 (1:200). Slides were incubated for 1 hour at room temperature with secondary antibodies (diluted in block solution): Alexa 568 Donkey anti Rabbit (1:800), Alexa647 Donkey anti-Mouse (1:200), Alexa569 anti-Goat (1:800). Cells were visualized by Zeiss 510-confocal microscope after staining nuclei with DAPI for 10 min at room temperature.

Telomerase activity measurement

All the cells were cultured in triplicate on gelatin-coated dishes for 3 days in complete ES medium in the presence (Dox+) or absence of doxycycline (Dox−). Cell lysates were prepared from 10^6 cells per sample. Telomerase activity was measured by TRAP assay using a TRAPEZE Telomerase Detection Kit (Millipore) according to the manufacturer's instructions.

G-banding karyotype Analysis

ES cells were treated with 0.1 μg/ml colcemid (Invitrogen) for 2 hour to induce metaphase arrest. Metaphase chromosome spreads were prepared, slides were incubated overnight at 65°C, treated with 0.1% trypsin for 1 min, and stained with 3% Giemsa reagent for 15 min. Karyotype analysis was carried out by SmartType software for n 65 metaphases per sample (see Fig. 2e).

Sister Chromatid Exchange (SCE) assay

SCE assay was done as previously described in details 50. Briefly, all mouse ES cells were maintained in complete ES medium containing doxycycline. For Zscan4 induction, doxycycline was removed from the medium for total of 3 days. Medium was added with BrdU for the last 24 hours, allowing the cells to complete 2 cell cycles. Medium was supplemented with 0.1 μg/ml colcemid for the last 4 hours to arrest the cells in metaphase. Metaphase spreads were prepared and SCE were counted in n>50 metaphases per sample. 3–4 independent experiments were carried out for each sample (total of n>150 metaphases).

Immunohistochemistry

Cells were plated in 24-well plates on sterilized coverslips. Cells in duplicate were maintained in Dox+ condition, whereas cells in triplicate were maintained in Dox− condition for 3 days to induce Zscan4 overexpression. Medium was changed every day. Cells were either fixed in 4% PFA for 10 min at room temperature or taken for metaphase spreads as described above. Cells in PFA were permeabilized with 0.25% NP-40 for 10 min. Cells were blocked for 10 min at room temperature in 1% BSA, 10% fetal bovine serum, and 0.2% saponin and incubated overnight at 4°C with the primary antibodies in a blocking solution: anti-FLAG antibody (1:1000), anti-ZSCAN4 (1:1000), anti-SPO11 (1:200), anti DMC1 (1:200), anti-TRF1 (1:500), anti-TRF2 (1:400), γ-H2AX (1:1000). As negative controls, cells stained without primary antibody were used as well as the Dox+ cells stained with Anti-Flag antibody. The bound antibody was visualized with a fluorescent Alexa546 secondary antibody (Invitrogen) under a Zeiss 510-confocal microscope. Nuclei were visualized with DAPI (Roche) staining for 5 min at room temperature.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Generation of Zscan4 antibodies

Custom-made polyclonal Rabbit anti-Zscan4 antibodies were generated (Genscript) against the C-terminal epitope of Zscan4: CSTYHRHLRNYHRSD (the C-terminal cysteine was added for KLH conjugation). This peptide is specific and common for predicted amino acids sequence of all Zscan4 paralogs, and thus, the antibody can recognize all the ZSCAN4 paralogs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Toshio Hamatani, Yuhki Nakatake, Manuela Monti, Li Xin, Bernard Binder, and Alexei A. Sharov for discussion; Yulan Piao, Cuong Nguyen, David Eckley, Ilya Goldberg, Dawood Dudekula, and Ilaria Stanghellini for technical assistance; Philippe Soriano for providing ROSA26-Floxed LacZ cells; and Hitoshi Niwa for providing ROSA-tet-inducible system. This work was entirely supported by the Intramural Research Program of the National Institute on Aging, National Institutes of Health.

References

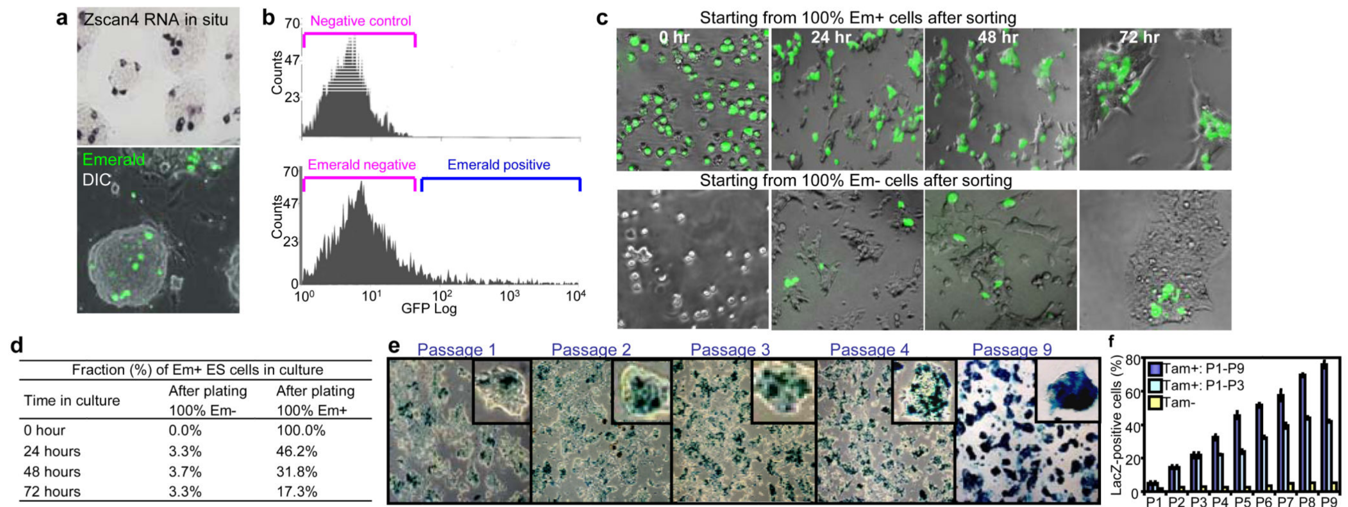
1. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981; 78:7634–7638. [PubMed: 6950406]
2. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981; 292:154–156. [PubMed: 7242681]
3. Yoshikawa T, et al. High-throughput screen for genes predominantly expressed in the ICM of mouse blastocysts by whole mount in situ hybridization. Gene Expr Patterns. 2006; 6:213–224. [PubMed: 16325481]
4. Niwa H. How is pluripotency determined and maintained? Development. 2007; 134:635–646. [PubMed: 17215298]
5. Suda Y, Suzuki M, Ikawa Y, Aizawa S. Mouse embryonic stem cells exhibit indefinite proliferative potential. J Cell Physiol. 1987; 133:197–201. [PubMed: 3667706]
6. Rebuzzini P, Neri T, Zuccotti M, Redi CA, Garagna S. Chromosome number variation in three mouse embryonic stem cell lines during culture. Cytotechnology. 2008; 58:17–23. [PubMed: 19002773]
7. Longo L, Bygrave A, Grosveld FG, Pandolfi PP. The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism. Transgenic Res. 1997; 6:321–328. [PubMed: 9322369]
8. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A. 1993; 90:8424–8428. [PubMed: 8378314]
9. Cervantes RB, Stringer JR, Shao C, Tischfield JA, Stambrook PJ. Embryonic stem cells and somatic cells differ in mutation frequency and type. Proc Natl Acad Sci U S A. 2002; 99:3586–3590. [PubMed: 11891338]
10. Blelloch RH, et al. Nuclear cloning of embryonal carcinoma cells. Proc Natl Acad Sci U S A. 2004; 101:13985–13990. [PubMed: 15306687]
11. Brimble SN, et al. Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. Stem Cells Dev. 2004; 13:585–597. [PubMed: 15684826]
12. Falco G, et al. Zscan4: a novel gene expressed exclusively in late 2-cell embryos and embryonic stem cells. Dev Biol. 2007; 307:539–550. [PubMed: 17553482]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

13. Carter MG, et al. An in situ hybridization-based screen for heterogeneously expressed genes in mouse ES cells. Gene Expr Patterns. 2008; 8:181–198. [PubMed: 18178135]
14. Storm MP, et al. Characterization of the phosphoinositide 3-kinase-dependent transcriptome in murine embryonic stem cells: identification of novel regulators of pluripotency. Stem Cells. 2009; 27:764–775. [PubMed: 19350676]
15. Edelstein LC, Collins T. The SCAN domain family of zinc finger transcription factors. Gene. 2005; 359:1–17. [PubMed: 16139965]
16. Feil R, Wagner J, Metzger D, Chambon P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem Biophys Res Commun. 1997; 237:752–757. [PubMed: 9299439]
17. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999; 21:70–71. [PubMed: 9916792]
18. Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol. 1985; 87:27–45. [PubMed: 3897439]
19. Nishiyama A, et al. Uncovering early response of gene regulatory networks in ESCs by systematic induction of transcription factors. Cell Stem Cell. 2009; 5:420–433. [PubMed: 19796622]
20. Cawthon RM. Telomere measurement by quantitative PCR. Nucleic Acids Res. 2002; 30:e47. [PubMed: 12000852]
21. Callicott RJ, Womack JE. Real-time PCR assay for measurement of mouse telomeres. Comp Med. 2006; 56:17–22. [PubMed: 16521855]
22. Poon SS, Martens UM, Ward RK, Lansdorp PM. Telomere length measurements using digital fluorescence microscopy. Cytometry. 1999; 36:267–278. [PubMed: 10404142]
23. Liu L, et al. Telomere lengthening early in development. Nat Cell Biol. 2007; 9:1436–1441. [PubMed: 17982445]
24. Bailey SM, Goodwin EH, Meyne J, Cornforth MN. CO-FISH reveals inversions associated with isochromosome formation. Mutagenesis. 1996; 11:139–144. [PubMed: 8671729]
25. Wang Y, et al. An increase in telomere sister chromatid exchange in murine embryonic stem cells possessing critically shortened telomeres. Proc Natl Acad Sci U S A. 2005; 102:10256–10260. [PubMed: 16000404]
26. Dronkert ML, et al. Mouse RAD54 affects DNA double-strand break repair and sister chromatid exchange. Mol Cell Biol. 2000; 20:3147–3156. [PubMed: 10757799]
27. Tateishi S, et al. Enhanced genomic instability and defective postreplication repair in RAD18 knockout mouse embryonic stem cells. Mol Cell Biol. 2003; 23:474–481. [PubMed: 12509447]
28. Keeney S, Giroux CN, Kleckner N. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. Cell. 1997; 88:375–384. [PubMed: 9039264]
29. Mahadevaiah SK, et al. Recombinational DNA double-strand breaks in mice precede synapsis. Nat Genet. 2001; 27:271–276. [PubMed: 11242108]
30. Reinholdt LG, Schimenti JC. Mei1 is epistatic to Dmc1 during mouse meiosis. Chromosoma. 2005; 114:127–134. [PubMed: 15928951]
31. Revenkova E, et al. Cohesin SMC1 beta is required for meiotic chromosome dynamics, sister chromatid cohesion and DNA recombination. Nat Cell Biol. 2004; 6:555–562. [PubMed: 15146193]
32. Chong L, et al. A human telomeric protein. Science. 1995; 270:1663–1667. [PubMed: 7502076]
33. Tanaka TS. Transcriptional heterogeneity in mouse embryonic stem cells. Reprod Fertil Dev. 2009; 21:67–75. [PubMed: 19152747]
34. Toyooka Y, Shimosato D, Murakami K, Takahashi K, Niwa H. Identification and characterization of subpopulations in undifferentiated ES cell culture. Development. 2008; 135:909–918. [PubMed: 18263842]
35. Hayashi K, Lopes SM, Tang F, Surani MA. Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. Cell Stem Cell. 2008; 3:391–401. [PubMed: 18940731]

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

36. 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]
37. Niida H, et al. Telomere maintenance in telomerase-deficient mouse embryonic stem cells: characterization of an amplified telomeric DNA. Mol Cell Biol. 2000; 20:4115–4127. [PubMed: 10805753]
38. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med. 1997; 3:1271–1274. [PubMed: 9359704]
39. Cesare AJ, Reddel RR. Telomere uncapping and alternative lengthening of telomeres. Mech Ageing Dev. 2008; 129:99–108. [PubMed: 18215414]
40. De Boeck G, Forsyth RG, Praet M, Hogendoorn PC. Telomere-associated proteins: cross-talk between telomere maintenance and telomere-lengthening mechanisms. J Pathol. 2009; 217:327–344. [PubMed: 19142887]
41. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J. 1995; 14:4240–4248. [PubMed: 7556065]
42. Gonzalo S, et al. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol. 2006; 8:416–424. [PubMed: 16565708]
43. Laud PR, et al. Elevated telomere-telomere recombination in WRN-deficient, telomere dysfunctional cells promotes escape from senescence and engagement of the ALT pathway. Genes Dev. 2005; 19:2560–2570. [PubMed: 16264192]
44. Ding H, et al. Regulation of murine telomere length by Rtel: an essential gene encoding a helicase-like protein. Cell. 2004; 117:873–886. [PubMed: 15210109]
45. Aiba K, et al. Defining developmental potency and cell lineage trajectories by expression profiling of differentiating mouse embryonic stem cells. DNA Res. 2009; 16:73–80. [PubMed: 19112179]
46. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126:663–676. [PubMed: 16904174]
47. Olson LE, et al. Protection from doxorubicin-induced cardiac toxicity in mice with a null allele of carbonyl reductase 1. Cancer Res. 2003; 63:6602–6606. [PubMed: 14583452]
48. Masui S, et al. An efficient system to establish multiple embryonic stem cell lines carrying an inducible expression unit. Nucleic Acids Res. 2005; 33:e43. [PubMed: 15741176]
49. Goodwin E, Meyne J. Strand-specific FISH reveals orientation of chromosome 18 alphoid DNA. Cytogenet Cell Genet. 1993; 63:126–127. [PubMed: 8467711]
50. Perry P, Wolff S. New Giemsa method for the differential staining of sister chromatids. Nature. 1974; 251:156–158. [PubMed: 4138930]
51. Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, R. Manipulating the Mouse Embryo: A Laboratory Manual. New York, New York: Cold Spring Harbor Laboratory Press; 2003.
52. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–408. [PubMed: 11846609]

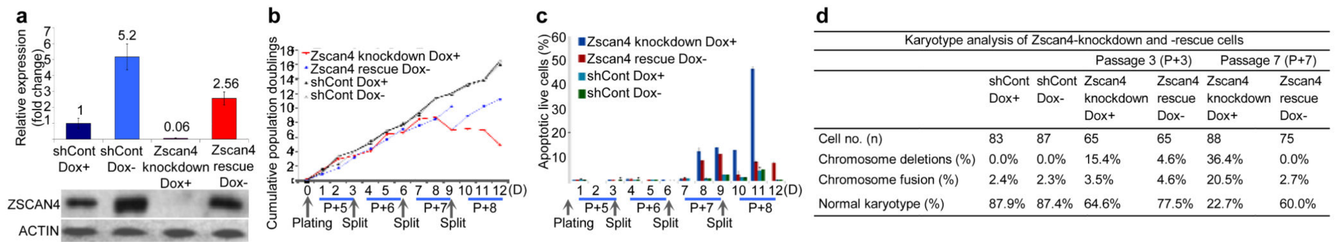


Time in culture	Fraction (%) of Em+ ES cells in culture: After plating 100% Em-	After plating 100% Em+
0 hour	0.0%	100.0%
24 hours	3.3%	46.2%
48 hours	3.7%	31.8%
72 hours	3.3%	17.3%

Figure 1. Zscan4 is transiently expressed in ES cells

a, Zscan4 expression visualized by RNA in situ hybridization and GFP-Emerald reporter gene (*green*). **b**, FACS analysis of pZscan4-Emerald cells. **c**, Time-course fluorescent/DIC images of pZscan4-Emerald cells after sorting and culturing only Em+ cells or Em− cells. **d**, Flow cytometry analysis of cells shown in **c**. **e**, Images of pZscan4-CreERT2 cells maintained in tamoxifen and stained with X-gal at each passage. Inserts: a single colony. **f**, A % fraction of LacZ+ cells measured by flow cytometry analysis using CMFDG fluorescence LacZ assay: tamoxifen was present continuously (Tam+: P1–P9), only for the first three passages (Tam+: P1–P3), or absent (Tam−, control).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript



Karyotype analysis of Zscan4-knockdown and -rescue cells

			Passage 3 (P+3)		Passage 7 (P+7)	
	shCont Dox+	shCont Dox-	Zscan4 knockdown Dox+	Zscan4 rescue Dox-	Zscan4 knockdown Dox+	Zscan4 rescue Dox-
Cell no. (n)	83	87	65	65	88	75
Chromosome deletions (%)	0.0%	0.0%	15.4%	4.6%	36.4%	0.0%
Chromosome fusion (%)	2.4%	2.3%	3.5%	4.6%	20.5%	2.7%
Normal karyotype (%)	87.9%	87.4%	64.6%	77.5%	22.7%	60.0%

Figure 2. Characterization of Zscan4 knockdown cells

a, Confirmation of Zscan4 knockdown and rescue by qPCR analysis using a common primer for all Zscan4 paralogs (top) and Western blot analysis (bottom). Cells were cultured for 3 days in Dox+ or Dox− conditions. shCont (control shRNA in the same parental cells) was used to exclude off-target effects. See Supplementary Fig 7 and 8 for additional controls. **b**, Reduction of proliferation by Zscan4 knockdown, until cells died abruptly at passage 8 (P +8). Rescue improved proliferation. Assays were done in triplicate in four independent experiments. **c**, Annexin-V Apoptosis assay performed by flow cytometry. **d**, Karyotype deterioration seen after Zscan4 knockdown.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

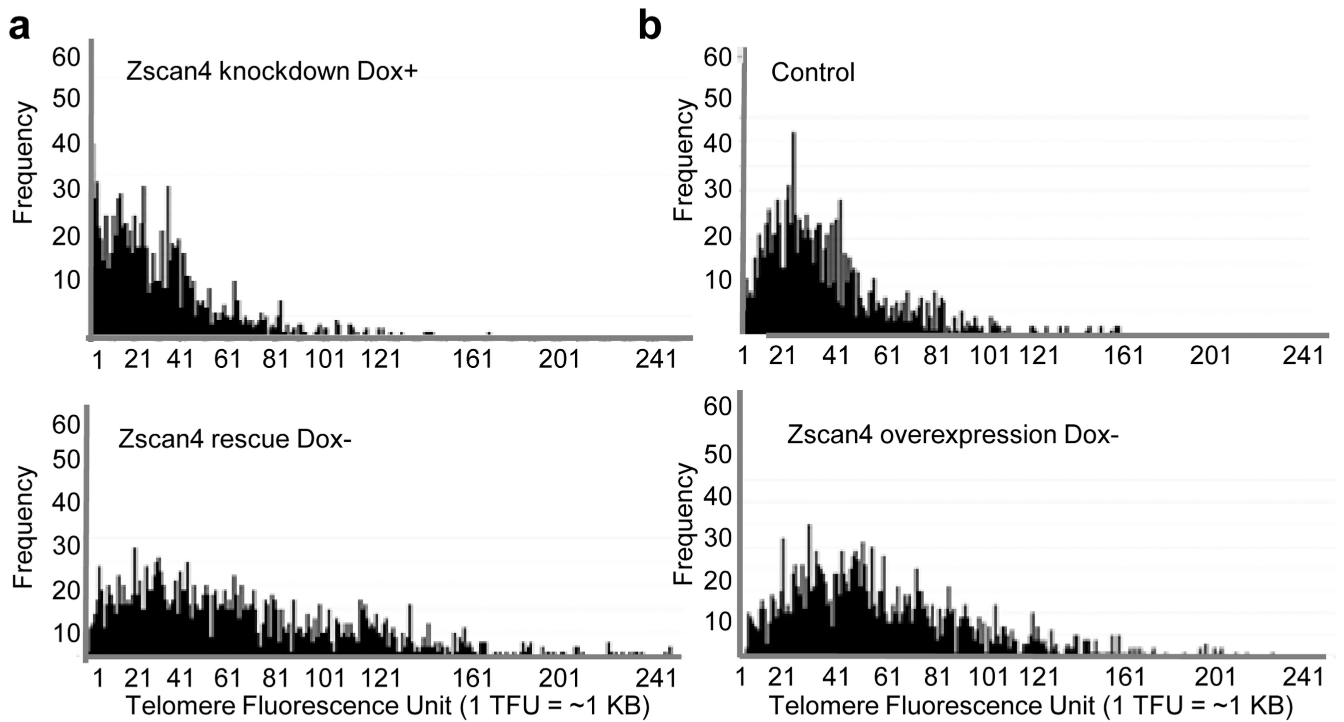
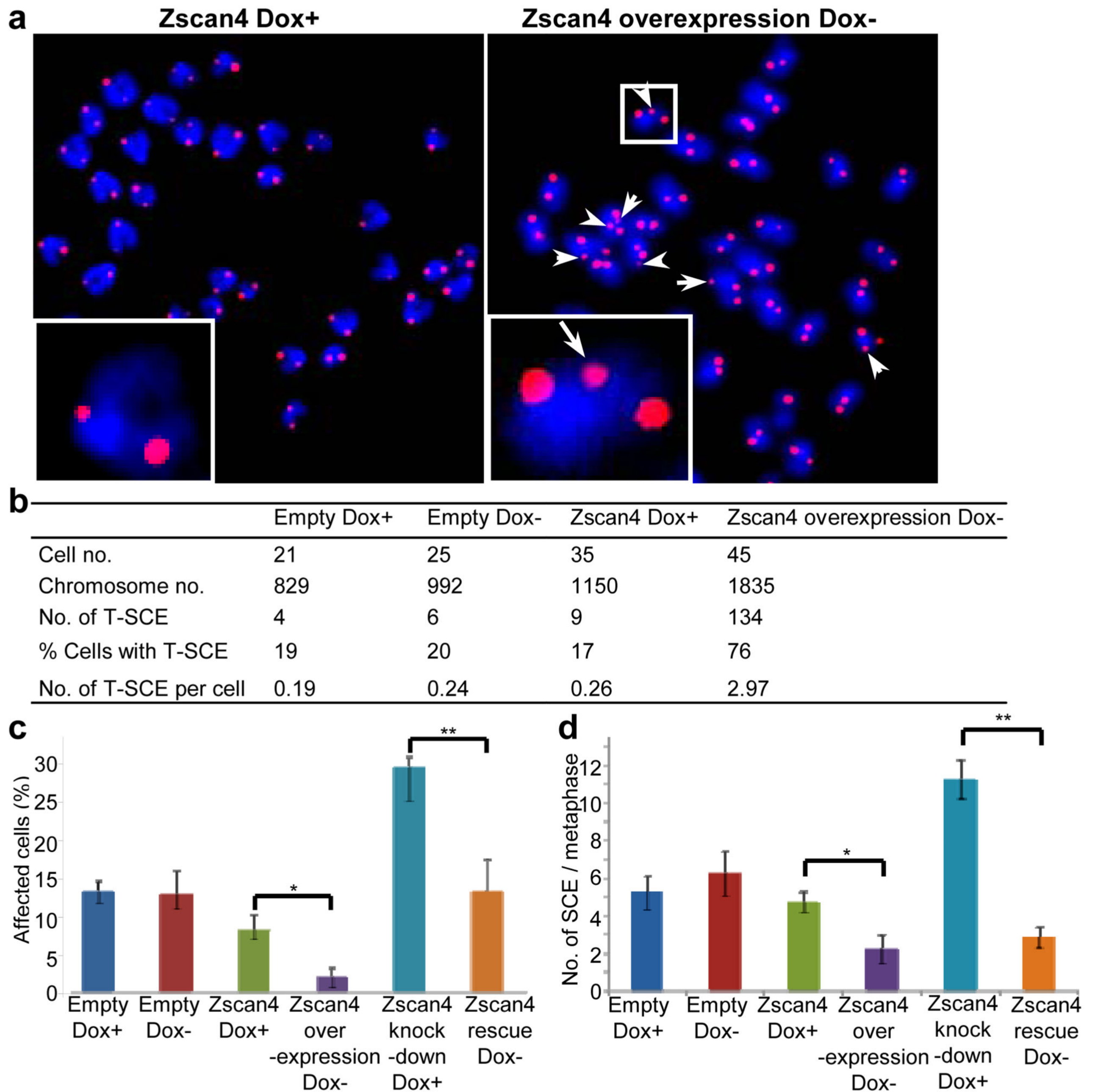


Figure 3. Zscan4 regulates telomere length

a, A distribution diagram of relative telomere length, analyzed by Q-FISH and TFL-Telo software (results of 10 pooled nuclei, totaling 1,600 telomeres). **b**, A distribution of relative telomere length in Zscan4c-overexpressing cells.

Author Manuscript



	Empty Dox+	Empty Dox-	Zscan4 Dox+	Zscan4 overexpression Dox-
Cell no.	21	25	35	45
Chromosome no.	829	992	1150	1835
No. of T-SCE	4	6	9	134
% Cells with T-SCE	19	20	17	76
No. of T-SCE per cell	0.19	0.24	0.26	2.97

Figure 4. Zscan4 promotes T-SCE but inhibits spontaneous SCE in non-telomeric regions

a, T-SCE (arrows) shown by chromosome orientation FISH (CO-FISH): chromosomes stained with DAPI (*blue*); telomeres marked with a Cy3-cojugated telomere probe (*red*). Left, tet-Zscan4c cells (Dox+). Right, Zscan4-overexpressing cells (Dox−). **b**, A summary of total T-SCE events in >20 nuclei/sample based on three independent experiments. **c**, The number of cells with spontaneous genomic SCEs was decreased by Zscan4 overexpression (*P=0.01) but increased by knockdown (**P=0.005). **d**, The number of SCEs per metaphase was reduced by Zscan4 overexpression (*P=0.02) but elevated by knockdown

(**P=0.0016). Assays were done in three independent experiment with 50 metaphases/ sample (total n=150). Error bars indicate s.e.m.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

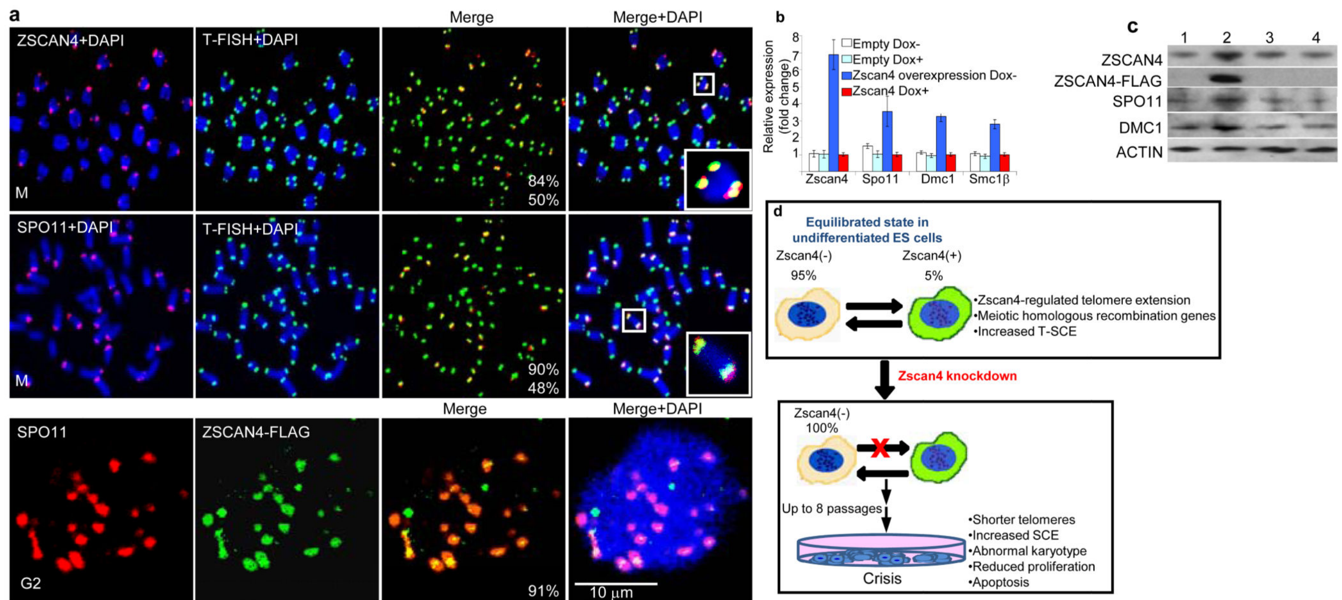


Figure 5. ZSCAN4 forms foci on telomeres along with meiosis specific homologous recombination mediators

a, confocal microscope images with chromosomal DNAs stained with DAPI (*blue*). Top, colocalization of ZSCAN4 (*red*, immunostaining) and telomeres (*green*, T-FISH using Alexa488-conjugated DNA probe). Middle, SPO11 localized on telomeres following Zscan4c induction for 3 days. Bottom, colocalization of ZSCAN4-FLAG foci (*green*: immunostaining with antibodies against FLAG, fused to ZSCAN4 ORF19) and SPO11 (*red*). **b**, qPCR analysis showing upregulation of meiosis-specific homologous recombination genes, following Zscan4c overexpression. **c**, Western blot confirmed **b** at protein levels. Lanes 1, Zscan4 Dox+; 2, Zscan4 overexpression Dox−; 3, Empty Dox+; and 4, Empty Dox−. **d**, A model for Zscan4 action in ES cells.