**Zscan5b Deficiency Impairs DNA Damage Response and Causes Chromosomal Aberrations during Mitosis**

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**SUMMARY**

Zygotic genome activation (ZGA) begins after fertilization and is essential for establishing pluripotency and genome stability. However, it is unclear how ZGA genes prevent mitotic errors. Here we show that knockout of the ZGA gene Zscan5b, which encodes a SCAN domain with C2H2 zinc fingers, causes a high incidence of chromosomal abnormalities in embryonic stem cells (ESCs), and leads to the development of early-stage cancers. After irradiation, with C2H2 zinc fingers, causes a high incidence of chromosomal abnormalities in embryonic stem cells (ESCs), and leads to the development of early-stage cancers. After irradiation, Zscan5b-deficient ESCs displayed significantly increased levels of γ-H2AX despite increased expression of the DNA repair genes Rad51L3 and Bard. Re-expression of Zscan5b reduced γ-H2AX content, implying a role for Zscan5b in DNA damage repair processes. A co-immunoprecipitation analysis showed that Zscan5b bound to the linker histone H1, suggesting that Zscan5b may protect chromosomal architecture. Our report demonstrates that the ZGA gene Zscan5b is involved in genomic integrity and acts to promote DNA damage repair and regulate chromatin dynamics during mitosis.

**INTRODUCTION**

The occurrence of de novo chromosomal abnormalities is a major problem in the use of assisted reproductive technology. Understanding when and how these genomic errors occur is essential to the field of reproductive medicine. In a preimplantation genetic testing for aneuploidies (PGT-A) using 24-chromosome single-nucleotide polymorphisms, approximately 40% of human embryos were found to be aneuploid (Demko et al., 2016). Aneuploidy as a consequence of meiotic chromosome malsegregation is mainly the result of events in oocytes and not spermatoocytes: the frequency of maternally derived aneuploidy increases from about 10%–20% to >60% with increasing maternal age (reviewed in Hassold and Hunt, 2001).

Another source of chromosomal anomalies in embryos is postfertilization mitotic division errors that result in embryonic mosaicism (Vanneste et al., 2009). In contrast to meiotic errors, it was found that the incidence of mitotic aneuploidy was not associated with maternal age in either day 3 blastomere biopsies or day 5 trophectodermal biopsies (Capalbo et al., 2013; McCoy et al., 2015). A PGT-A of 28,052 day 3 human embryos indicated that more than 25% harbored aneuploidies of mitotic origin; moreover, the incidence of mitotic aneuploidy exceeded maternally derived meiotic aneuploidy in embryos of women less than 40 years old (reviewed in McCoy, 2017). These mitotic errors produce karyotypically distinct cell lineages within an embryo, which is then termed a mosaic embryo. Mosaicism occurs in ~15%–90% of all cleavage stage human embryos (Daphnis et al., 2008; Harper et al., 1995; Rubio et al., 2007). Kort et al. (2016) showed that 48% (43/89) of cleavage-stage embryos contained two or more abnormal nuclei; moreover, DNA damage, as indicated by γ-H2AX (phosphorylated [Ser139] histone H2A.X) and replication protein A (RPA) staining, was significantly elevated in cells with micronuclei (74/85, 62/85) compared with cells with normal nuclear morphology (60/642, 36/642) (Kort et al., 2016). Nevertheless, little is known about the molecular basis of mitotic errors and the consequences for mosaic embryonic survival.

After fertilization, the maternal genetic program governed by maternally derived RNAs and proteins is switched to the embryonic genetic program by de novo transcription; this switch is termed zygotic genome activation (ZGA) (Hamatani et al., 2004). Nuclear transfer experiments in human somatic cells have demonstrated that ZGA failure can lead to embryonic developmental arrest (Noggle et al., 2011; Yamada et al., 2014). Therefore, ZGA is one of the first and most critical events in animal development. At present two ZGA genes that are specifically expressed during the activation process in embryonic stem cells (ESCs) have been identified, namely, Zscan4 and Hmgpi. Zscan4 is essential for long-term culture of ESCs and...
Figure 1. Zscan5b Is a Zygotic Genome Activation Gene which Is Specifically Expressed during Preimplantation Stages and in Embryonic Stem Cells

(A) Real-time qPCR analysis of Zscan5b expression in adult tissues and during postimplantation development (E7–E17). mRNA was isolated from mouse tissues (B, brain; H, heart; K, kidney; Li, liver; Lu, lung; M, muscle; S, spleen; T, testis). The expression levels were normalized against the reference gene Gapdh. Values are means ± SEM from three technical replicates.

(B) Real-time qPCR analysis of Zscan5b expression during preimplantation development. Three sets of 10 pooled embryos were collected from unfertilized eggs (U), fertilized eggs (F), two-cell embryos (2), four-cell embryos (4), eight-cell embryos (8), morulae (M), and blastocysts (B) are shown after normalization to an internal reference gene (mouse Chuk). Values are means ± SEM from four separate experiments. The total number of samples and the number of individual experiments (in parentheses) contributing to a particular experiment are indicated above each column.

(C) Zscan5b is transcribed in embryonic stem cells (ESCs), but not in differentiated embryoid bodies (EBs). The expression levels were normalized against the reference gene Gapdh. Values are means ± SEM from three separate experiments. The total number of samples and the number of individual experiments (in parentheses) contributing to a particular experiment are indicated above each column.

(D) De novo (zygotic) transcription of the Zscan5b gene. Application of α-amanitin, an RNA polymerase II inhibitor, showed that Zscan5b is transcribed zygotically, but not maternally. Zscan5b expression was not observed before the two-cell stage, and α-amanitin completely inhibited de novo transcription at the two-cell stage. The expression levels were normalized using H2afz as a reference gene. Values are means ± SEM from two separate experiments. The total number of samples and the number of individual experiments (in parentheses) contributing to a particular experiment are indicated above each column.

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maintenance of chromosomal integrity during telomere elongation (Falcon et al., 2007; Zalzman et al., 2010). Hsgpi is responsible for DNA replication and has a role in peri-implantation development and ESC isolation (Yamada et al., 2010).

Another ZGA gene, Zscan5b, is predicted to encode a SCAN domain with C2H2 zinc fingers (C2H2-ZF). Zinc-finger genes represent a significant portion of the genes in the vertebrate genome (reviewed in Edelstein and Collins, 2005). The DNA-binding sites, interacting proteins, and transcriptional responses to genetic perturbation of C2H2-ZF proteins have recently been characterized (Schmitges et al., 2016). The SCAN domain, a leucine-rich region, functions as a protein interaction domain that mediates self-association or selective association with other proteins (Edelstein and Collins, 2005). These structural characteristics suggest that Zscan5b plays a role in genomic stability either independently or in combination with other factors for telomere elongation, such as Zscan4 (Zalzman et al., 2010), or for apoptosis-dependent DNA damage response, such as Zscan10 (Skamagki et al., 2017).

Although the origins of chromosomal abnormalities are well documented in the literature and are known to contribute to female infertility, the molecular mechanisms of these abnormalities are not well understood. In the present study, we hypothesized that the ZGA gene Zscan5b is involved in genome stability during mitosis. We tested this hypothesis by determining whether Zscan5b-deficient mice could act as a model for spontaneous mitotic chromosomal errors, and also investigated the roles of this ZGA gene in genome stability after fertilization and in developmental competency. Our analyses show that Zscan5b deficiency causes chromosomal abnormalities, and leads to the growth of early-stage cancerous lesions.

RESULTS

Gene Structure and Expression of the ZGA Gene Zscan5b

An in silico analysis showed that mouse Zscan5b was expressed specifically in preimplantation embryos. Gene expression profiling indicated that Zscan5b transcript levels were upregulated during ZGA at the one-to four-cell embryo stages (Boroviak et al., 2018; Hamatani et al., 2004; Wang et al., 2004) (Figures S1A and S1B). RNA sequencing analysis of preimplantation embryos indicated that human ZSCAN5B levels peaked at the eight-cell stage (Figure S1C) (Boroviak et al., 2018), suggesting that human ZSCAN5B is also transcribed zygotically during the major burst of ZGA. Three primate-specific paralogs, ZSCAN5A, ZSCAN5C, and ZSCAN5D, have been identified that are divergent from the parental gene (Sun et al., 2016); the level of primate ZSCAN5A peaked at the eight-cell stage (Figure S1D) (Boroviak et al., 2018). Analysis of a public expressed sequence tag (EST) database identified 21 cDNA clones that were found exclusively in preimplantation embryos (two-cell to morula stages) (Figure S1E). One of these clones (AK13965.1) contained the full Zscan5b gene coding sequence, which spans 1,980 bp and has 2 exons; SMART domain prediction analysis indicated the gene encodes a putative protein of 468 amino acids (NP_001028965.1) that harbors a SCAN domain and five zinc-finger domains (Schultz et al., 1998) (Figure S1F). In the NCBI Gene database, the Zscan5b gene has three transcripts (splice variants). Two of three splice variants contain a full-length open reading frame encoding a single protein model. The Zscan5b gene is predicted to be conserved in Homo sapiens, and pairwise alignment scores by a BLAST search of amino acid sequences for the human ortholog indicated 54% similarity (Figure S1G).

Experimental analyses were carried out to confirm the results of the in silico analysis that indicated a preimplantation stage-specific expression pattern for Zscan5b. A real-time PCR analysis using cDNA isolated from mouse adult tissues and fetuses (embryonic day [E]7, E11, E15, and E17) detected Zscan5b expression in all tested tissues (Figure 1A). Real-time qPCR analysis of preimplantation embryos indicated that Zscan5b mRNA levels increased during the one-to two-cell stages, peaked at the four-cell stage, and then gradually decreased during the eight-cell to blastocyst stages (Figures 1B, S1H, and S1I). The real-time qPCR results were separately normalized against the internal standard genes Chuk, H2afz, and Gapdh (Falco et al., 2006; Mamo et al., 2007) to ensure their reproducibility. The normalization procedure confirmed the in silico prediction of a preimplantation stage-specific expression pattern of Zscan5b. Expression of Zscan5b was found in isolated mouse ESCs, whereas it was downregulated in differentiated tissues.
We then performed real-time qPCR analysis using α-amanitin, an RNA polymerase II inhibitor, to investigate de novo (zygotic) transcription of the Zscan5b gene. The presence of α-amanitin during in vitro culture from the one-cell stage significantly reduced Zscan5b mRNA expression in embryos at 43 and 53 h post-human chorionic gonadotropin (hCG) (early and late two-cell stages, respectively) (Figure 1D), suggesting that Zscan5b is transcribed zygotically during the major burst of ZGA. Our results contradict a previous report showing ubiquitous expression of Zscan5b (Sun et al., 2016), although both studies detected high expression in early embryos and in the testis.

**Level of the ZSCAN5B Protein**

To study the temporal and spatial distribution of the protein (ZSCAN5B) encoded by Zscan5b in mice, we raised a polyclonal antibody against ZSCAN5B peptides. Our gene expression analysis indicated that Zscan5b transcription started at the two-cell stage, peaked at the four-cell stage, and then gradually decreased until the blastocyst stage (Figure 1E); immunostaining with the polyclonal antibody showed the presence of ZSCAN5B from the two-cell stage until the blastocyst stage. ZSCAN5B was also present in both inner cell mass cells and trophectodermal cells of blastocysts.

ZSCAN5B was detected in the cell nucleus and cytoplasm throughout the preimplantation development (Figures 1E and 1F); however, it was exclusively localized to nuclei in eight-cell, morula, and late-blastocyst stages. ESCs isolated from blastocyst embryos were ZSCAN5B positive, with distribution mainly restricted to the nucleus, suggesting a specific role as a nuclear protein in preimplantation embryos and ESCs (Figure 1G). We also found that the OCT4 protein colocalized with ZSCAN5B in the nucleus of ESCs (Figure 1G). These results indicate that ZSCAN5B is a putative nuclear protein.

**Zscan5b Is Not Essential for Fertility**

Our in silico analysis demonstrated that the Zscan5b gene consisted of seven exons (Figure S1F). To investigate the function of the ZSCAN5B protein in ESCs, we constructed a plasmid, pNT1.1, carrying a modified Zscan5b sequence in which the second and third exons were replaced with a neomycin resistance cassette (Neo) (Figure 2A). The construct was introduced into C57BL/6 mouse ESCs by electroporation, and colonies with resistance to the drug G-418 were screened for homologous recombination. Using the Neo targeting vector, 192 colonies were screened by Southern blotting (data not shown), and 14 (7.3%) heterozygous ESCs were isolated. To study the in vivo function of Zscan5b, we injected five Neo heterozygous ESC lines into C57BL/6 blastocysts. Germline transmission was obtained from three independent ESC clones. Among 64 mice born from heterozygous intercrosses, 12 were wild-type, 27 were heterozygous, and 24 were homozygous. Litter sizes of the intercrossed homozygotes were not significantly lower than those of wild-type mice (Figures 2B and 2C). And intercrossed homozygous mice produced second-generation homozygotes.

**Zscan5b Is Not Essential for Acquiring Self-Renewal Potential in the Derivation of ESCs**

To identify ESCs homozygous for the introduced gene mutation, blastocysts were tested in an outgrowth assay. Zscan5b-deficient embryos showed vigorous outgrowth, leading to the derivation of ESCs that were alkaline phosphatase (ALP) positive (Figure 3A). Thirteen Zscan5b homozygous ESC lines were isolated, and these clones were confirmed...
Figure 3. Expression of Pluripotency Markers in Zscan5b-Deficient ESCs Is Comparable with Wild-Type ESCs

(A) A Zscan5b-deficient blastocyst and blastocyst outgrowth are shown in the upper panel. Morphological appearance of Zscan5-deficient ESCs and ALP staining (purple) of Zscan5b-deficient ESCs are shown in the lower panel. Scale bars represent 200 and 50 μm.

(B and C) Southern blotting analysis-based genotyping (B) and PCR-based genotyping (C) to distinguish between wild-type, Zscan5b heterozygous (+/-) and Zscan5b-deficient (-/-) ESCs.

(D) Reverse transcription PCR analysis of Zscan5b mRNA expression in wild-type and Zscan5b +/- ESCs.

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to be homozygous for the Zscan5b mutation by Southern blotting (Figure 3B) and genomic PCR (Figure 3C). Reverse transcription PCR and real-time qPCR analysis further confirmed the lack of Zscan5b expression in homozygous cells (Figures 3D and 3E). All of these cell lines were positive for pluripotency markers (Figures 3E–3I). To test whether Zscan5b contributes to normal development, a chimera assay was performed; this assay indicated that Zscan5b-deficient ESCs could contribute to the whole body (Figures 3J and 3K). These results suggest that Zscan5b does not contribute to ESC derivation or stemness as pluripotent stem cells.

**Differentiation Assays In Vitro and In Vivo**

In embryoid bodies, Zscan5b-deficient ESC lines gave rise to cell types in all three germ layers (Figure 4A). Surprisingly, on transplantation into immunocompromised mice, all Zscan5b homozygous ESC lines developed small malignant germ cell tumors (Figure 4B). The tumors primarily exhibited the histology of mature teratomas, and consisted of tridermic elements, with scattered foci of immature teratomas mainly consisting of neural tissues. Furthermore, adjacent to the immature teratoma components, a small number (1–3 per tumor) of tiny foci with malignant germ cell tumor components, histologically distinct from the immature teratoma cells, were present. The size of these lesions ranged from 0.5 to 3 mm in diameter, with most being around 1 mm. We classified these lesions as choriocarcinomas and embryonal carcinomas. However, the very small sizes and the multiplicity of these apparently malignant germ cell tumor components, in addition to the absence of metastatic lesions outside the tumor, might indicate that they were very early-stage cancerous lesions rather than authentic, fully malignant germ cell tumors with metastasizing potential. The tumor sizes developed from Zscan5b-deficient ESC lines was comparable with that from wild-type ESC lines (Figures 4C and 4D).

**DNA Repair Is Mediated through Zscan5b**

The mechanisms underlying the tumorigenic characteristics of Zscan5b-deficient ESCs were investigated by X-irradiating the cells and staining for the DNA damage markers γH2AX and RPA. γH2AX foci in nuclei were significantly more frequent in Zscan5b-deficient ESCs than in wild-type ESCs (Figures 5A and 5B). Also, the number of foci of RPA, a single-strand DNA-binding protein, in Zscan5b-deficient ESCs was increased (not significantly) (Figures 5C and 5D), indicating the presence of DNA damage such as DNA double-strand breaks during mitosis.

Global transcriptional changes, including those of DNA repair genes, were investigated in a microarray analysis of three wild-type ESC lines and three Zscan5b-deficient ESC lines (Tables S2 and S3). Using a clustering analysis, we found that the Zscan5b-deficient ESC lines grouped separately from the wild-type ESC cell lines (Figures 5E and 5F). In addition, all three Zscan5b-deficient ESC lines showed elevated expression of the DNA damage repair genes Rad51I3 and Bar1 (Table S2), indicating that Zscan5b deficiency induces susceptibility to DNA damage.

To determine whether DNA damage repair depends on Zscan5b, a piggyBac vector carrying wild-type Zscan5b-GFP driven by a CAG promoter for long-term stable expression was transfected into Zscan5b-deficient ESCs. We found a significant increase in DNA damage after irradiation in Zscan5b-deficient ESCs. However, a lower frequency of γH2AX foci was present in transfected Zscan5b-deficient ESCs (Figures 5G–5I), suggesting that restoration of Zscan5b expression in these cells was sufficient to restore genome stability. Therefore, we conclude that DNA damage can be resolved through Zscan5b-dependent repair pathways.

**Zscan5b Deficiency Increases Chromosomal Aberrations**

Chromosome instability is a feature of many cancers and may be a crucial factor in carcinogenesis. We carried out a chromosomal analysis of Zscan5b-deficient ESCs and somatic cells of homozygous-deficient adult mice. Analysis of Q-banded chromosomes showed that they displayed an increasing number of chromosome breaks, defined as a disruption in the chromosome, in Zscan5b-deficient ESCs (Figures 6A, 6B, and S3). Fusion of chromosome
fragments and unrepaired chromosome breaks are known to be associated with cell malignancy (reviewed in van Gent et al., 2001). Somatic cells from Zscan5b-deficient adults showed chromosome instability with a significantly increased frequency of chromosome gaps, derivative chromosomes, gain or loss of chromosomes, Robertsonian translocations, and chromosome breaks (Figure S4). In particular, the frequency of chromosome gaps in spleen cells of the Zscan5b-deficient mice was significantly higher than in those of wild-type mice (43.0% versus 1.3%, p < 0.001). The positions of chromosome gaps were random in all chromosomes except chromosome 11 (Figures S3 and S4; Table S4). These results imply that Zscan5b deficiency can lead to chromosome instability and stochastically derived chromosome gaps may induce other chromosomal abnormalities such as chromosome breaks.

**ZSCAN5B Binds to Histone H1**

From the results of our various analyses, we propose that Zscan5b likely protects cells against a range of de novo chromosomal abnormalities during mitosis. We further speculate that ZSCAN5B binds either directly or indirectly to
Figure 5. Genomic Instability in Zscan5b-Deficient Cells

(A and B) Immunostaining for γ-H2AX indicating DNA damage (A) and mean γ-H2AX-positive foci (B) in irradiated Zscan5b-deficient ESCs. Scale bars represent 10 μm. The number of analyzed cells is shown above each bar, and the number shown in the parentheses indicates two independent experiments. Error bars, SEM.

(C and D) Immunostaining for RPA (C) and mean RPA-positive foci (D) in Zscan5b-deficient irradiated ESCs. Scale bars represent 10 μm. The number of analyzed cells is shown above each bar, and the number shown in the parentheses indicates two independent experiments. Error bars, SEM. N.S., not significant.

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chromosomes, and helps to form nucleosome structures. To test these speculations, we performed a co-immunoprecipitation analysis using protein extracted from ESCs transfected with 3XFLAG-Zscan5b-EGFP (Figures 6C–6E). The components of bands from 3XFLAG-Zscan5b-EGFP combined proteins were analyzed and the histone H1 family, H1.1, H1.2, and H1.4, was specifically detected in bands 1, 2, and 4 of the antibody-added protein samples (Figure 6F). We cross-validated these results by an immunoprecipitation assay using cells transfected with a pcDNA3.1+C-eGFP plasmid carrying mouse Zscan5b cDNA and three pcDNA3.1-MYC-HIS A plasmids carrying mouse histone 1,1, 1.2, and 1.4 cDNAs (Figure S5). The sizes of the bands corresponded to the molecular sizes of histones H1.1, H1.2, and H1.4 in the antibody-added protein sample. These results support our suggestion that ZSCAN5B binds to histones H1.1, H1.2, and H1.4.

DISCUSSION

Here we have demonstrated that a ZGA gene, Zscan5b, contributes to the maintenance of DNA integrity against cellular DNA damage and de novo chromosomal aberrations during mitosis, thereby preventing development of cancers. Zscan5b-deficient ESCs did not differ from wild-type ESCs line.
ESC's in terms of pluripotency but did display a distinct range of abnormalities including elevated DNA stress and chromosomal abnormalities. Interestingly, spleen cells isolated from Zscan5b-deficient adult mice showed random distribution of gaps in their chromosomes; gaps were defined here as an unstained section in a chromatid. A co-immunoprecipitation analysis showed that ZSCAN5B bound to the linker protein histone H1, suggesting a role in chromatin dynamics during mitosis. Therefore, the Zscan5b gene can be classified as encoding a protein directly involved in DNA repair and protection of chromosomal architecture, and which contributes to preventing the formation of cancerous lesions.

**ZSCAN Family Might Contribute to the Maintenance of Genomic Integrity**

Nuclear reprogramming during the preimplantation developmental period is crucial for genomic integrity. Although induced pluripotent stem cells (iPSCs) have similar pluripotency to ESCs, those derived from aged donors (A-iPSCs) show diminished DNA damage responses and a defect in the apoptotic response to DNA damage; as a consequence, A-iPSCs show a greater number of cells with genetic abnormalities (Skamagki et al., 2017). Somatic cell nuclear transfer (SCNT) has shown that oocytes can reprogram somatic cells into pluripotent stem cells. Unlike A-iPSCs, stem cells derived by SCNT (ntESCs) using the genome of an aged donor and cytoplasm of a young donor (A-ntESCs) show normal DNA damage responses and normal genomic integrity (Skamagki et al., 2017). The key difference here is that there are more reprogramming factors stored in oocytes; moreover, ZGA genes, including Zscan5b, are transcribed more efficiently during preimplantation embryo development than the four “Yamanaka” factors (OCT4, SOX2, KLF4, and c-MYC) (Egli et al., 2008).

Another ZSCAN family gene, ZSCAN10, is specifically expressed in ESCs and iPSCs, and higher expression is shown in iPSCs from younger donors (Y-iPSCs) than in A-iPSCs. Overexpression of ZSCAN10 in conjunction with the four Yamanaka factors can restore impaired genomic integrity in A-iPSCs (Skamagki et al., 2017), implying that ZSCAN10 is responsible for the poor DNA damage response and genomic instability in A-iPSCs. Although it is unclear whether ZSCAN family genes interact with each other, overexpression of the zygotic gene Zscan5b could play a role in restoring genome integrity through DNA repair after the initial DNA damage response in A-iPSCs similar to that in A-ntESCs, as shown here in the rescue experiments.

**Origins of Chromosome Abnormalities in Zscan5b-Deficient Somatic Cells**

A clue to the origin of chromosome abnormalities in Zscan5b-deficient somatic cells can be obtained from the consequences of nuclear translocation of the ZSCAN5B protein. Our immunocytochemical analysis showed that the ZSCAN5B protein was present in both nuclei and cytoplasm at early preimplantation stages, but became predominantly localized to the nucleus from the eight-cell stage. Although the peptide antibody raised against synthetic peptides of ZSCAN5B did not demonstrate high specificity (Figures S2A and S2B), a bipartite nuclear localization signal peptide (RFQCTECKSKLYSFRDLHQRSHTGERPFCKLC) was present in the ZSCAN5B sequence based on the *in silico* analysis (Kosugi et al., 2009). Thus, the nuclear localization of ZSCAN5B after the eight-cell stage implies a spatial requirement. The precise timing of formation of chromosomal abnormalities is unclear. Chromosome breaks were observed in one of the three Zscan5b-deficient ESC lines isolated from the inner cell mass of Zscan5b-deficient blastocysts. In addition, spleen cells from Zscan5b-deficient mice showed a range of chromosomal abnormalities. These observations imply that formation of chromosomal abnormalities is initiated from the peri-implantation period. Because chromosome fragments can induce other chromosome aberrations (reviewed in van Gent et al., 2001), these abnormalities might be accumulated during mitosis without Zscan5b expression.

**Zscan5b Works as a DNA Damage Repair Gene**

Zscan5b-deficient ESCs differentiated into germ cell tumors with multilineage, differentiated cell types. Karyotype analysis of ESCs and spleen cells of Zscan5b-deficient adults identified chromosomal abnormalities, ranging from small insertions or deletions to large chromosomal alterations. The frequency of γ-H2AX foci in Zscan5b-deficient irradiated ESCs was significantly elevated compared with wild-type ESCs. Zscan5b-deficient irradiated ESCs also displayed a trend to increased frequency of RPA foci. RPA relocates into distinct nuclear foci and colocalizes with γ-H2AX at the sites of DNA damage in a time-dependent manner (Balaje and Geard, 2004). These results suggest that DNA repair processes after the initial DNA damage response do not work in Zscan5b-deficient ESCs. However, our rescue experiment showed the presence of a lower frequency of γH2AX foci (Figures S5H and S1), suggesting that restoration of Zscan5b expression in Zscan5b-deficient ESCs was sufficient to restore genome stability. A global transcriptional analysis showed elevated expression of Rad51B and Bard1 in Zscan5b-deficient ESCs (Table S2). RAD51 is a key component of the homologous recombination mechanism of DNA damage repair and is associated with the activation of double-strand break DNA repair (Forget and Kowalczykowski, 2010). BRCA1–BARD1 interacts with RAD51 directly, and BRCA1 performs several functions at the stalled fork and in double-strand break repair (Zhao et al., 2017). In addition, chromatin immunoprecipitation
sequencing (ChIP-seq) showed that ZSCAN5B binds with the promoter region of RAD51 (Sun et al., 2016). These results suggest that Zscan5b regulates Rad51 expression and is involved in postdamage DNA repair during mitosis.

**Zscan5b Binds to Linker Histone H1 and Protects Chromatin Structure**

Immunoprecipitation assays using proteins extracted from ESCs and HeLa cells transfected with Zscan5b and vectors expressing histone proteins H1.1, H1.2, and H1.4 showed that the ZSCAN5B protein binds to histones H1.1, H1.2, and H1.4. Hashimoto et al. (2010) reported that the linker protein histone H1 plays an important role in nucleosome spacing and interphase nuclear compaction. In addition, histone H1-null somatic mouse cells show an ~7-fold increase in the rate of chromosomal aberrations, including chromosomal gaps and breaks, compared with wild-type cells (Hashimoto et al., 2010). These results suggest that ZSCAN5B is associated with histone H1 and contributes to the maintenance of chromosomal architecture.

**Zscan5b-Deficient Mouse Is a Model of the Human Disease, Chromosome Instability Syndrome**

The Zscan5b-deficient mouse cannot repair postreplication DNA damage, and shows an increase in chromosome aberrations including gaps, breaks, gain or loss of chromosomes, and Robertsonian translocations. Defective DNA repair is a common feature of chromosome instability syndromes, also known as chromosome breakage syndromes (Sun et al., 2016). The classic chromosome instability syndromes are Fanconi pancytopenia syndrome, Bloom syndrome, and ataxia-telangiectasia. The diagnostic cytogenetic finding in Bloom syndrome is a marked increase in the level of spontaneous sister chromatid exchanges (SCEs). The normal rate of SCEs in human cells is 6–10; however, in Bloom syndrome, more than 50 SCEs per cell are found (Ellis et al., 2001). A Q-banding analysis of Zscan5b-deficient spleen cells showed increased SCEs in total numbers; this phenotype resembles that of chromosomal gaps and breaks, compared with wild-type cells (Hashimoto et al., 2010). These results suggest that ZSCAN5B is associated with histone H1 and contributes to the maintenance of chromosomal architecture.

**Conclusion**

To our knowledge, this is the first report showing that the ZGA gene Zscan5b is involved in the maintenance of genomic integrity during mitosis. We propose that abnormal mitosis without the presence of Zscan5b induces genome instability and causes altered stem cell differentiation leading to tumorigenesis. In addition, Zscan5b might be involved in maintaining DNA structure in combination with histone H1 and for packaging chromosomes into a high order of chromatin structure to prevent chromosomal abnormalities. Testing this hypothesis will be carried out in subsequent investigations.

**EXPERIMENTAL PROCEDURES**

**Identification of the Mouse Zscan5b Gene by In Silico Analysis**

Preimplantation-specific genes were identified by global gene expression profiling of oocytes and preimplantation embryos (Hamataki et al., 2004) and EST frequencies in the Unigene database. SMART (Schultz et al., 1998) was used for domain prediction analysis.

**Collection and Manipulation of Oocytes**

Female B6D2F1 mice were superovulated at 6–8 weeks old by injection of 5 IU of pregnant mare serum gonadotropin (Sigma-Aldrich, St Louis, MO, USA), followed 48 h later by 5 IU of hCG (Sigma-Aldrich). Unfertilized eggs were harvested 18 h after the hCG injection by the standard method (Nagy et al., 2003), and cumulus cells were removed by incubation in M2 medium (Embryo-OLMax M2 Powdered Mouse Embryo Culture Medium; Millipore, Billerica, MA, USA) supplemented with 300 mg/mL hyaluronidase (Sigma-Aldrich). The eggs were then thoroughly washed, selected for good morphology, and collected. Fertilized eggs were also harvested from mated superovulated mice in the same way as unfertilized eggs; embryos with two pronuclei were collected to synchronize in vitro embryo development. Fertilized eggs were cultured in synthetic oviductal medium enriched with potassium
After two more passages, mouse ESC lines were isolated using Accutase (Invitrogen), and passaged onto new MEF plates. Photographed daily. After 1 week, proliferating cells were trypsinized.

**Blastocyst Outgrowth and ESC Isolation**

The zona pellucida of blastocysts at 3.5 d.p.c. was removed using acidic Tyrode's solution (Sigma). The blastocysts were cultured individually in ES medium: KnockOut DMEM (Invitrogen) containing 15% KnockOut Serum Replacement (Invitrogen), 2,000 U/mL ESGRO (mouse leukemia inhibitory factor [mLIF]; Chemicon, Temecula, CA, USA), 0.1 mM nonessential amino acids, 2 mM GlutaMax (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), and penicillin/streptomycin (50 U/50 mg/mL; Invitrogen) on irradiated mouse embryonic fibroblast (MEF) cells at 37°C in an atmosphere of 5% CO₂ according to a standard procedure (Nagy et al., 2003). The cultured cells were examined and photographed daily. After 1 week, proliferating cells were trypsinized using Accutase (Invitrogen), and passaged onto new MEF plates. After two more passages, mouse ESC lines were isolated.

**Blastocyst Injections to Produce Chimeras**

To visualize the in vivo contribution of Zscan5b-deficient ESCs, Zscan5b-deficient ESCs were electroporated with the piggyBac transposon gene expression vector and transposase-expressing vector using a 4D-nucleofector device (Ikawa et al., 1999). We isolated the GFP-positive Zscan5b-deficient ESCs (EGFP-Zscan5b-deficient ESCs) line, which was continuously cultured on feeder layers in mouse ESC medium with LIF. EGFP-Zscan5b-deficient ESCs were injected into blastocysts of B6D2F1 mice, then transferred to the uteri of pseudopregnant ICR mice. Embryos were dissected on E14.5 and the contribution of ESCs to embryos was assessed using a fluorescence stereomicroscope (MVX10, OLYMPUS, Japan).

**Irradiation**

Irradiation of mouse ESCs was performed as previously described (Fukawatase et al., 2014). In brief, cells were given 4 Gy of X-rays, using a CELLRAD (Faxitron Biopics, Tuscon, AZ, USA). Immediately after irradiation, cells were returned to the incubator at 37°C in a humidified atmosphere containing 95% air and 5% CO₂, and incubated until further processing. Cells numbers were counted using a TC20 automated cell counter (Bio-Rad Laboratories, Hercules, CA, USA).

**Transfection with Zscan5b Constitutive Expression Plasmid**

Zscan5b-deficient ESCs were washed with PBS once, detached by Accutase and suspended in ESC derivation medium. Cells were dissociated into a single-cell suspension by vigorous pipetting and counted. Pellets were made and mixed with 1 µg of transposase-expressing vector and 1 µg of the piggyBac transposon gene expression vector (pPB-CAG-mZscan5b-P2A-EGFP) (Niwa et al., 1991) purchased from VectorBuilder in 100 µL of P2 primary cell nucleofector solution (Lonza, Switzerland). The cell suspension was transferred to a cuvette and electroporated using a 4D-nucleofector device (Lonza) with program CG-104 following the manufacturer's protocol. The electroporated cells were plated onto a six-well plate on MEF in mouse ESC medium. The GFP-positive colonies were picked up and the clonal lines were maintained as rescued Zscan5b-deficient mouse ESCs (res-Zscan5b-deficient ESCs).

**Statistical Analysis**

A chi-square test or a one-way ANOVA with Bonferroni’s multiple comparison testing was used for statistical analyses. Statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). p < 0.05 was considered to indicate statistical significance (*p < 0.05, ****p < 0.0001).

**ACCESSION NUMBERS**

The GEO accession number for the data reported in this paper are GSE124354.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2019.05.002.

**AUTHOR CONTRIBUTIONS**

S.O., M.Y., and T.H. designed the study, and performed the data analysis and interpretation with K.M., Y.Y., M.T., and A.U. M.Y. wrote the manuscript with input from all authors. S.O., Akihiro Nakamura, M.Y., R. Ooka, and R. Okawa performed gene expression analysis. H.T. performed the knockout analysis. S.O. derived the ESCs, and performed characterization and differentiation of ESCs with assistance from M.Y., Akari Nakamura, and H.A. J.M. and A.U. performed and interpreted the pathological analysis. T.S. and Akari Nakamura performed the rescue analysis. S.M. and Akari Nakamura produced chimeras. S.O., M.Y., and T.H. performed the microarray analysis. M.Y. performed the statistical analysis.

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