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Role of histone chaperone for chromatin reorganization in early embryo

(初期胚のクロマチン再構成におけるヒストンシャペロンの役割)

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Abstract

In mammals, dynamic chromatin reorganization and epigenetic reprogramming takes place soon after fertilization. The chromosomal structure of the pericentromeric regions changes from a ring structure to dot-like structure during 2-cell stage. This structural alternation is called chromocenter formation (CF) and is required for preimplantation development. Although reverse transcripts of major satellite repeats at pericentromeric regions are known to play roles in CF, its underlying mechanism is not fully understood. We previously reported that Stella (also known as PGC7 and Dppa3) deficiency led to developmental arrest at the preimplantation stage, accompanied by frequent abnormal chromosome segregation (ASC), suggesting that Stella plays a critical role in the epigenetic reprogramming.

In this study, I further investigated the effect of Stella deficiency on chromatin regulation. The Stella-null embryos exhibited impaired CF and down-regulation of transcription from reverse strand of major satellite repeats. Accumulation of histone variants H3.3, which is normally enriched at major satellite region, was also aberrant in Stella-null embryo. Moreover, histone chaperone Daxx, which is necessary for the H3.3 deposition, was decreased in the Stella-null embryos and overexpression of Daxx rescued the impaired CF of the Stella-null embryos. It has been reported that 2-cell-embryo-like state cell (2CLC) presented in embryonic stem (ES) cells at very low frequency plays an important role in the maintenance of pluripotent state and genomic integrity. I found that the 2CLC population was increased in Daxx-depleted ES cells. Therefore, Daxx regulated chromocenter formation through activating the expression of major satellite through the H3.3 deposition in early embryos, and may have important role for the regulation of the 2-cell-embryo-like state in ES cells.

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Introduction

The establishment and maintenance of DNA methylation

Epigenetics modifications are heritable changes in gene regulation that occur without altering DNA sequence itself [1]. DNA methylation on the cytosine of CpG dinucleotides is one of the major epigenetic modifications playing an important role in the physiological events such as embryonic development [1]. In mammalian genome, almost all CpG dinucleotide is methylated. The exception of this pattern is the CpG-rich regions, termed CpG islands, normally remains unmethylated [1]. When the CpG island located in the gene promoter is methylated, expression of the gene is typically silenced (Figure I1A). DNA methylation pattern is primarily established by *de novo* DNA methyltransferases Dnmt3a and Dnmt3b, and maintained through the DNA replication by the maintenance DNA methyltransferase, Dnmt1, which is recruited to replication foci and binds to hemimethylated DNA [2-5] (Figure I1B). During the development, tissue-specific DNA methylation patterns are established and maintained by the function of Dnmts, playing a fundamental role in achieving tissue-specific gene expression profiles.

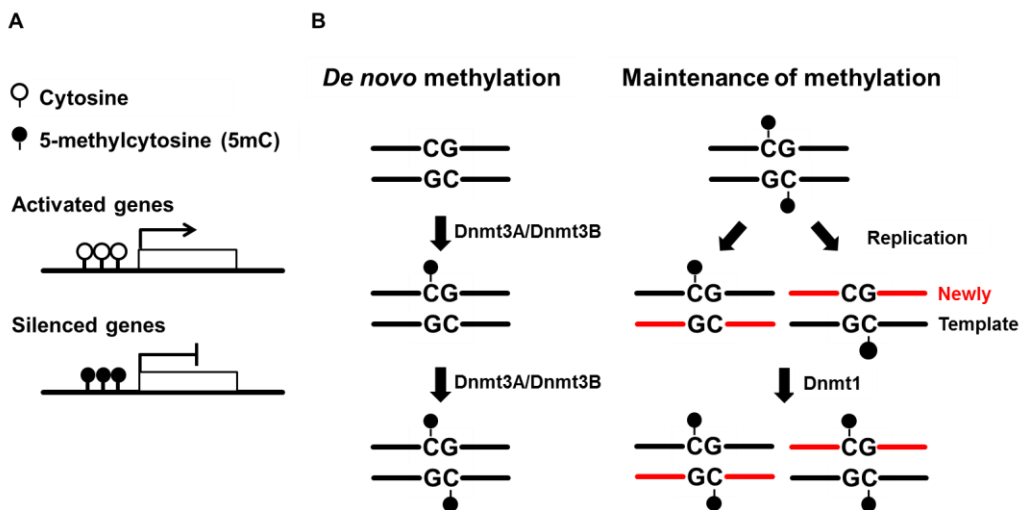


Fig. I1 The function and the processes of DNA methylation establishment and maintenance

(A) The gene expression of unmethylated and methylated genes. (B) The pathway of *de novo* and maintained DNA methylation through DNA replication.

DNA demethylation in mouse zygotes

DNA methylation is important to stabilize gene expression patterns in specialized cell types so that cellular identity and lineage fidelity is preserved. However, soon after the fertilization, almost of DNA methylation transferred from gametes was wiped out until the blastocyst stage [6,7]. Although both paternal and maternal genomes are demethylated during this process, the molecular mechanisms underlying these processes are different. In the paternal genome, Tet-protein-mediated 5-methylcytosine (5mC) oxidation is occurred before DNA replication [8-10] (Figure. I2). Among the Tet family proteins, Tet3 is the only gene highly expressed in zygote, and responsible for this process [10]. In contrast, 5mC of the maternal genome is protected from Tet3-mediated oxidation and diluted through DNA replication, which is called as passive demethylation (Figure. I3) [9].

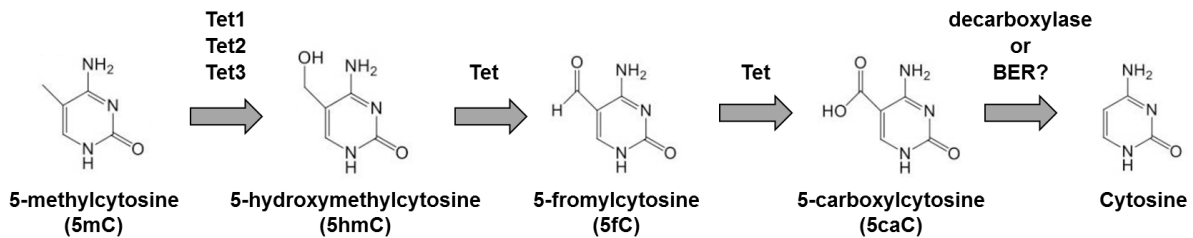


Fig. I2 Active DNA demethylation through by Tet family protein-mediated oxidation.

Scheme showing the active demethylation of 5-methylcytosine triggered by Tet-mediated oxidation to 5-hydroxycytosine, 5-formylcytosine and 5-carboxycytosine, followed presumably by decarboxylation/base excision repair (BER).

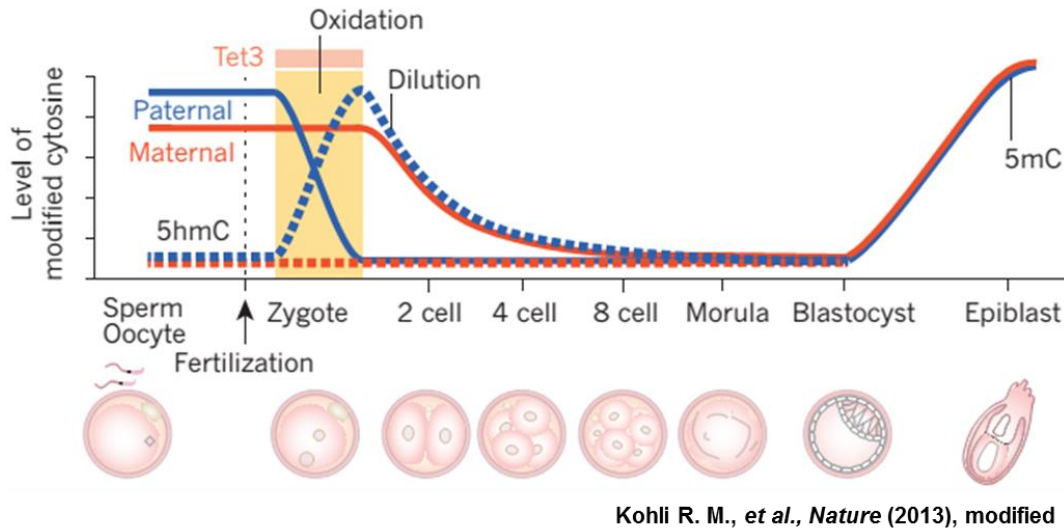


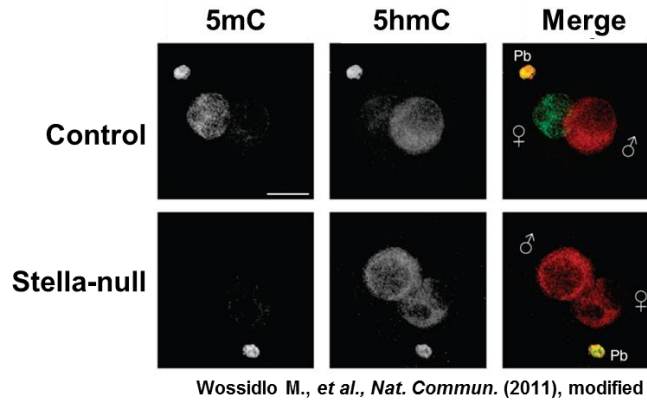
Fig. I3 DNA methylation dynamics during early development

Dynamics of 5mC and 5hmC in pre-implantation embryos.

Function of Stella in zygotes

Stella/PGC7/Dppa3 is originally reported as the PGC marker, and later known to be the essential maternal factor for the normal development [11,12]. Stella plays a role for protecting the maternal genome from oxidative conversion by inhibiting the Tet3-binding to maternal genome, accordingly maternal 5hmC and aberrant DNA demethylation was observed in Stella-null zygotes [11,13,14,15] (Figure. I4). Previously, we found that phosphorylation of the histone variant H2AX (γ H2AX) was accumulated in paternal chromatin with Tet3-dependent manner, and aberrantly observed in maternal chromatin of Stella-null zygotes [16]. It has been demonstrated that γ H2AX inhibits DNA replication [17,18]. Consistent with this report, the delayed DNA replication was observed in Stella-null embryos [16] (Figure. I5A). Moreover, Stella-null blastomere frequently exhibited abnormal chromosome segregation (ACS) [16] (Figure. I5B, C). Although these abnormalities are likely to be one of the main causes of the

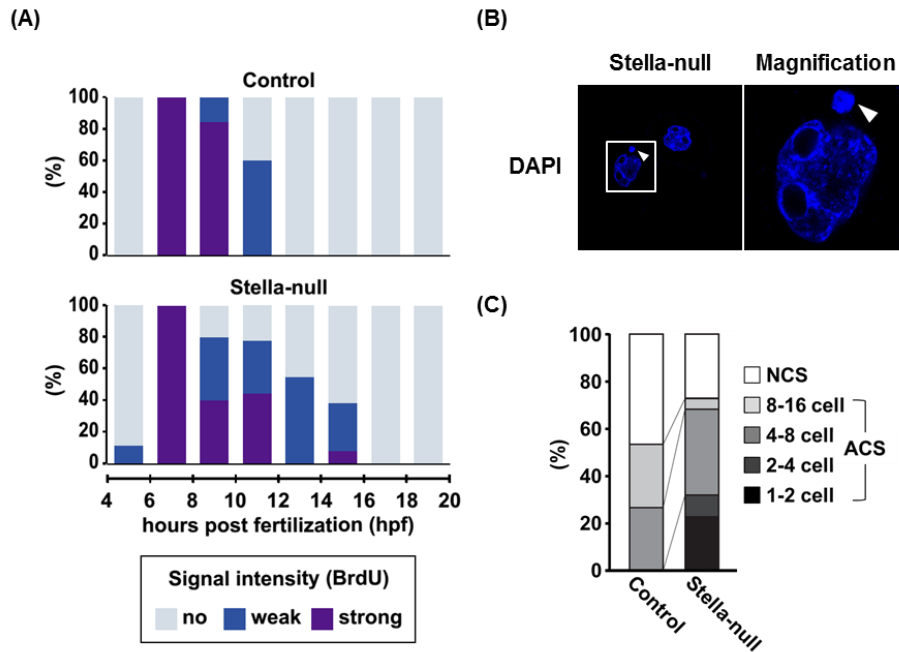
developmental arrest of Stella-null embryos, other causes are suggested to play a role as well, since about 30% of Stella-null embryos exhibited normal chromosome segregation (Fig. I5C).



Wossidlo M., et al., *Nat. Commun.* (2011), modified

Fig. I4 Increase of 5hmC in the maternal genome of Stella-null zygotes.

Immunostaining of 5mC and 5hmC in zygotes. The control and Stella-null zygotes at the G2-phase were stained with an anti-5mC and 5hmC antibody. Pb, polar body.



Nakatani T., et al., *EMBO Report* (2015), modified

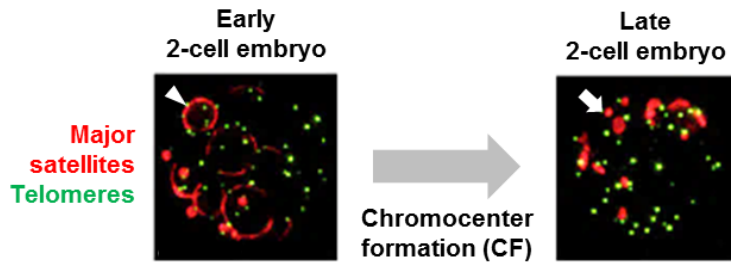
Fig. I5 Delayed DNA replication and frequent occurrence of ACS in Stella-null embryos.

(A) The DNA replication timing in control and Stella-null embryos. DNA synthesis was analyzed by signal intensity of 5-bromo-2'-deoxyuridine (BrdU). (B) Lower and higher magnification images of nuclear structure of Stella-null 2-cell embryo. Arrowhead pointed Ectopic micronuclei frequently observed in Stella-null embryos. (C) The percentage of normal chromosome segregation (NCS) and abnormal chromosome segregation (ACS) in control and Stella-null embryos.

Chromocenter formation during early development

Chromocenter is subnuclear structure which contains a cluster of constitutive heterochromatic regions consisting of transposable element and repetitive element, including major satellite repeat [19]. Major satellite, which is tandem arrays of AT-rich sequence of up to 2 mega-base pairs, located in the pericentromeric region flanking the centromeric domains where kinetochores form. In most somatic cells, pericentromeric region is organized in chromocenter [20,21]. These regions are generally maintained transcriptionally silenced state throughout the cell cycles as opposite to euchromatic regions [19]. In contrast to the somatic cell, pericentromeric regions in zygotes show a morphologically distinct ring-like structure surrounding the nucleolus precursor bodies (NPBs). This structure is reorganized to dot-like chromocenter in the late 2-cell stage [22,23] (Figure. I6). This structural change is termed as chromocenter formation (CF) and known to play an important role in the early development [24] (Figure. I7). CF is triggered by the transcripts from major satellite repeats. [22]. Transcription of major satellite repeats occurs from both the forward and reverse strands in a strand-specific manner, and transcriptional activity from the forward and reverse strand are highest during the early and late 2-cell stage, respectively [19,22] (Figure. I8). It is reported that transcripts from reverse but forward strand of major satellite is important for the normal CF [24] (Figure. I7).

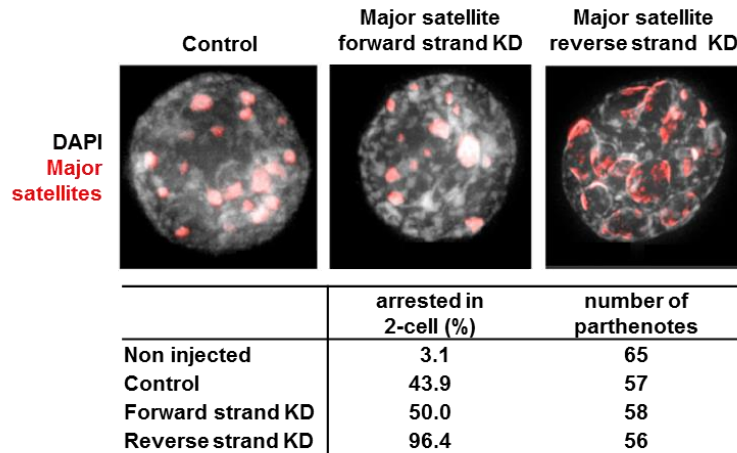
However, underlying molecular mechanism how these transcriptions are regulated has remained unclear.



Probst A. V., et al., *Dev. Cell* (2010), modified

Fig. I6 Chromocenter formation in 2-cell stage embryos.

The images of the conformational dynamics of major satellite repeat regions (red) during 2-cell stage. The ring structures of major satellite (arrowhead in the left image) in the early 2-cell embryo changes to condensed dot-like structures (arrow in the right image) at the late 2-cell stage.



Casanova, M., et al., *Cell Rep.* (2013), modified

Fig. I7 Essential role of reverse strand of major satellite transcripts for CF and normal development.

(Top) Nuclear structure of control (left image), the knockdown (KD) of forward strand of the major satellite

(middle image) and, the KD of reverse strand (right image) 2-cell embryos.

(Bottom) A summary of developmental rate of the KD embryos.

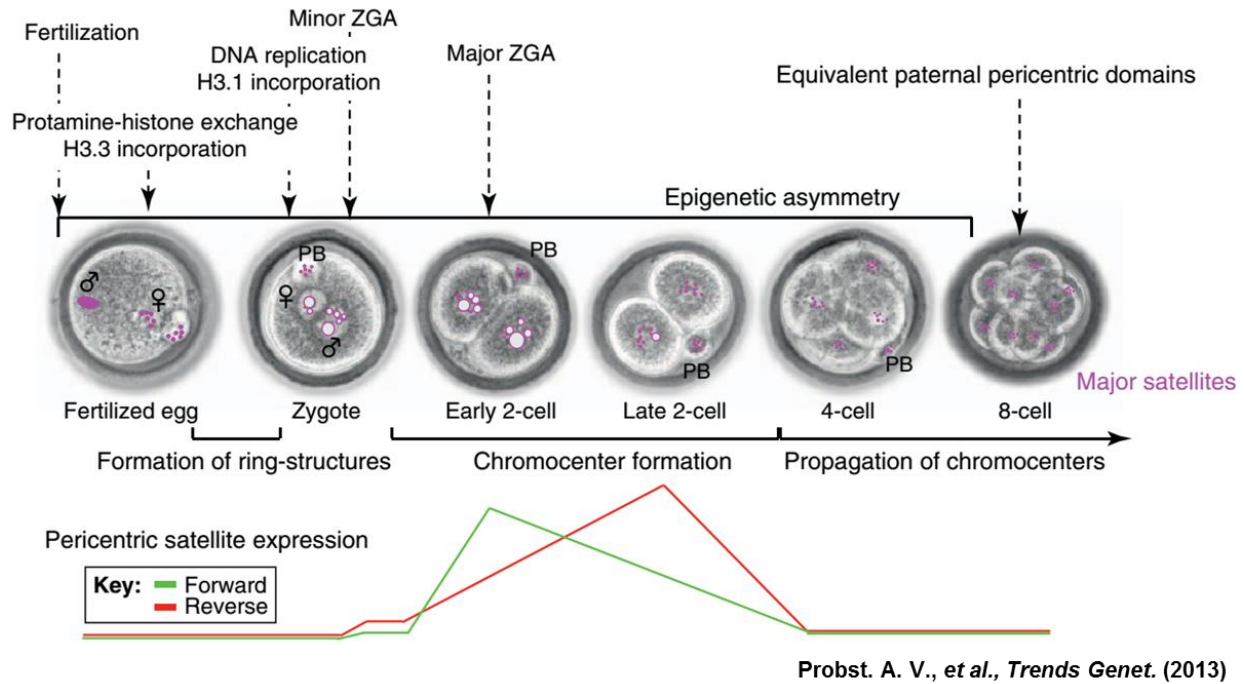


Fig. I8 Dynamics reorganization and expression of major satellites during preimplantation development

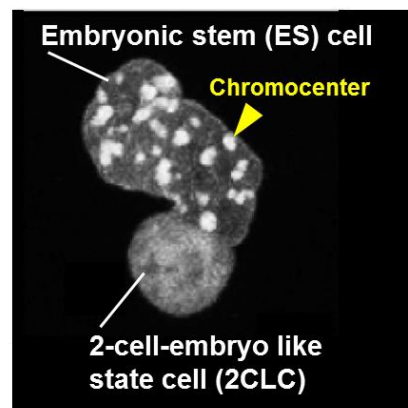
(Top) Schematic illustrating the organization of major satellite (purple) and the events occurred in early development. ZGA, zygotic gene activation; PB, polar body.

(Bottom) Transcription dynamics during early development of the forward (green) and the reverse (red) major satellite transcripts.

Disruption of chromocenter in totipotent cells

In mice, only the zygote and 2-cell stage embryo can form all the cell types including extraembryonic and placental cells. This ability is referred to as totipotency. Embryonic stem (ES) cell and induced pluripotent stem (iPS) cell can differentiate into embryonic lineage, but not

extraembryonic lineages, indicating they are pluripotent but totipotent cells [25]. Totipotent cells are reported to exhibit unique features, such as low expression of Oct4, activation of retrotransposons and the lack of chromocenter structure [26,27]. Recently, minor population retaining above features has been identified in ES cell [27]. The cell, termed as 2-cell-embryo-like state cell (2CLC), are reported to contribute to both embryonic and extraembryonic lineages of chimeric embryo [25,27]. Similar to early embryo, chromocenter is disrupted during transition ES cell to 2CLC (Figure. I9). This result suggested the correlation of chromocenter disruption and acquisition of totipotency but their causal relationship remains unclear.



Ishiuchi T., et al., *Nat Struct Mol Biol.* (2015), modified

Fig. I9 Chromocenter disruption in 2-cell-embryo-like state cell (2CLC)

Chromatin structures of embryonic stem (ES) cell and 2-cell-embryo like state cell (2LC) visualized by DAPI staining. The yellow arrowhead indicates Chromocenter.

The features of mouse histoneH3 variants H3.3

The fundamental unit of chromatin is the nucleosome, consisting of 147bp DNA and four types of histone proteins, H2A, H2B, H3 and H4 [28]. In addition to these canonical histone

proteins, recently, it has been shown that histone variants play an important role in the transcriptional regulation, as well as DNA and histone modifications [29,30]. In mouse, five major histone H3 variants have been reported. H3.1 and H3.2 are commonly referred to as canonical H3. In addition, three replacement variants are presented, such as H3.3, CENP-A and H3t [31]. Although differences between H3.3 and canonical H3s are at only five or four amino acid, H3.3 is specifically distributed around the transcriptionally active regions and is closely associated with global gene expression in somatic cells [32]. The incorporation of H3.3 into the nucleosome is regulated by two major histone chaperone complexes, HIRA and Daxx, in a replication-independent manner [31]. HIRA is responsible in the incorporation of H3.3 into euchromatic regions, while Daxx plays a role in pericentromeric and telomeric heterochromatin regions [31,33,34] (Table. I1). Daxx-deficient mouse embryonic fibroblasts show reduced H3.3 incorporation and up-regulation of major satellite expression [35]. In zygote, H3.3 localizes to pericentromeric regions of the paternal pronucleus predominantly [36,37]. However, its molecular significance remains unclear.

In this study, we found that CF and transcription of the reverse strand of major satellite repeats were impaired in Stella-null embryos. Overexpression of Daxx improved the incorporation of H3.3 and rescued abnormal CF in the Stella-null embryos. In addition, KD of Daxx in ES cells led to significant increase in the number of 2-cell-embryo-like state (2CLC). Therefore, Daxx ensures chromatin reorganization through the H3.3 deposition in early embryos, and is likely to play a role in the transition to 2-cell-embryo-like state.

Table. I1 Mouse histone H3 variant and their chaperone

Histone H3 variant		Chaperone	Deposition	Enrichment
Canonical	H3.1	CAF-1 complex	DNA replication dependent	Replication and repair
	H3.2			
Replacement	H3.3	HIRA complex	DNA replication independent	Euchromatic region
		Daxx complex		Pericentromeric and Telomeric regions
	CENP-A	HJURP	DNA replication independent	Centromeric region
	H3t	ND	ND	Testis chromatin
ND, not determined				Ray-Gallet D., <i>Essays Biochem.</i> (2010), modified

Results

Impairment of chromocenter formation and transcription of major satellite in the Stella-null embryos

I first analyzed the nuclear structures of 4-8-cell embryos by DAPI staining to examine the effect of Stella deficiency during preimplantation development. Chromocenter, which is a dot-like heterochromatic region heavily stained with DAPI, was obvious in the control embryos. In contrast, appearance of chromocenter was not observed in Stella-null embryos, suggesting the impairment of CF in Stella-null embryos (Fig. 1A). To trace the localization of major satellite repeat regions in more detail, I used TALE technology [38,39]. Consistent with previous report, major satellite dynamics could be visualized by TALE-mClover_MajSat in Stella-deficient ES cells [40] (Fig. 1B). I injected *in vitro* synthesized mRNA of *TALE-mClover_MajSat* into the control and Stella-null zygotes and examined CF based on dot-like structures of major satellite repeats at the late 2-cell stage. The number of embryos harboring the ring structures of major satellite repeats was significantly increased in Stella-null embryos compared to control (Fig. 1C, D and E). These results clearly indicated that CF was impaired in Stella-null embryos.

Next, to evaluate the cause of impaired CF, I analyzed transcription of the reverse strand of the major satellite repeats. Although the levels of forward transcription of the control and Stella-null embryos did not differ significantly, the reverse transcripts were significantly reduced in Stella-null embryos (Fig. 2). These results suggested that the decreased expression of the reverse strand of major satellite repeats was the major cause of impaired CF in the Stella-null embryos.

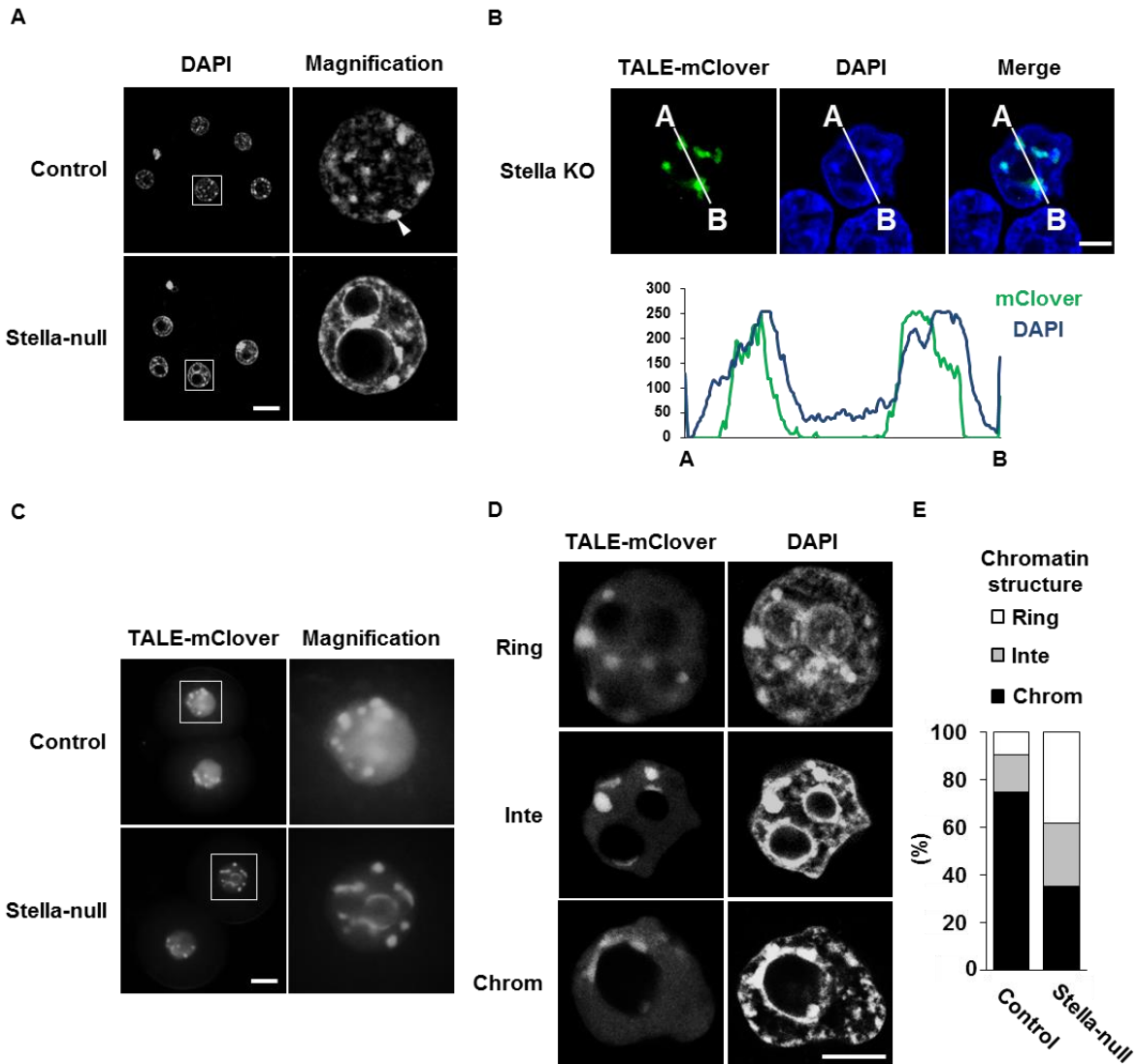


Fig. 1 Chromocenter formation in Stella-null embryos.

(A) Chromatin structures of control and Stella-null embryos visualized by DAPI staining at 4-8 cell stage. The white arrowhead indicates Chromocenter. Scale bars = 20 μ m. (B) Validation of TALE-mClover_MajSat and DAPI are shown in the upper panels. The plasmid used to express TALE-mClover_MajSat was transfected into Stella-knockout (KO) ES cells. Line-profiles of fluorescence intensities for DAPI (blue) and the TALE-mClover_MajSat (green) from the A-B line drawn in images are shown in the right-hand panels. Scale bars = 5 μ m. (C-E) Distribution of major satellite repeats visualized by TALE-mClover_MajSat in Stella-null late 2-cell embryos. The control and Stella-null zygotes were injected with TALE-mClover_MajSat mRNA and

observed at 48 h after hCG injection. Representative image (C) and chromatin structures of major satellite repeats in each classification in late 2-cell embryos (D) are shown. The chromatin structure was classified as shown in the Material and Methods. Quantitative analysis of chromatin structure in the late 2-cell embryos (E) is shown. Ring, Chrom, and Inte indicate the ring, chromocenter, and the intermediate structures, respectively. A total of 32 control and 34 Stella-null embryos were analyzed. $p < 0.01$ by χ^2 -test (Control vs. Stella-null in the ring category). Scale bars = 20 μm .

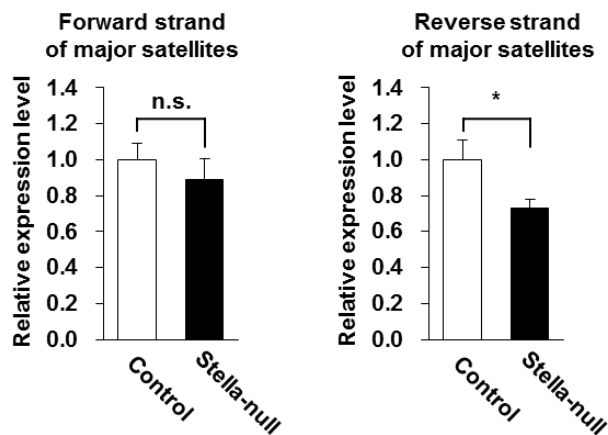


Fig. 2 The expression of major satellite repeats in Stella-null zygotes.

Expression levels of the forward and reverse strands of major satellite repeats. Strand-specific RT-qPCR was performed using the control and Stella-null zygotes at the PN3-4 stage. Fourteen to 30 zygotes were subjected to each experiment. Mean \pm SEM. (n = 4). * $p < 0.05$, n.s., not significant by t -test.

Reduced H3.3 accumulation on major satellite repeats in Stella-null zygotes

Major satellite repeats are aligned to the regions surrounding NPBs soon after fertilization [37]. At the same time, H3.3, a H3 variant associated with active transcription, is predominantly incorporated into the region around paternal NPBs [36]. Since it was reported that H3.3 was important in major satellite transcription [35], I hypothesized that abnormality in H3.3 was the cause of impaired CF in the Stella-null embryos. To this end, *H3.3-GFP* mRNA was injected and

dynamics of H3.3 was analyzed. Although GFP signal around paternal NPBs was detected in both control and Stella-null zygotes, the signal intensity in the Stella-null zygotes was significantly low (Fig. 3A, B and C), suggestive of impairment of H3.3 incorporation.

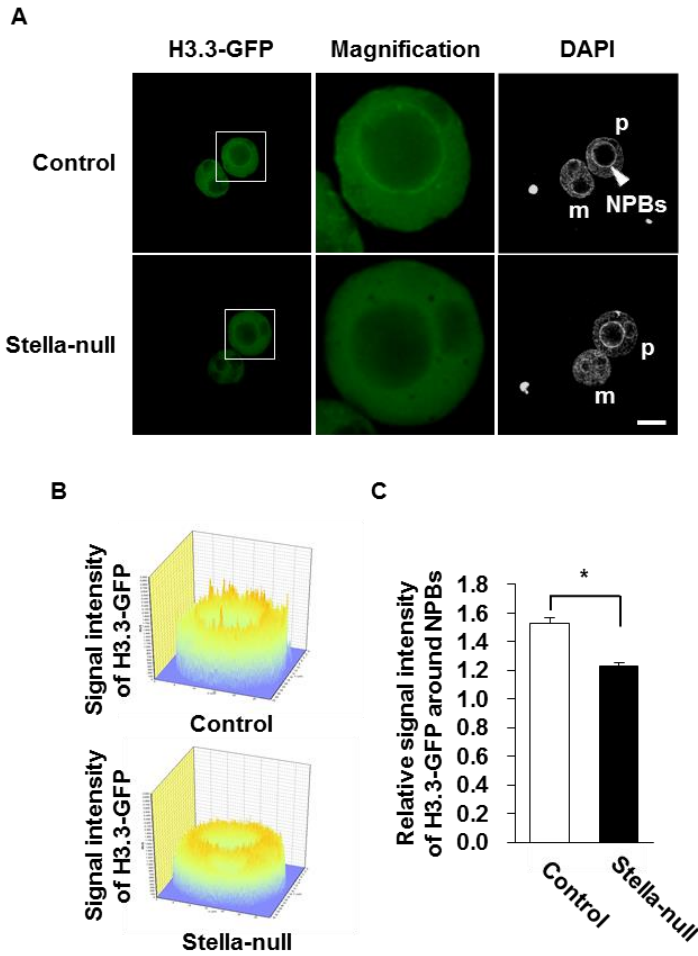


Fig. 3 Reduced H3.3 accumulation around paternal NPBs in the Stella-null zygotes

(A) Representative images of H3.3-GFP-injected zygotes. (B) Fluorescence distribution analysis of H3.3-GFP in the paternal pronucleus. The control and Stella-null zygotes were injected with *H3.3-GFP* mRNA and were observed at the PN3-4 stage. (C) Quantification of the signal intensity of H3.3-GFP around paternal NPBs. Relative signal intensity was normalized to the mean signal intensity of the nucleoplasmic regions in the paternal pronucleus, and relative values. Fourteen control and 16 Stella-null zygotes were analyzed. p: paternal

pronucleus, m: maternal pronucleus, NPB: nucleolus precursor bodies. * $p < 0.0001$ by t -test. Scale bars = 20 μm .

The essential and sufficient role of Daxx in reverse transcription of major satellite and subsequent chromocenter formation

Since it has been reported that H3.3 is incorporated by Daxx into pericentromeric regions in somatic cells [37], I hypothesized that defect in H3.3 incorporation into pericentromeric regions was caused by the defect in Daxx. To test this hypothesis, I examined the distribution and expression of Daxx in the Stella-null zygotes by the immunohistochemistry. Although Daxx was detected in the pronuclei of both control and Stella-null zygotes, the signal intensity was significantly lower in the Stella-null zygotes than the control (Fig. 4A and B). Western blotting analysis confirmed that the protein levels of Daxx decreased in the Stella-null zygotes significantly (Fig. 4C and D). Immunohistochemical analysis of GV-stage oocyte demonstrated that the number of oocytes with no Daxx staining was significantly increased in Stella-null oocyte (Fig. 5). This result suggested that maternal store of Daxx protein was reduced in Stella-null mice.

I next examined whether the reduced expression of Daxx was the cause of abnormal chromatin reorganization in Stella-null embryos. Overexpression of Daxx by the microinjection of mRNA in Stella-null zygotes restored the reverse transcripts of major satellite repeats and H3.3 accumulation, but the forward transcript level was unaffected (Fig. 6A, B and C). The number of embryos that formed chromocenter was significantly increased by the expression of Daxx in Stella-null embryos (Fig. 6D). To investigate whether overexpression of Daxx rescued the developmental arrest in Stella-null embryos through the restoration of CF, I analyzed the developmental rate. However, none of Daxx-overexpressed Stella-null embryos reached

blastocyst stage, indicating other causes of embryonic lethality (Fig. 6E). Based on these results, I concluded that the reduced expression of Daxx was the major cause of impaired CF in Stella-null embryos, but the complementation of Daxx was not enough to recover developmental arrest in Stella-null embryos.

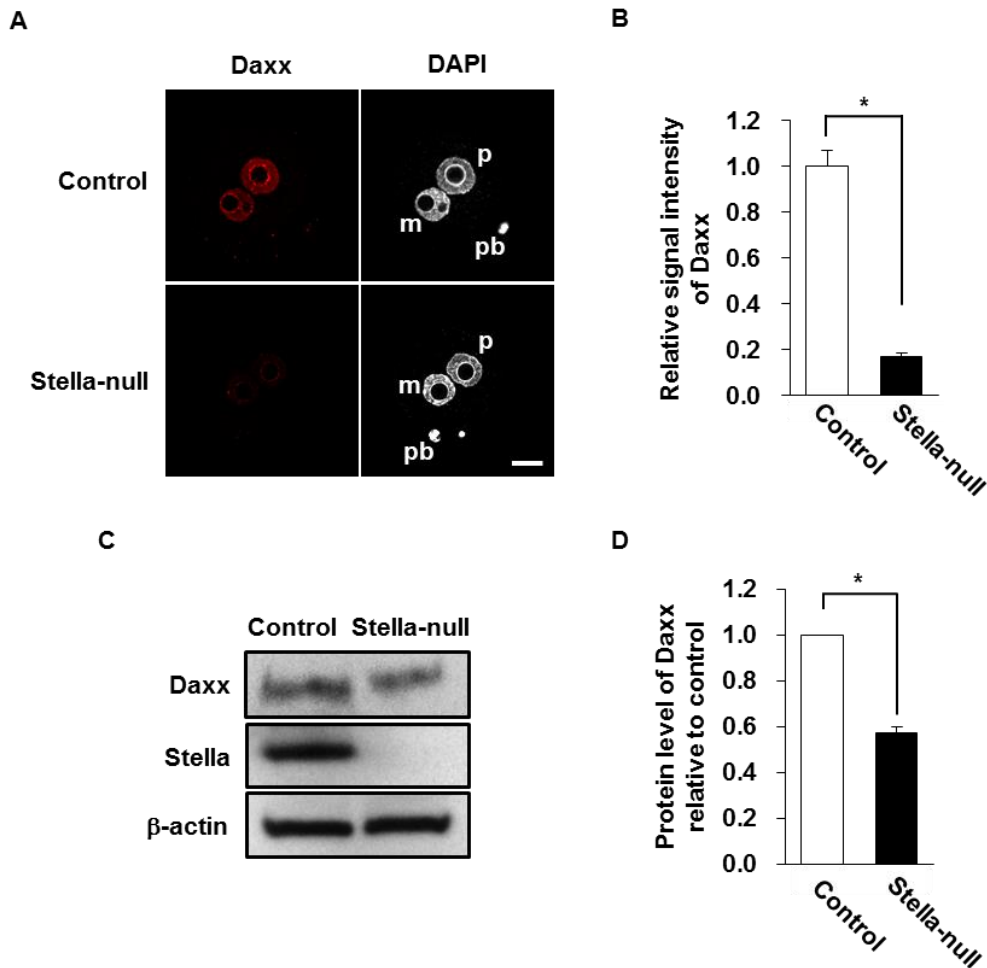


Fig4. Decreased expression of Daxx in Stella-null zygotes.

(A, B) Immunostaining of Daxx in zygotes. The control and Stella-null zygotes at the PN3-4 stage were stained with an anti-Daxx antibody and counter-stained with DAPI. Representative images of Daxx in zygotes (A) and quantitative data of the signal intensity of Daxx in the zygotes (B) are shown. Thirteen control and

Stella-null zygotes were analyzed. $*p < 0.0001$ by *t*-test. (C, D) Reduced expression levels of Daxx in the Stella-null zygotes. A representative result of Western blotting using 54 to 155 of the control and Stella-null zygotes (C) and-quantification results of Western blotting normalized to β -actin (D) are shown. Mean \pm SEM. (n = 5). $*p < 0.0001$ by *t*-test. Scale bar = 20 μ m.

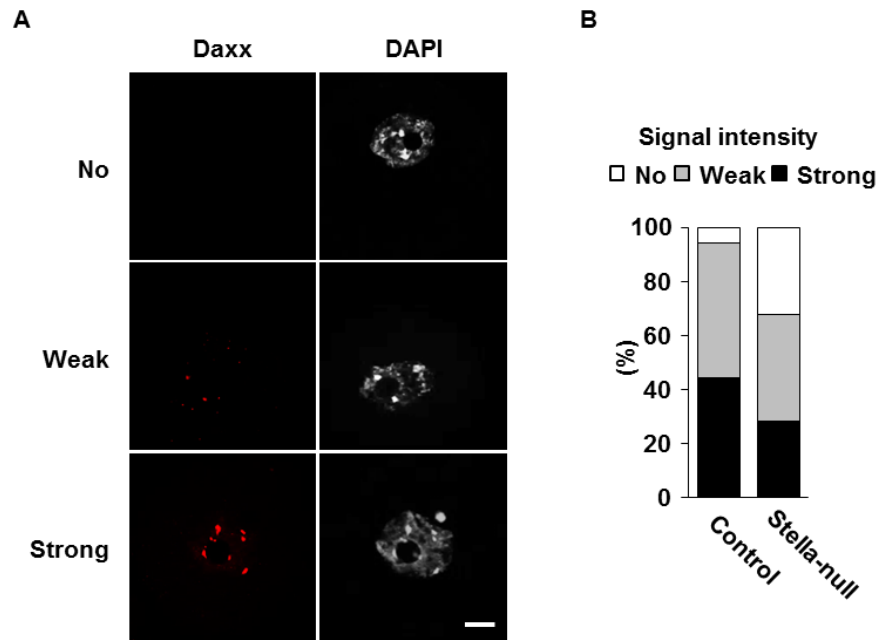
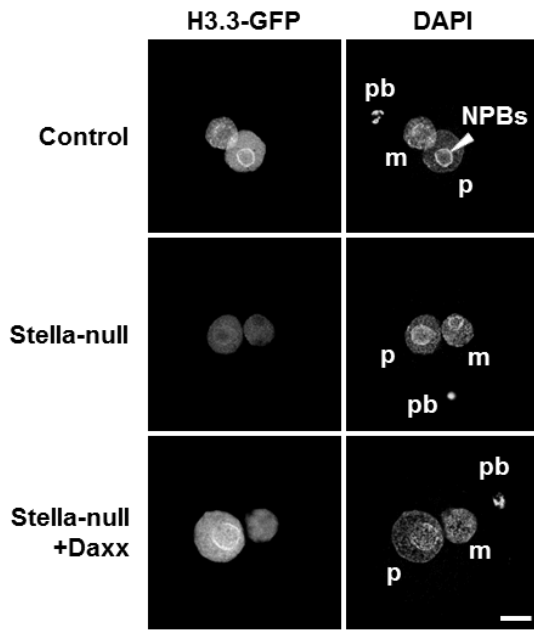


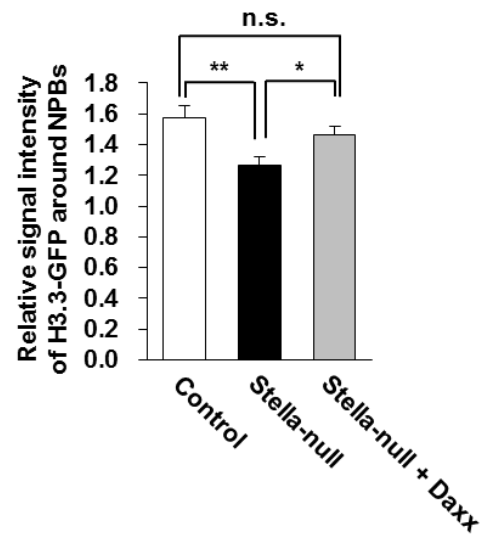
Fig5. Decreased expression of Daxx in Stella-null oocytes and 2-cell embryos.

(A) Representative images of Daxx in GV oocytes. The control and Stella-null GV oocytes were stained with an anti-Daxx antibody and counter-stained with DAPI. (B) Quantification results of the signal intensity of Daxx in the GV oocytes. The Daxx signals were classified as shown in the Material and Methods. Eighteen control and 25 Stella-null GV oocytes were analyzed. $p < 0.01$ by χ^2 -test (Control vs. Stella-null in no category). Scale bar = 20 μ m.

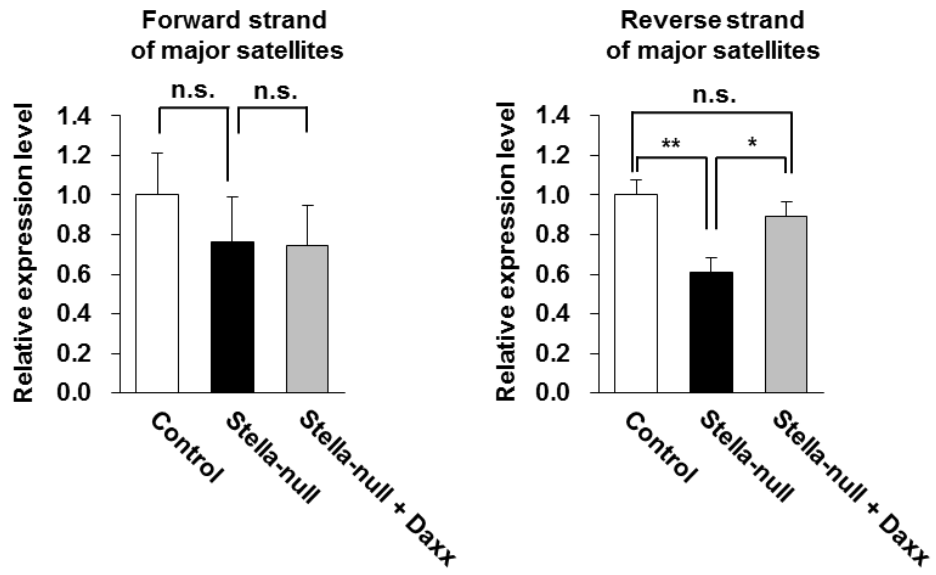
A



B



C



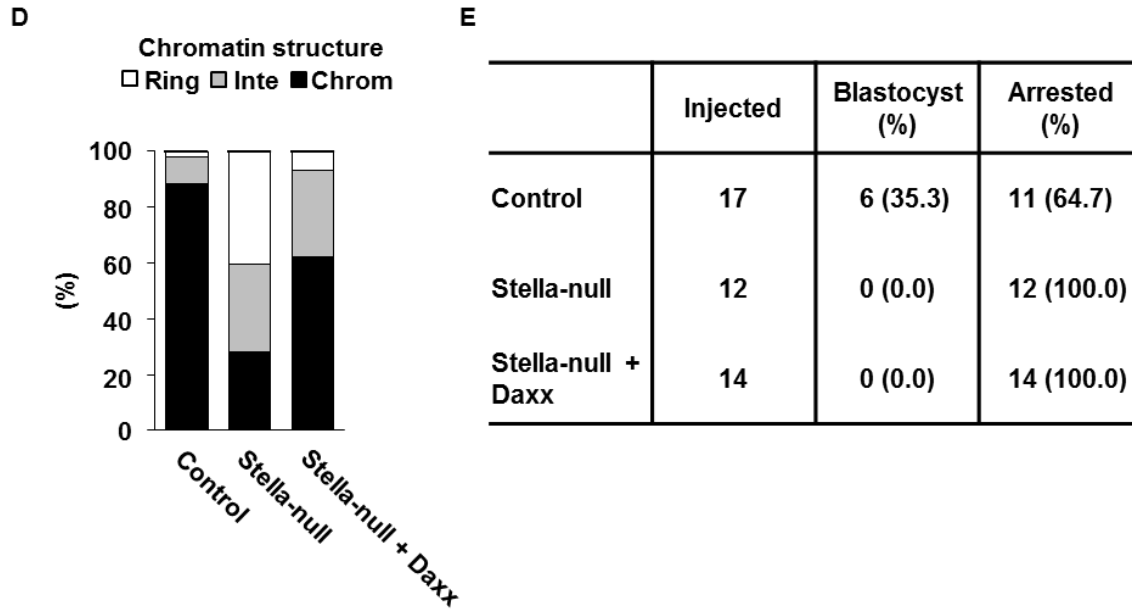


Fig. 6 Restored chromocenter formation by the expression of Daxx in Stella-null embryos.

(A, B) The distribution of H3.3-GFP in the Stella-null zygotes was restored by the expression of Daxx. The control and Stella-null zygotes were injected with *H3.3-GFP* mRNA and *Daxx* mRNA, and observed at the PN3-4 stage. Representative images (A) are shown. The signal intensity of H3.3-GFP around paternal NPBs was normalized to the mean signal intensity of the nucleoplasmic regions in paternal pronucleus, and relative values (B) are shown. Seven control, 8 Stella-null, and 9 Daxx-expressing Stella-null zygotes were analyzed. * $p < 0.05$, ** $p < 0.01$, n.s., not significant by *t*-test. (C) The expression levels of the reverse strands of major satellite repeats in the Stella-null embryos were restored by the expression of Daxx. Strand-specific RT-qPCR was performed using the control, Stella-null, and Daxx-expressing Stella-null zygotes at PN3-4 stage. Ten to 22 zygotes were used for each experiment. Mean \pm SEM. (n = 4). * $p < 0.05$, ** $p < 0.01$, n.s., not significant by *t*-test. (D) Restored chromocenter formation in Daxx-expressing embryos. Quantification of the chromatin structure in late 2-cell embryos. *DsRed* mRNA was used to compensate for the total amount of mRNA for injection. Fifty-two control, 57 Stella-null, and 29 Daxx-expressing Stella-null embryos were analyzed. $p < 0.01$ by χ^2 -test (control vs. Stella-null; Stella-null vs. Daxx-expressing Stella-null in the ring category). (E) The developmental rate of control, Stella-null and Daxx-expressing Stella-null embryos. *DsRed* mRNA was used to compensate for the total amount of mRNA for injection. Scale bar = 20 μ m.

Reduced Daxx expression induces 2-cell embryo like cells

Absence of chromocenter was observed not only in early embryos, but also in totipotent-like 2CLC [26]. To investigate whether Daxx is involved in the regulation of 2-CLC to non-2CLC transition, I performed knockdown experiment of Daxx in ES cells. In order to visualize 2CLC population, 2C::tdTomato reporter ES cell was used. This ES cell harbors tdTomato gene under MERV-L long terminal repeat which is specifically expressed in 2CLC [27]. Efficient knockdown of Daxx was confirmed by RT-qPCR and Western blotting analysis (Fig. 7A and B). In Daxx-knockdown ES cells, the genes specifically expressed in 2-cell-embryos were significantly up-regulated (Fig. 7C). Up-regulation of 2-cell-specific transcripts were likely to be caused by the increase in 2CLC, as the number of the cells of expressing 2C::tdTomato reporter was obviously increased in Daxx-knockdown ES cells (Fig. 7D). The quantitative analysis through FACS confirmed 6 to 28 times increase of 2CLC in Daxx-depleted ES cell (Fig. 7E). Taken together with the function of Daxx in early embryos, these results suggested that Daxx has a role for the transition 2CLC to non-2CLC through the progression of CF.

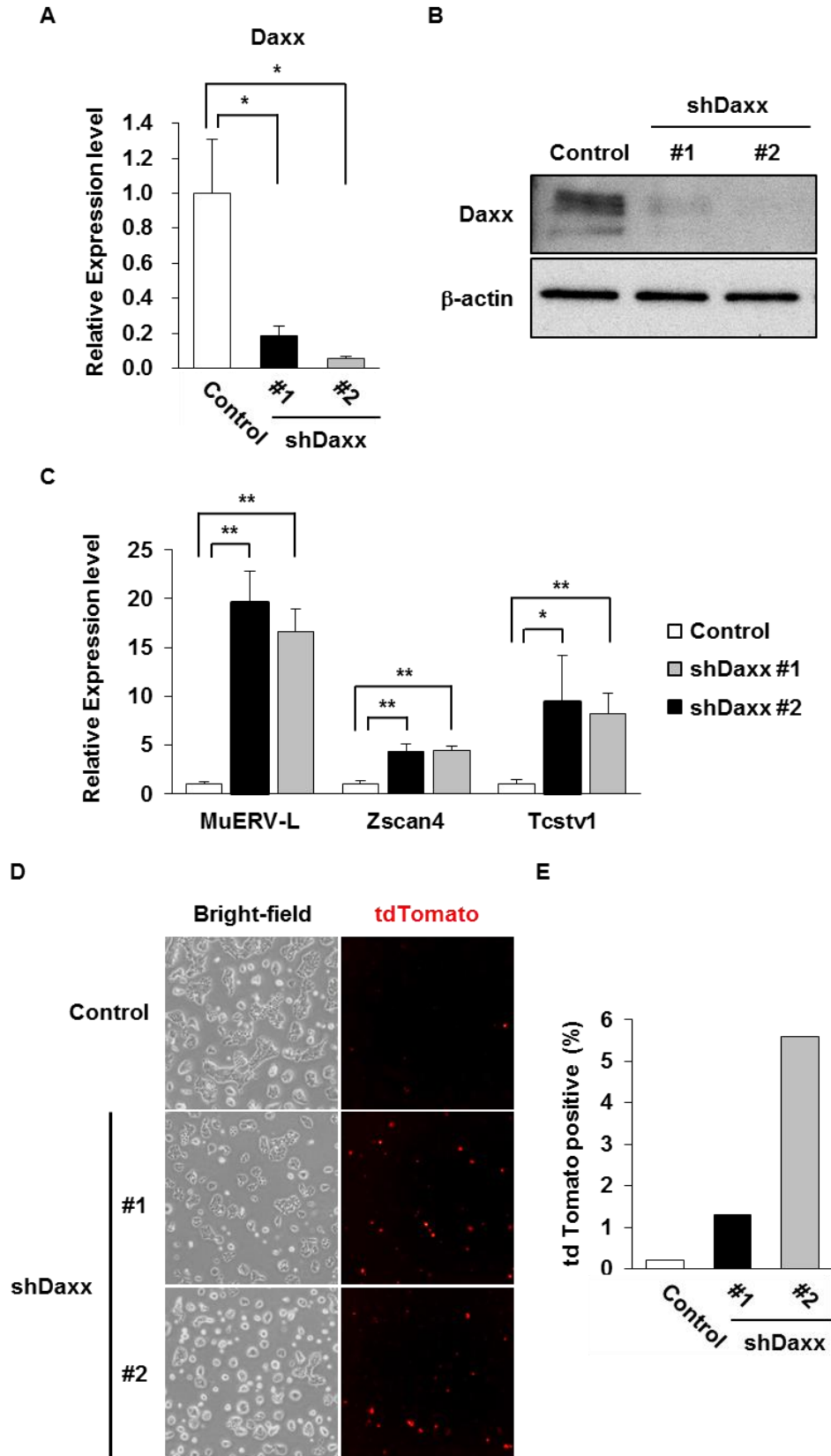


Fig. 7 Down-regulated Daxx expression induces 2-cell like cells

(A) Knockdown efficiency of Daxx analyzed by RT-qPCR. RNA was harvested at day8 of knockdown. n=3 Mean \pm SEM. (n = 3). * p < 0.01 by t -test. (B) A representative result of Western blotting analysis of Daxx knockdown ES cells. Total protein was harvested at day10 of knockdown. (C) RT-qPCR analysis of 2-cell embryo specific genes after knockdown of Daxx. Mean \pm SEM. (n = 3). * p < 0.05, ** p < 0.01 by t -test. (D, E) Increased number of tdTomato-positive ES cells. Representative images of tdTomato-expressing ES cells after knockdown of Daxx (D) and quantification of tdTomato-positive cells by FACS (E) are shown.

Discussion

Our previous result indicated that the loss of epigenetic asymmetry and delayed DNA replication in Stella-null zygotes [16]. Since Daxx can restore CF without altering the 5hmC status, epigenetic asymmetry is suggested to be dispensable for CF (Fig. 6D and Fig. 8). This is consistent with previous reports that parthenogenetic embryos consist of only maternal genome with exhibit no clear abnormality in CF [24]. In addition, impaired CF is likely to be independent to the delayed DNA replication because it has been reported that CF was not affected by treatment with a DNA replication inhibitor [24]. Based on these results, I concluded that the reduced H3.3 accumulation around NPBs was the major cause of impaired CF in Stella-null embryos.

H3.3 in zygote derives from two sources, maternal H3.3 which has been stored during oogenesis and zygotic H3.3 which is transcribed after fertilization [41]. Since maternal H3.3 is required for early embryogenesis, rapid and appropriate H3.3 incorporation is critical for the normal development [41]. Under Stella-null conditions, H3.3 accumulation was impaired in zygotes due to the decreased expression of Daxx (Fig. 3, 5). However, even in Stella-null embryos, H3.3 was deposited to the nuclear similar to the wild type at 2-cell stage (Fig. 9). This indicated that the maternal storage of Daxx is responsible for prompt H3.3 incorporation soon after fertilization, but it is compensated by the newly synthesized Daxx at 2-cell stage. Therefore, maternal Daxx plays a critical role in the narrow time window, but it is critical for the trigger of CF. A recent report demonstrated that knockdown of H3.3 led to decreased expression of several genes which is essential for early embryogenesis [41]. Deposition of H3.3 at appropriate timing is likely to be critical not only for CF but also for the preimplantation development through the

regulation of developmental gene expression.

Although previous study reported that Daxx-KO mouse exhibited embryonic lethality at E9.5, physiological function of Daxx in the preimplantation embryo was unclear [42]. This is because maternally stored Daxx was not affected in the oocyte of heterozygous mouse and transmitted to the early embryo of conventional Daxx-KO mouse. In this study, I took advantage of Stella-null embryo that shows reduced Daxx expression, and demonstrated that maternal Daxx is critical for the H3.3 accumulation, transcription of the reverse strand of major satellite repeats, and normal CF in the early embryo (Fig. 6). Although little is known about the physiological function of CF in other stages, it is possible that the defects in post-implantation developmental stages in Daxx-KO mouse are caused by the abnormality in CF [35].

Moreover, I also demonstrated that knockdown of Daxx in ES cells led to increased the number of 2-cell like reporter cells (Fig. 7). The features of 2CLC include silencing of Oct4 expression, reactivation of retrotransposon and major satellite repeats, higher chromatin mobility and lack of chromocenter [26,27]. In addition, 2CLC has the capacity for differentiating both embryonic and extraembryonic lineages like totipotent cells [26]. Thereby, underlying mechanism of the transition between 2CLC and non-2CLC is one of the most important questions to understand the molecular basis of the acquisition and the maintenance of pluripotent state. In this study, I demonstrated that Daxx promoted the transition of 2CLC to non-2CLC. Taken the role of Daxx in CF in early embryos into consideration, Daxx could modulate the transition of 2CLC through CF.

Although normal CF was almost completely restored in Stella-null embryos by Daxx expression, the developmental arrest of Stella-null embryos was not rescued (Fig. 6E). This result suggested that impairment of CF in Stella-null embryos was not the major cause of

developmental abnormality. We have recently shown that ectopic 5hmC in maternal chromatin leads to γ H2AX accumulation and induce abnormal chromosome segregation in Stella-null zygotes [16]. Since 5hmC levels in Stella-null embryos was not affected by the expression of Daxx, developmental arrest is likely to be caused by the abnormal chromosome segregation and other unknown mechanisms (Fig. 8).

In addition, I previously reported that Stella is an essential factor for the production of functional oocytes through transcriptional repression and chromatin condensation [43]. I herein revealed novel Stella function for chromatin reorganization. Taken together with previous finding, Stella plays a critical role in chromatin regulation before and after fertilization to ensure occurrence of early embryogenesis.

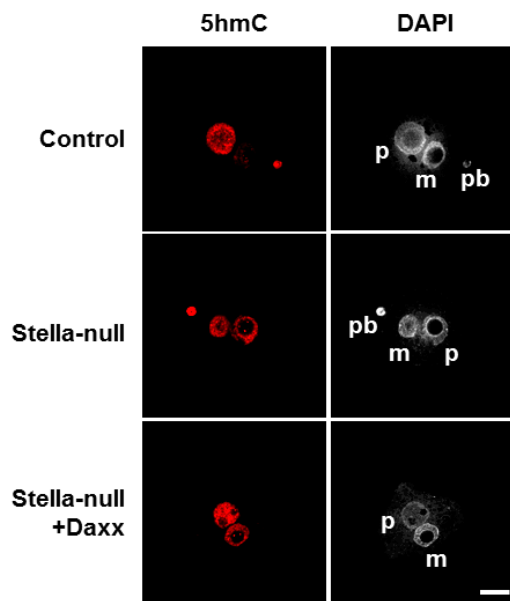


Fig. 8 Abnormal enrichment of 5hmC in the Daxx-expressing Stella-null zygotes.

Distribution of 5hmC in zygotes. The control, Stella-null and Daxx-expressing Stella-null at the PN3-4 stage were stained with an anti-5hmC antibody and counter-stained with DAPI. Scale bar = 20 μ m.

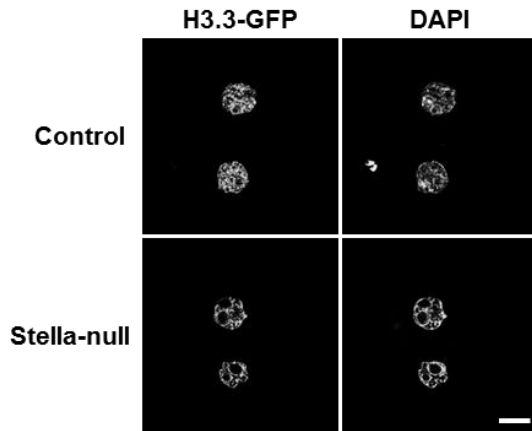


Fig9. H3.3 accumulation in Stella-null 2-cell embryos.

Representative images of H3.3-GFP and DAPI in 2-cell embryos are shown. The control and Stella-null zygotes were injected with *H3.3-GFP* mRNA and observed at 48h after hCG injection. Scale bar= 20 μ m.

Materials and Methods

Zygote collection and culture

Female Stella^{+/-} and *Stella*^{-/-} mice were generated by homologous recombination described previously [11]. Female *Stella*^{+/-} and *Stella*^{-/-} mice > 12 weeks of age were super-ovulated by injecting 5 U of human chorionic gonadotropin (hCG) 48 h after injection of 5 U of pregnant mare serum gonadotropin (PMSG), and then mated with male B6D2F1 mice. Fertilized eggs were collected from the oviduct 15 h after hCG injection, placed in 100 µl drops of KSOM (Millipore) and cultured at 37°C with 5% CO₂. Experiments were performed in accordance with the guidelines of the Osaka University Animal Care and Use Committee.

Cell culture

Embryonic stem (ES) cells were cultured in Glasgow modified Eagle's medium (GMEM) supplemented with 10% fetal calf serum, 2-mercaptoethanol, and LIF.

Immunohistochemistry

Embryos and cells were washed in PBS, fixed in 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature, and permeabilized in 0.2% Triton X-100 in PBS for 15 min at room temperature. The embryos and cells were blocked in 5% normal goat serum in PBS for 1 h at room temperature and incubated overnight at 4°C with the primary antibodies described below.

After washing in PBS, the embryos and cells were incubated with the secondary antibodies described below. DNA was stained with 1 $\mu\text{g/ml}$ 4',6-diamidino-2-phenylindole (DAPI). Immunofluorescence was visualized using an FV1000-D confocal laser-scanning microscope (Olympus).

Plasmids

pTALYM3B15 was a gift from Maria-Elena Torres-Padilla (Addgene plasmid # 47878). The pTALYM3B15 plasmid was used to express transcription activator-like factor (TALE) against major satellite repeats, which were fused with the monomeric GFP mClover (TALE-mClover_Majsat) [40]. 2C::tdTomato reporter was gift from Samuel Pfaff (Addgene plasmid # 40281) [27]. The *H3.3-GFP* cDNA was cloned into pcDNA4mycHisA (Invitrogen).

Classification of the major satellite structure

The chromatin structure of major satellite repeats was visualized by TALE-mClover_MajSat. The structures were divided into three categories: ring, intermediate, and chromocenter (ring: localization of major satellite repeats on periphery of NPBs and no dot-like structure, intermediate: a mixed state of the ring and chromocenter, chromocenter: only dot-like structure) .

Quantification of fluorescence signals

The fluorescence signals acquired from immunohistochemical analysis were quantified using

Image J software. The signal intensity of Daxx in GV oocytes was divided into three categories: no, weak, and strong. The arbitrary threshold was set between strong and weak.

***In vitro* transcription and mRNA injection**

Capped mRNA was synthesized using a T7 mMessage mMachine kit (Ambion). Poly (A) tails were added to the capped mRNA using a Poly (A) Tailing Kit (Ambion) according to the manufacturer's protocol. The synthesized mRNAs were subjected to gel filtration using NucAway Spin Columns (Invitrogen) to remove unreacted substrates and then stored at -80°C. The synthesized mRNAs were microinjected into the cytoplasm of early pronuclear zygotes and were incubated in KSOM at 37°C with 5% CO₂.

Strand-specific reverse transcription-quantitative PCR (RT-qPCR)

Total RNAs were obtained from embryos using a Picopure RNA isolation kit (Life technologies). An equal amount (0.5 pg/embryo) of control *GFP* mRNA was added into each sample containing an equal number of embryos before RNA extraction. Genomic DNA was removed from the samples by treatment with Turbo DNase (Ambion) at 37°C for 1 h. The cDNA was synthesized using a ThermoScript RT-PCR system (Invitrogen). The strand-specific primers conjugated with adapter sequences were used for RT reactions for major satellite repeats [44]. RT-qPCR was performed using a ViiA7 Real-Time PCR system (Applied Biosystems) using SYBR Green (Applied Biosystems). Relative gene expression levels were normalized to the external control *GFP*.

Western blotting

Embryos and ES cells were lysed in the sample buffer (62.5 mM Tris-HCl, 700 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 5% sucrose, and 0.01% bromophenol blue) and denatured at 98°C for 3min. The samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked in 5% skim milk for 1 h and incubated overnight at 4°C with primary antibodies described below. The HRP-linked IgG was used as the secondary antibody and the level of Daxx was detected using ECL Western blotting detection reagent (GE Healthcare). Primary and secondary antibodies used in this study are listed below. The band intensity was determined using Image J software.

The induction of 2C::tdTomato reporter in ES cells

2C::tdTomato Reporter (Addgene plasmid #40281) was transfected into J1 ES cells using Lipofectamine RNAi MAX Regent (Thermo Fisher Scientific). Two days after transfection, cells were selected by the use of 150µg/ml hygromycin for 8days, and survived cells were used to experiments as 2C::tdTomato reporter cells.

The knockdown of Daxx in ES cells

MISSION shRNA Vectors (SIGMA-ALDRICH) were co co-transfected into 293T cells with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev vector using Lipofectamine RNAi MAX Regent (Thermo Fisher Scientific). The medium was replaced 24 h after transfection. After additional 24

h, virus-containing supernatants derived from these 293T cell cultures were filtered through a 0.45 µm cellulose acetate filter (Pall Corporation) and supplemented with 8 µg/mL polybrene (Sigma). 2C::tdTomato reporter cells were incubated in the virus/polybrene-containing supernatants for overnight. After infection, the cells were replaced with fresh medium. Two days after infection, cells were selected by the use of 1 µg/mL puromycin (InvivoGen) for 8 days and survived cells were used to experiments. The RNAi Consortium (TRC) ID of shRNA vectors were TRCN0000077383 (shDaxx #1) and TRCN0000077383 (shDaxx #2).

RT-qPCR of ES cells

Total RNA was extracted from J1 ES cells transfected 2C::tdTomato reporter using RNeasy plus Mini Kit (QIAGEN), according to the manufacturer's protocol. Genomic DNA was removed from the samples by treatment with Turbo DNase (Ambion) at 37°C for 30min. The cDNA was synthesized using a ThermoScript RT-PCR system (Invitrogen). RT-qPCR was performed using a ViiA7 Real-Time PCR system (Applied Biosystems) using SYBR Green (Applied Biosystems). Relative gene expression levels were normalized to Gapdh.

Quantification of tdTomato-positive cells

The Daxx-knockdown 2C::tdTomato reporter cells were suspended in PBS, and sorted by BD FACS Aria II (BD Biosciences).

Antibodies

Antibodies used in this study were as following. Primary antibodies: rabbit anti-Daxx (1:1000 dilution; Santa Cruz Biotechnology, sc-7152), rabbit anti-Stella (1:3000 dilution; described previously [43]), and mouse anti- β -actin (1:6000 dilution; Sigma-Aldrich, A5441). Secondary antibodies: anti-rabbit IgG-Alexa568 (1:500 dilution; Invitrogen, A11036), anti-mouse IgG-Alexa488 (1:500 dilution; Invitrogen, A21042), anti-rat IgG-Alexa568 (1:500 dilution; Invitrogen, A11077), HRP-linked anti-mouse IgG (1:3000 dilution; GE Healthcare, NA9310V), and HRP-linked anti-rabbit IgG (1:3000 dilution; GE Healthcare, NA9340V).

Table. 1 Primers used in this study

Name	Sequence (5' to 3')	Application
Major satellite forward strand-RT	CTGATCTAGAGGTACCGGATCC GACTCGAGTCGACATCGTTTTTC CTCGCCATATTTTAC	RT
Major satellite reverse strand-RT	CTGATCTAGAGGTACCGGATCC GACTCGAGTCGACATCGACGAC TTGAAAAATGACGAAATC	RT
EGFP forward	ACGTAAACGGCCACAAGTTC	RT, qPCR
EGFP reverse	AAGTCGTGCTGCTTCATGTG	RT, qPCR
Major satellite forward strand-qPCR	CTTGACGACTTGAAAAATGACG AAATCAC	qPCR
Major satellite reverse strand-qPCR	CCTTTTTCAGTTTTCTCGCCAT ATTTTAC	qPCR
Major satellite adapter sequence-qPCR	AGAGGTACCGGATCCGACTCGA GTCGACATC	qPCR
Daxx forward-qPCR	AGTAGGAAGTGCTACAAGTTGGA	qPCR
Daxx reverse-qPCR	AGAGGCCAGAAACACAGACTG	qPCR
MuERV-L forward-qPCR	CTCTACCACTTGACCATATGAC	qPCR
MuERV-L reverse-qPCR	GAGGCTCCAAACAGCATCTCT	qPCR
Zscan4 forward-qPCR	AAATGCCTTATGTCTGTTCCCTATG	qPCR
Zscan4 reverse-qPCR	TGTGGTAATTCCTCAGGTGACGAT	qPCR
Tcstv1 forward-qPCR	TGAACCCTGATGCCTGCTAAGACT	qPCR
Tcstv1 reverse-qPCR	AGATGGCTGCAAAGACACAAGTGC	qPCR
Gapdh forward-qPCR	AAGGTCATCCCAGAGCTGAA	qPCR
Gapdh reverse-qPCR	CTGCTTACCACCTTCTTGA	qPCR

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Achievement

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1. Arakawa T, Yoshimi T, Azuma M, Tachibana T. Production of a Monoclonal Antibody specific for Pou5f1/Oct4. ***Monoclonal Antibodies in immunodiagnosis and immunotherapy***. (2013), 32 (3); 229-231

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以下の論文においては抗体の作製などに関与しました。

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以下の論文においてはサンプル採取などに関与しました。

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6. Higashi K, Yagi M, Arakawa T, Asano K, Kobayashi K, Tachibana T, Saito K. A novel marker for undifferentiated human embryonic stem cells. *Monoclonal Antibodies in immunodiagnosis and immunotherapy*. (2015), 466 (1); 60-65

学会発表

1. Role of histone chaperone for chromatin reorganization in early embryo

~From the analysis of DNA demethylation regulator, Stella, -null embryo as a model~

大阪大学医学系研究フォーラム、第3回若手研究フォーラム、2014年7月

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日本分子生物学会、第37回日本分子生物学会年会、2014年11月

3. 初期胚のクロマチン再構成におけるヒストンシャペロンの役割

~DNA 脱メチル化制御因子 Stella 欠損胚をモデルとして~ 新学術領域研究；生殖細胞の

エピゲノムダイナミクスとその制御、若手勉強会 2015, 2015年7月

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