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Stella controls chromocenter formation through regulation of Daxx expression in 2-cell embryos

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ABSTRACT

In mammals, the structure of the pericentromeric region alters from a ring structure to a dot-like structure during the 2-cell stage. This structural alteration is termed 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 chromosome segregation. In this study, we further investigated the effect of Stella deficiency on chromatin reorganization. The Stella-null embryos exhibited impaired CF and reduced expression of the reverse strand of major satellite repeats. In addition, the accumulation of H3.3, a histone H3 variant associated with transcriptional activation, at the pericentromeric regions and expression of the H3.3-specific chaperone Daxx were reduced in Stella-null embryos. These abnormalities were restored by the enforced expression of Daxx in Stella-null embryos. Thus, Stella controls the expression of Daxx and ensures chromatin reorganization in early embryos.

1. Introduction

Maternal and paternal chromatin in zygotes possess a different type of epigenetic mark, termed epigenetic asymmetry, which is known to be crucial for gene regulation during early development. A maternal factor, Stella/PGC7/Dppa3, is essential for preimplantation development presumably by contributing to epigenetic asymmetry between paternal and maternal chromatin in zygotes [1–3]. Previously, we found that the Stella-null blastomere frequently exhibited abnormal chromosome segregation during early mitosis [4].

Chromocenter is a cluster of constitutive heterochromatic regions, in which repetitive elements and transposable elements are transcriptionally silenced [5] and stably conserved, even during the cell cycle in somatic cells. In zygotes, pericentromeric regions show a morphologically unique ring structure around the nucleolus precursor bodies (NPBs). Chromocenter formation (CF) is the dynamic reorganization of ring structures to the chromocenter during development to the 2-cell stage [6,7]. CF is triggered by transcripts from major satellite repeats, which are tandem arrays of sequences of up to 2 M base pairs in the pericentromeric regions [5,8,9]. Transcription of major satellite repeats occurs from both the forward and reverse strands in a strand-specific manner, and

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transcriptional activity from the forward and reverse strand are highest during the early and late 2-cell stage, respectively [6]. Reverse transcripts have been reported mainly to contribute to CF and preimplantation development [10].

Recently, it has been shown that histone variants play important roles in the transcriptional states, as well as DNA and histone modifications [11,12]. Histone H3.3, one of the histone H3 variants, is distributed around the transcriptionally active regions and is closely associated with global gene expression in somatic cells [13]. The incorporation of H3.3 is regulated by the histone chaperone Daxx in a replication-independent manner [14]. Daxx-deficient mouse embryonic fibroblast cells show reduced H3.3 incorporation and major satellite expression [15]. In contrast, H3.3 is incorporated predominantly into the heterochromatic regions of the paternal pronucleus prior to the maternal pronucleus in zygotes [16,17]. 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. In addition, we found that Daxx rescued the abnormal CF that was caused by abnormal incorporation of H3.3 in the Stella-null embryos.

2. Materials and Methods

2.1. Zygote collection and culture

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% CO_2. Experiments were performed in accordance with the guidelines of the Osaka University Animal Care and Use Committee.

2.2. 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.

2.3. 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 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Immunofluorescence was visualized using an FV1000-D confocal laser-scanning microscope (Olympus).

2.4. 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) [18]. The *H3.3-GFP* cDNA was cloned into pcDNA4mycHisA (Invitrogen).

2.5. 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) (Supplemental Fig. S1).

2.6. 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.

2.7. 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% CO_2.

2.8. 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 [19]. 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*. RT-qPCR primers are listed in Supplemental Table S1.

2.9. Western blotting

Embryos 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.

2.10. 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

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