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令和2年度 博士論文

***Tet1*-dependent chromocenter clustering in DNA hypomethylated cells**

低メチル化細胞におけるTet1依存的なクロモセンタークラスタリング

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令和3年3月修了

Abstract

Pericentromeric heterochromatin (PCH), constitutive heterochromatin of pericentric regions, plays crucial roles in various cellular events, such as cell division and DNA replication. PCH forms chromocenters in the interphase nucleus and chromocenters further cluster at the prophase of meiosis. This chromocenter clustering has been reported to be critical for normal meiosis progression, especially for synapsis formation. However, the underlying molecular mechanism of chromocenter clustering remains elusive. In this study, I found that global DNA hypomethylation, 5hmC enrichment in PCH, and chromocenter clustering of *Dnmt1*-KO ESCs were similar to those of female meiotic germ cells. I revealed *Tet1* was essential for the deposition of facultative histone marks of H3K27me3 and H2AK119ub at PCH and the chromocenter clustering in *Dnmt1*-KO ESCs. In this situation, RING1B, one of the core components of PRC1 was recruited to PCH by *Tet1* and PRC1 played a critical role in the chromocenter clustering. Rearrangement of chromocenter under the hypomethylated condition was mediated by liquid-liquid phase separation in *Dnmt1*-KO ESCs. I also found *Tet1*-KO mice showed chromocenter clustering abnormality at the meiotic prophase. Therefore, I demonstrated a novel role of *Tet1* in the chromocenter rearrangement in DNA hypomethylated cells.

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1. Introduction

1.1. Pericentric heterochromatin and chromocenter organization

Pericentromere, a chromosomal region located on both sides of centromere contains species- and chromosome-specific sequences that differ in nucleotide sequence and repetitive length. Yeast (*S. pombe*) pericentromeric region consists of dg and dh repetitive sequence in all 3 chromosomes (P. C. Li et al., 2013). The mouse genome is consisting of 20 chromosomes, and they share the 234-bp AT-rich repetitive sequence, called major satellite repeat (Fig.1A) (Almouzni & Probst, 2011; Choo et al., 1989). Unlike the mice genome, the human genome contains different classes of repetitive sequences which are divided in to six families: 1 to 3 and α to γ (Francstel & Magdinier, 2019). In spite of the difference across species, it is conserved that the pericentromeric region plays a role in genomic stability, suppression of abnormal recombination during meiosis, and formation of the silent region in nuclear (Francstel & Magdinier, 2019).

In mammals, the pericentromeric region harbors high levels of trimethylation of histone H3 lysine 9 (H3K9me3), trimethylation of histone H4 lysine 20 (H4K20me3), and DNA methylation at the C-5 Position of Cytosine (5mC). These epigenetic marks interact with the heterochromatin protein (HP1) family and form silent regions as constitutive heterochromatin. At the interphase of the cell cycle, pericentromeric heterochromatin (PCH) of different chromosomes aggregate and form the chromocenter, which is visible as 4'6-diamidino-2-

phenylindole (DAPI)-dense foci in the nucleus (Fig.1B) (Almouzni & Probst, 2011). The organization of chromocenters including their position, size, and number in the nucleus are different among individual cell-types and are subject to change during the differentiation (Brero et al., 2005; Novo et al., 2016).

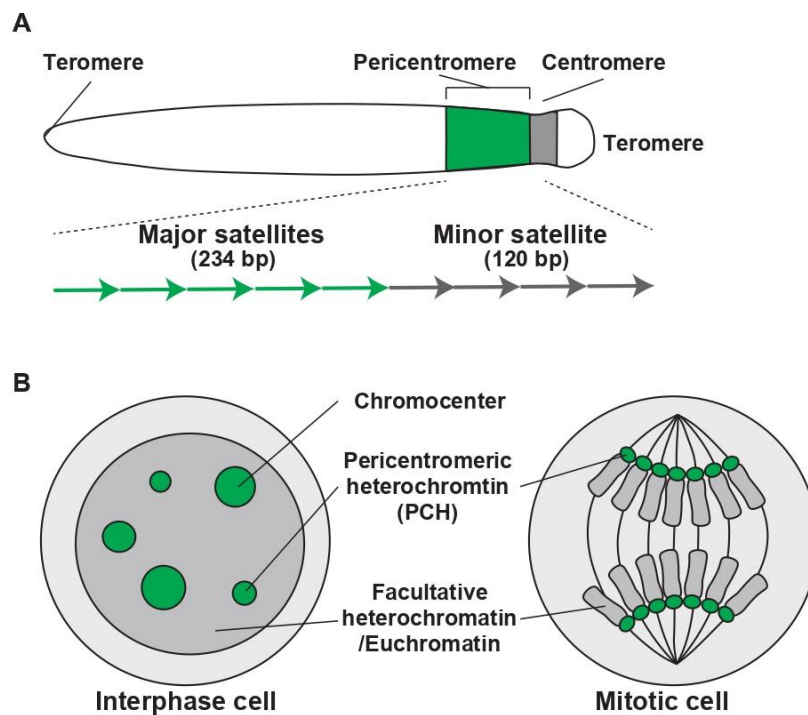


Figure 1. Mouse chromatin architecture

(A): Schematic diagram of major and minor satellite repeats in pericentromeric and centromeric compartments. (B): Pericentromeric heterochromatin of different chromosomes cluster together in chromocenter during interphase.

1.2. Biological significance of chromocenter clustering

One of the most striking re-organization is called chromocenter clustering, an accumulation, and gathering of chromocenters. A couple of factors has been identified to affect the chromocenter clustering. For example, MeCP2 and MBD2 localize to chromocenter and induce

the clustering during myogenesis, and NANOG is recruited to chromocenter by SALL1 and controls chromocenter clustering via its transactivation domain in embryonic stem cells (ESCs) (Brero et al., 2005; Novo et al., 2016). The biological significance of these particular chromocenter organization remains unclear. However, chromocenter clustering has been reported to be important for functional acquisition or normal differentiation in specific type of cells, such as rod cells and meiotic germ cells. Rod cells of rodents are observed as a single chromocenter in the center of the nucleus with highly clustered chromocenters, which is known as inverted chromocenter (Solovei et al., 2009). This clustered chromocenter is common in nocturnal and crepuscular animals, such as opossum, cat, and rabbit, and acts as collecting lenses that efficiently transmit light to the light-sensing rod outer segment (Solovei et al., 2009).

Chromocenter clustering is also functional in the germ cells at the meiotic prophase, in which clustering occurs preceding centromere clustering. (Takada et al., 2011). Chromocenter clustering occurs in both males and females and HP1 γ and G9a have been reported as critical factors in males (Takada et al., 2011). In male meiosis, HP1 γ regulates G9a localization to chromocenter, and this cascade is critical for chromocenter clustering at the meiotic prophase. Depletion of HP1 γ affects the subsequent meiotic process of centromere clustering, homologous chromosome pairing, and synapsis formation, suggesting chromocenter clustering is a crucial step for normal meiosis progression (Takada et al., 2011). However, the underlying molecular mechanism of chromocenter clustering of female meiotic germ cells remains elusive.

1.3. DNA methylation cycle and Tet family

DNA methylation level at PCH has been reported to link nuclear organization, including chromocenter clustering (Brero et al., 2005; Gilbert et al., 2007). Three types of DNA-methyltransferases (DNMTs) have been reported to catalyze 5mC in mice; de novo methylase DNMT3A and DNMT3B, and maintenance methylase DNMT1 (E. Li & Zhang, 2014). Loss of DNMT family causes global hypomethylation of CpG sites. In the wild-type ESCs, 82% of CpG sites are methylated, while it is reduced to 18% in the *Dnmt1*-KO ESCs and 0.4% in the *Dnmt1/3a/3b*-TKO ESCs (triple knock out of *Dnmt1*, *Dnmt3a*, and *Dnmt3b*) (Tsumura et al., 2006). Ten-eleven translocation (TET) family (TET1, TET2, and TET3) have been identified as a dioxygenase that converts 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (X. Wu & Zhang, 2017). TET-mediated iterative oxidation is reported to play a role in the loss of 5mC either through the glycosylation by TDG followed by a base excision repair or DNA replication-dependent dilution (Fig.2A) (X. Wu & Zhang, 2017).

TET proteins show cell-type-specific expression: *Tet1* expresses in ESCs, primordial germ cells (PGC), and neuronal cells, *Tet2* is also detected in ESCs and differentiated cells such as hematopoietic stem cells, *Tet3* highly expresses in the zygote and neuronal cells (Yang et al., 2020). TET1 and TET3 have CXXC domains that can bind to unmethylated CpG but TET2 lacks CXXC domain during evolution and separated CXXC domain express as independent

gene *Idax* (Fig.2B). In addition, TET proteins have multiple roles not only in a catalytic activity-dependent but also -independent manner. For example, TET1 associates with SIN3A and NuRD complex as well as pluripotency factors such as NANOG, PRDM14, and LIN28, and regulates the specific genes expression (Costa et al., 2013; Okashita et al., 2014; Shi et al., 2013; Zeng et al., 2016). Considering that enrichment of 5hmC in PCH of meiotic germ cells became undetectable in *Tet1*-KO mice, TET1 would access to PCH locus under this circumstance but its biological function is poorly understood (Yamaguchi et al., 2012)

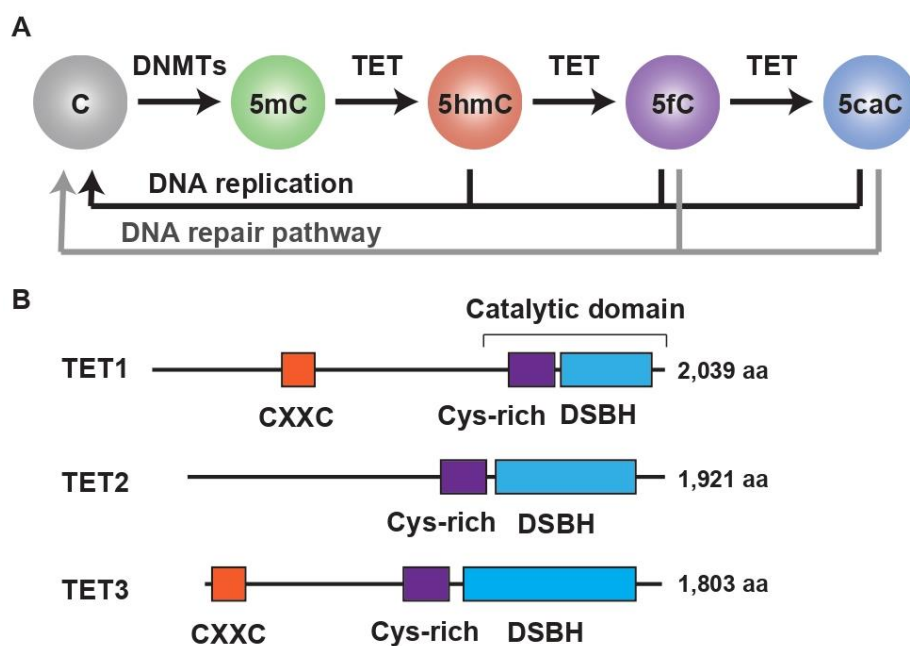


Figure 2. DNA methylation cycle and TET family structure

(A): The cycle of DNA methylation. DNMT family converts unmethylated cytosine to 5mC. 5mC is converted to 5hmC, 5fC, and 5caC by TET-mediated oxidation. Through DNA repair pathway and DNA replication-dependent dilution, modified cytosine back to its unmodified state. (B): Domain structure of mouse TET1 proteins. TET1 and TET3 have the CXXC domain at N-terminal. All TET proteins have Cysteine-rich (Cys-rich) domain and double-stranded β -helix (DSBH) domain which possess oxidation catalytic activity.

1.4. The role of Polycomb group proteins in spatial genome organization

Polycomb group (PcG) repressor proteins play important roles in developmental gene regulation and facultative heterochromatin formation (Simon & Kingston, 2013). Polycomb repressive complex 1 (PRC1) has an activity to ubiquitinate the lysine 119 of histone H2A (H2AK119ub), which is catalyzed by ubiquitin E3 ligase RING1A and RING1B (RNF2). PRC2 deposited histone H3 lysine 27 trimethylation (H3K27me3) through the methyltransferase EZH2.

PRC1 and PRC2 act synergistically to suppress the transcription of target genes; PRC2-mediated H3K27me3 is recognized by PRC1, while H2AK119ub leads to recruitment of PRC2 (Blackledge et al., 2014; Cooper et al., 2014). Accumulation of PRC1 and PRC2 establish a long-range interaction between PcG-bound loci and form a huge protein-DNA complex named PcG body, which is detectable as puncta in the nucleus (Denholtz et al., 2013; Isono et al., 2013; Pachano et al., 2019; Schoenfelder et al., 2015). PcG proteins have been reported to associate with nuclear structural proteins, Lamin A/C but not Lamin B, this interaction contributes to PcG body assembly and spatial chromatin organization (Cesarini et al., 2015). Moreover, ectopic PcG localization to chromocenters has been reported in DNA hypomethylated ESCs and cancer cells but their affection to chromocenter organization remains unknown (Cooper et al., 2014; Hall et al., 2017; Saksouk et al., 2014).

1.5. Liquid-liquid phase separation and heterochromatin organization

Liquid-liquid phase separation (LLPS) is characterized by spontaneous demixing of homologous solution into two phases. Proteins with low complexity intrinsically disordered domain (LCDR) mediate multivalent weak interaction, such as hydrophobic and electrostatic (π/π , cation/ π , and etc.) interactions, and these interactions promote and stabilize LLPS. LLPS is involved in multiple biological phenomena through the formation of membrane-less organelles and functional drops; transcriptional regulation through super-enhancers and PcG bodies, stimulation response of DNA damage repair and autophagy, and developmental process such as homologous chromosome pairing (Ding et al., 2019; Noda et al., 2020; Plys et al., 2019; Sabari et al., 2018; Tataavosian et al., 2019; Wheeler et al., 2016).

LLPS is also believed to contribute to the formation of constitutive heterochromatin formation through H3K9me3 binding proteins (L. Wang et al., 2019). Heterochromatin protein α (HP1 α), one of the major chromocenter proteins, has been reported to form LLPS-driven droplets *in vitro* (Larson et al., 2017; Strom et al., 2017). However, HP1 α foci were resistant to the treatment of 1,6-hexanediol, aliphatic alcohol that disrupts the hydrophobic interaction of LLPS (Strom et al., 2017). Therefore, established heterochromatin is believed to comprise mostly immobile compartments rather than purely liquid structures.

In this study, I found that ESCs with hypomethylated DNA showed deposition of facultative heterochromatin marks in PCH and chromocenter clustering. Deposition of 5hmC, H2AK119ub, and H3K27me3 in PCH of the hypomethylated ESCs were all abrogated by the deletion of *Tet1*. Moreover, I found TET1-PRC1 axis was critical for the chromocenter clustering in the hypomethylated ESCs. I also demonstrated DNA hypomethylation-dependent chromocenter clustering was driven by LLPS. *Tet1* also plays a critical role in female germ cells at meiotic prophase. Thus, these finding revealed a novel function of *Tet1* on the rearrangement of heterochromatin triggered by the loss of DNA methylation.

2. Material and method

2.1. ESCs culture

Wild-type and KO ESCs (J1 strain) were maintained in the Glasgow modified Eagle's medium (GMEM) containing high glucose supplemented with 10% fetal calf serum, 100 U/mL mouse LIF, 2-mercaptoethanol, L-glutamine, and penicillin/streptomycin, 3 μ M CHIR99021 (Chemscene) and 1 μ M PD0325901 (CAYMAN) on gelatin-coated culture plates. *Dnmt1*-KO and *Dnmt1/3a/3b*-TKO ESCs were provided by Dr. Okano (Tsumura et al., 2006) (Kumamoto University, Japan).

2.2. Generation of knockout cell lines

All knockout ESC lines were established through the CRISPR/Cas9 system. ESCs were transfected with a mixture of px330-puro vectors containing specific sgRNA targeting each gene (Table.1). Successful depletion of target genes was verified using genotyping PCR, western blotting, and immunocytochemical analyses.

As for the depletion of *Ring1A/1B*, *Dnmt1*-KO ESCs was transfected with a mixture of px330-Puro vector containing sgRNA targeting *Ring1A/1B* (Table S1). For selection, ESCs were cultured with 1 μ g/mL puromycin for 2 d. Subsequently, the cells were cultured for recovery without puromycin for 1 d. The cells were then harvested and used for surface spread preparation.

2.3. Epigenome editing experiments

The fragment for sgRNA targeting the major satellite repeat (Table.1) was inserted into the pPlatTET-gRNA2 vector (addgene #82559). The scFv-only vector was generated by removing the Tet1-CD domain from this plasmid. These plasmids were linearized by ApaL1 and transfected into E14 ESCs to establish a stably expressing cell line. ESCs were maintained in GMEM containing high glucose supplemented with 10% fetal calf serum, 100 U/mL mouse LIF, 2-mercaptoethanol, L-glutamine, and penicillin/streptomycin.

Table.1 sgRNAs used in this study

sgRNA target	Target sequence (5' to 3')	Application
<i>Tet1</i> exon 4	GGCTGCTGTCAGGGAGCTCA	KO
Major satellite repeat	GAAATGTCCACTGTAGGACG	dCas9 recruitment
<i>Ezh2</i> upstream of 5'UTR	AGTATGGCACGCCGGAAGAAG	KO
<i>Ezh2</i> upstream of 3'UTR	TCCCCAGGGTACAAGTTGGC	KO
<i>Ring1a</i> upstream of 5'UTR	TGCAGCTGCGGCCGGCTTTC	KO
<i>Ring1a</i> upstream of 3'UTR	TCACCTTCCTATTTACAACC	KO
<i>Ring1b</i> exon 4	TCAACCATTAAGCAAAACAT	KO
<i>Ring1b</i> exon 8	AAAAGCTCATTGTGCTCCT	KO

2.4. Surface spread and immunocytochemistry

ESCs and gonadal cells were dissociated and placed on a glass slide dipped in a fixation solution of 1% paraformaldehyde (PFA) in distilled water (DW) (pH 9.2) containing 0.15% Triton X-100 and 3 mM dithiothreitol. Slides were then incubated overnight at 4 °C for a fixation. The slides were then washed in 0.4% Photoflo (Kodak) in DW and dried at room

temperature. The samples were washed with 0.1% Triton X-100/PBS (PBS with Tween 20; PBST) and treated with 0.5% Triton X-100 for 20 min. After washing with PBST, the samples were incubated with 3% BSA and 2% goat serum in PBST (blocking buffer). Samples were then incubated with primary antibodies diluted in blocking buffer, washed, and incubated with appropriate secondary antibodies. Immunofluorescence was visualized using an LSM880 (Carl Zeiss).

2.5. Mice and surface spread for germ cells

Tet1 heterozygous mice (*Tet1*^{+/-}) with Oct4-GFP reporter were established by the mating with *Oct4-GFP* strain (Yoshimizu et al., 1999). Germ cells were detected by Oct4-GFP expression and purified by FACS. Then, cells were washed with PBS once and fixed on the slide as described above. Meiotic germ cells at E15.5 and E16.5 were dissociated by trypsin and washed with PBS, followed by a fixation on the slide.

2.6. Immunocytochemistry for RING1B and TET1 detection using culture slide

For immunostaining against RING1B and TET1, ESCs were plated in a culture slide (Corning, #354118) and cultured for 1 d before immunostaining. ESCs were fixed with 4% PFA for 5 min at room temperature, followed by permeabilization with 0.5% Triton X-100/PBS for 20 min and 45 min for RING1B and TET1, respectively. They were then incubated with the

primary antibodies diluted in the blocking buffer at 4 °C overnight and 3 d for RING1B and TET1, respectively. The samples were washed with PBST and incubated with the secondary antibodies.

2.7. Antibodies

Primary antibodies used in this study include rabbit anti-5hmC (Active Motif, #39769), mouse anti-5mC (Active Motif, #39649), rabbit anti-H3K9me2 (Abcam, ab1220), rabbit anti-H3K9me3 (Abcam, ab8898), mouse anti-H3K27me3 (Wako, MABI0323), rabbit anti-H2AK119Ub (Cell signaling, #8240), mouse anti- γ H2AX (Sigma-Aldrich, 07-164), mouse anti-SCP3 (Santa Cruz, sc-74569), rabbit anti-SCP3 (Novus, NB-300-232), mouse anti-HP1 γ (Invitrogen, MA3-054), human anti-Centromere Protein (CREST) (Antibodies Incorporated, 15-235), rabbit anti-TET1 (Millipore, 09-872), rabbit anti-EZH2 (Cell signaling, #5246) and rabbit anti-RING1B (Cell signaling, #5694). Secondary antibodies used in this study include Alexa Flour 488 goat anti-mouse IgG, Alexa Flour 488 goat anti-rabbit IgG, Alexa Flour 568 goat anti-mouse IgG, Alexa Flour 568 goat anti-rabbit IgG, Alexa Flour 647 goat anti-human IgG (Invitrogen).

2.8. Live-cell imaging

The Dnmt1-KO ESCs stably expressing hKO1-HP1 γ was established by the transfection of the linearized CAG- hKO1-HP1 γ expressing plasmid. Time-lapse fluorescence microscopy was performed using an upright OLYMPUS confocal microscope (SpinSR10) with an x100 oil immersion lens. Images were taken every 2 min and processed using cellSens (OLYMPUS) and IMARIS (Carl Zeiss) software.

2.9. Hexanediol treatment

To disrupt LLPS, 1,6-hexanediol (Nacalai Tesque, 17913–72) or 2,5-hexanediol (TCI, H0100) at a final concentration of 10% was added to the ESC suspension. After 1 min of incubation at room temperature, the cells were placed on a glass slide dipped in the fixative. For the recovery experiment, 1,6-hexanediol treated cells were diluted with a 100-fold volume of ESC culture medium and incubated for 1 h in a humidified incubator at 37 °C, followed by the fixation on a glass slide.

3. Results

3.1. Epigenetic and chromocenter remodeling of female germ cells and *Dnmt1*-KO ESCs

First, I analyzed HP1 γ and H3K9me2, which was catalyzed by G9a H3K9 methyltransferase at meiotic prophase in female germ cells. PCH forms clusters in the nuclei of both male and female germ cells at the meiotic prophase. HP1 γ and G9a, both of which are highly enriched in the chromocenter in male germ cells, play a critical role in the clustering of PCH and homologous chromosome pairing in male gonocytes (Takada et al., 2011). In contrast, although HP1 γ showed specific and consistent localization to PCH, H3K9me2 was depleted in PCH of female germ cells (Figure 3A and 3B). This distinct enrichment pattern of epigenetic marks suggests that the clustering of PCH in male and female germ cells is regulated by different mechanisms.

I then hypothesized that the difference in DNA methylation levels at the meiotic stage would contribute to the sex difference of chromocenter clustering, because it affects the nuclear architecture (Madakashira & Sadler, 2017). To test this hypothesis, I analyzed the *Dnmt1*-KO ESCs (Tsumura et al., 2006). PCH of the *Dnmt1*-KO ESCs was highly clustered to form large chromocenters in the nucleus, and their number was significantly lower than that of the wild-type ESCs (Fig.4A and 4B). This result was consistent with the previous report showing that hypomethylation caused chromocenter clustering (Gilbert et al., 2007). Condensed

chromocenter of the *Dnmt1*-KO ESCs retained strong enrichment of HP1 γ , which is similar to meiotic germ cells (Fig.3A and 4A).

Meanwhile, 5hmC was enriched in the PCH of the *Dnmt1*-KO ESCs, which was also observed in the primordial germ cells (PGCs) during the epigenetic reprogramming phase (Fig.4C) (Yamaguchi et al., 2013). The relative enrichment score of 5hmC at PCH in the *Dnmt1*-KO ESCs was about two times more than that of the wild-type ESCs (1.21 v.s. 0.62) (Fig.4D). These results demonstrated that the *Dnmt1*-KO ESCs exhibited similar features to that of female germ cells, such as global hypomethylation, PCH-5hmC, and clustered chromocenter, to some extent. Therefore, I assumed that *Dnmt1*-KO ESCs is a good cell model to study the molecular mechanism of epigenetic state of meiotic germ cells.

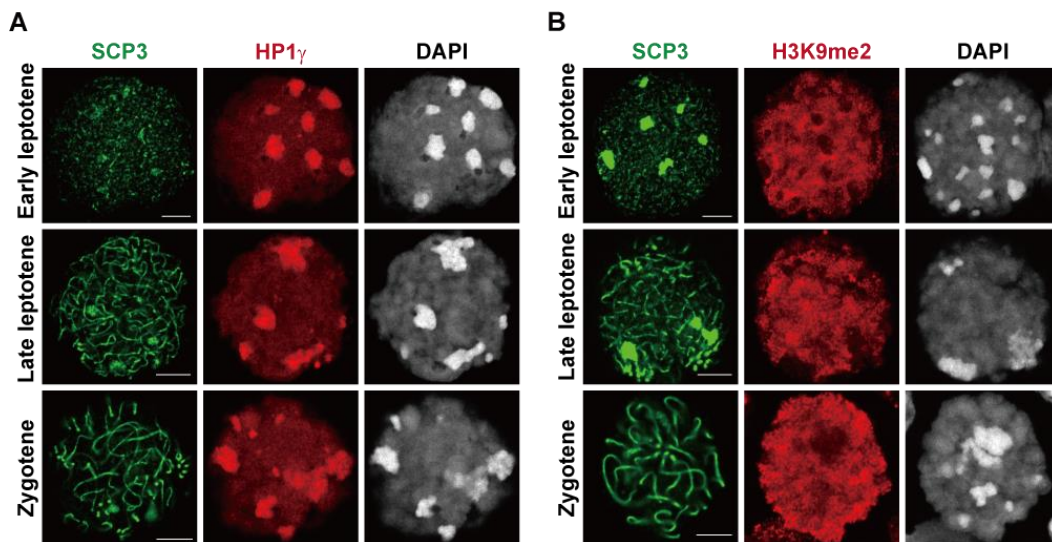


Figure 3. The enrichment of HP1 γ and the depletion of H3K9me2 from the chromocenter of female meiotic germ cells

(A), (B): Representative immunostaining images of surface-spread nuclei at individual stages of the first meiotic prophase. Female germ cells were harvested from E15.5-E16.5 embryos. Scale bar, 5 μ m.

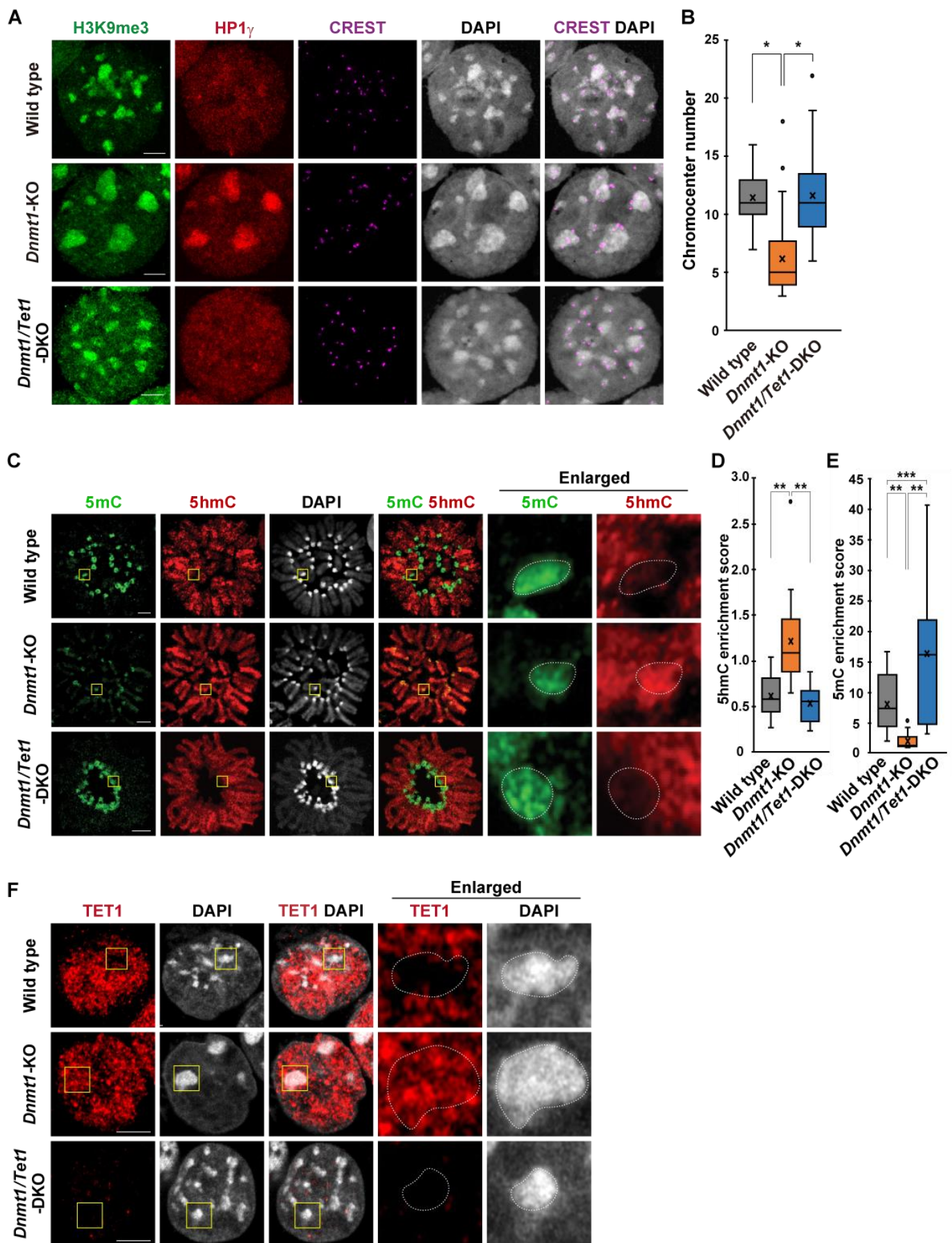


Figure 4. *Tet1*-dependent chromocenter clustering and 5hmC deposition at PCH in DNA hypomethylated ESCs

(A): Representative immunostaining images of surface-spread nuclei of the wild-type, *Dnmt1*-KO and *Dnmt1/Tet1*-DKO ESCs. (B): Boxplot showing the number of distinct chromocenter in the nuclei of ESCs, related to (A). In boxplots, the enter lines show the medians; box limits indicate the 25th and 75th percentiles, and whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Actual values are indicated by circle. Wild-type, n = 55; *Dnmt1*-KO, n=64; *Dnmt1/Tet1*-DKO, n = 64. (C): Representative immunostaining images of surface-spread nuclei of the wild-type, *Dnmt1*-KO and *Dnmt1/Tet1*-DKO ESCs. The yellow squares indicate the enlarged areas shown on the right. The white dashed circles indicate the PCH. (D), (E): Quantification of immunostaining of 5hmC (D) and 5mC (E), related to (C). The enrichment score is calculated by the average signal intensity of 5hmC and 5mC at PCH that is labeled as a strong DAPI signal, normalized by the entire chromosome. An average of 3 chromosomes was calculated for each cell. n = 20 per cell line. (F): Representative immunostaining images of the wild-type, *Dnmt1*-KO and *Dnmt1/Tet1*-DKO ESCs. The yellow squares indicate the enlarged areas shown on the right. The white dashed circles indicate the chromocenter. ESCs were plated in a culture slide and cultured for 1 d before immunostaining. P values were calculated using the Mann—Whitney U-test. *P < 0.001, **P < 0.01, ***P < 0.05. Scale bar, 5 μ m.

3.2. *Tet1* is critical for PCH-5hmC deposition and chromocenter clustering

Considering that TET1 is critical for the acquirement of PCH-5hmC in the PGCs and highly expressed in ESCs, I focused on the function of TET1 (Yamaguchi et al., 2012). Immunocytochemical analysis demonstrated that TET1 was slightly but significantly enriched in the chromocenter of the *Dnmt1*-KO ESCs compared to the wild-type ESCs (Fig.4F). Taken together, I hypothesized that TET1 was responsible for the gain of 5hmC at PCH and play a role in chromocenter clustering in DNA hypomethylated cells. To validate this hypothesis, I knocked out *Tet1* in the *Dnmt1*-KO ESCs by CRISPR-mediated mutagenesis and the depletion of TET1 protein was confirmed by immunocytochemistry (Fig.4F) (H. Wang et al., 2013).

Enrichment of 5hmC in PCH of the *Dnmt1/Tet1*-DKO ESCs was significantly decreased compared to the *Dnmt1*-KO ESCs and was comparable to that of the wild-type ESCs (0.54 v.s. 0.62) (Fig.4D). In contrast, 5mC enrichment in PCH of the *Dnmt1/Tet1*-DKO ESCs was about twice more than that of the wild-type ESCs (16.4 v.s. 8.1) (Fig.4E). These results exhibited that TET1 actively oxidizes 5mC to 5hmC at PCH in the *Dnmt1*-KO ESCs. Remarkably, *Tet1* depletion brought about the dispersed pattern of chromocenters and the increase of the number was comparable to the wild-type ESCs (Fig.4A and 4B). These results demonstrated that *Tet1* is critical for chromocenter clustering in DNA hypomethylated ESCs.

To investigate whether the chromocenter clustering is mediated by catalytic activity-dependent or -independent function of TET1, was important for the chromocenter clustering, I analyzed the *Dnmt1/3a/3b*-TKO ESCs which harbor no detectable 5mC nor 5hmC (Szwagierczak et al., 2010; Tsumura et al., 2006). Chromocenter clustering of the *Dnmt1/3a/3b*-TKO ESCs was similar to those of the *Dnmt1*-KO ESCs (Fig.4A and 5A). In contrast, chromocenters of *Dnmt1/3a/3b/Tet1*-QKO (quarto knock out of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Tet1*) ESCs were dispersed in the nucleus and its number was significantly more than that of the *Dnmt1/3a/3b*-TKO ESCs (Fig.5B). The number of chromocenter of the *Dnmt1/3a/3b/Tet1*-QKO ESCs was essentially same as that of the wild-type and the

Dnmt1/Tet1-DKO ESCs (Fig.4B and 5B). These results demonstrate that 5hmC and the catalytic activity of TET1 were dispensable for the chromocenter clustering.

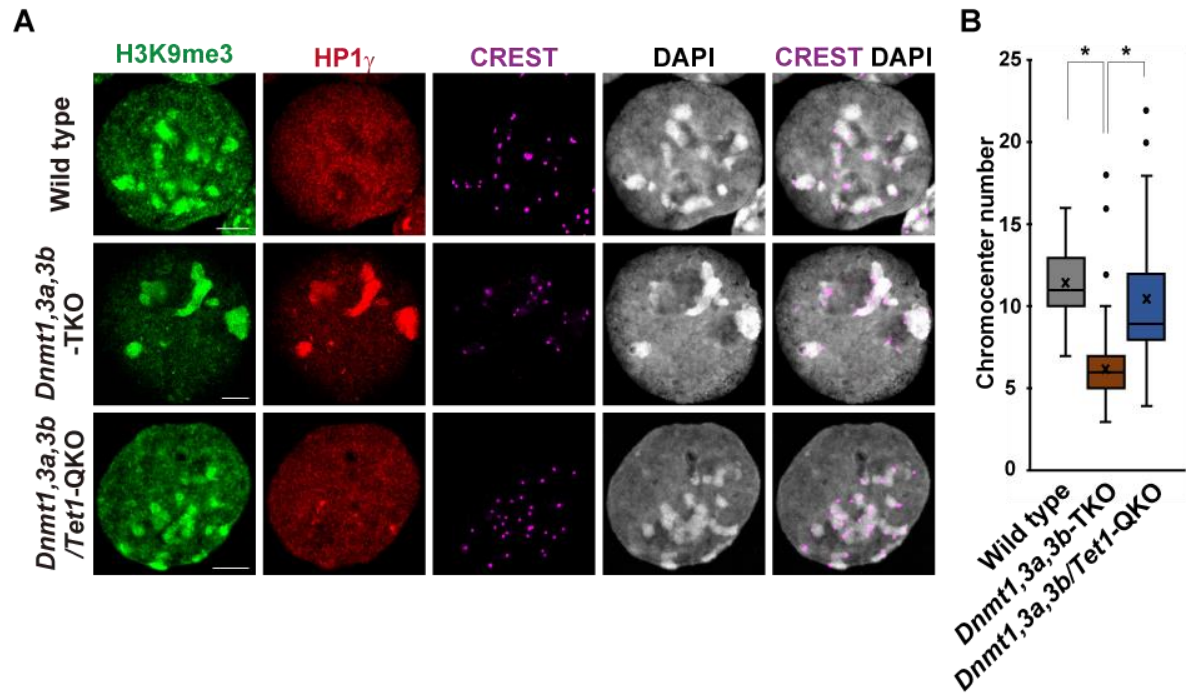


Figure 5. 5hmC was not dispensable for *Tet1*-dependent chromocenter clustering

(A): Representative immunostaining images of surface-spread nuclei of the wild-type, *Dnmt1/3a/3b*-TKO, and *Dnmt1/3a/3b/Tet1*-QKO ESCs. Scale bar, 5 μ m. (B): Boxplot showing the number of distinct chromocenter in the nuclei of ESCs, related to (B). Wild-type, n = 55; *Dnmt1/3a/3b*-TKO, n = 99; *Dnmt1/3a/3b/Tet1*-QKO, n = 55. P values were calculated using the Mann—Whitney U-test. *P < 0.001.

3.3 Induction of chromocenter clustering by the forced recruitment of TET1

To address TET1 localization to PCH is sufficient to induce chromocenter clustering, I carried out an experiment recruiting TET1 to PCH by the epigenome editing method (Morita et al., 2016). This dCas9-SunTag system recruits TET1 catalytic domain (CD) to single-guide RNA (sgRNA) targeted regions through anti-GCN4 scFv domains by tethering dCas9-fused GCN4 (Fig.6A) (Morita et al., 2016). I designed sgRNA targeting major satellite repeats to recruit TET1-CD to PCH. A whole cassette without sgRNA and scFv-only cassettes were used as negative controls (Fig.6B and 6D).

As shown in Fig.6B and 6C, 5hmC was strongly enriched in PCH of the TET1-CD recruited ESCs but was absent in PCHs of negative control ESCs and TET1 catalytic dead mutant (CD-mut) recruited ESCs. These results verified that TET1-CD was successfully recruited to PCH in a sgRNA-dependent manner. Chromocenters were clustered and their number was significantly decreased in not only TET1-CD recruited ESCs but also TET1-CD-mut recruited ESCs (Fig.6D and 6E). These results clearly showed that the recruitment of TET1-CD to PCH was sufficient to induce chromocenter clustering in its catalytic activity-independent manner.

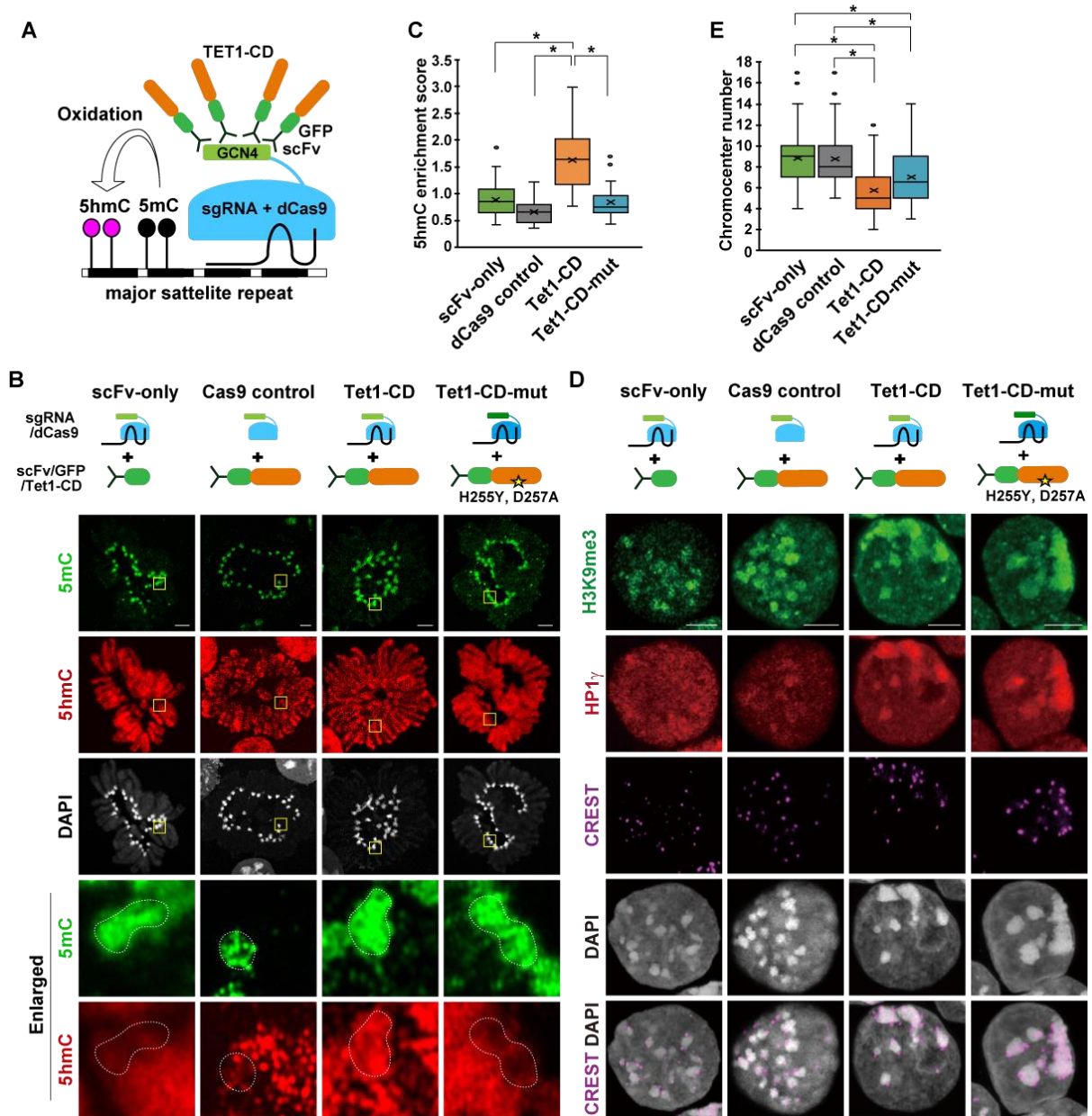


Figure 6. Forced recruitment of TET1-CD to PCH induces 5hmC deposition at PCH and chromocenter clustering

(A): Schematic illustration of the epigenome editing experiment. GCN4 was conjugated with catalytically-dead Cas9 (dCas9) which was recruited to PCH by the sgRNA targeting major satellite repeat. GCN4 was targeted by scFv conjugated with TET1-CD. (B): Representative immunostaining images of surface-spread nuclei of the ESCs expressing scFv-only, dCas9 control, Tet1-CD and Tet1-CD-mut cassette. The yellow squares indicate the enlarged areas shown on the bottom. The white dashed circles indicate the PCH. (C): Quantification of immunostaining of 5hmC, related to (B). n = 30 per cell lines.

(Legend continued on next page)

(D): Representative immunostaining images of surface-spread nuclei of the ESCs expressing individual cassette. (E): Boxplot showing the number of distinct chromocenter in the nuclei of ESCs expressing individual cassette, related to (C). scFv-only, n = 56; dCas9 control, n = 103; Tet1-CD, n = 105; Tet1-CD-mut, n = 34. P values were calculated using the Mann—Whitney U-test. *P < 0.001. Scale bar, 5 μ m.

3.4. Contribution of PRCs to chromocenter clustering in the DNA hypomethylated ESCs

Polycomb factors would be the core of chromocenter clustering mediated by *Tet1* because of the aggregate-forming ability and the co-localization with TET1 (Neri et al., 2013). TET1 distribution overlaps with PRC2 and H3K27me3 in mouse ESCs (Neri et al., 2013). The loss of DNA methylation has been demonstrated to induce PRC2 and H3K27me3 enrichment in PCH (Cooper et al., 2014; Saksouk et al., 2014). Consistently, H3K27me3 at PCH, which was depleted in the wild-type ESCs, was increased in the *Dnmt1*-KO ESCs (Fig. 7A and 7B). In contrast, the immunocytochemical analysis showed that the level of H3K27me3 of the *Dnmt1/Tet1*-DKO ESCs was lower than that of the *Dnmt1*-KO ESCs and was similar to that of the wild-type ESCs (Fig. 7A and 7B). These results suggested that *Tet1* plays a role in recruiting PRC2 to PCH under the DNA hypomethylated condition.

I then depleted *Ezh2*, which is the core subunit of PRC2, in the *Dnmt1*-KO ESCs to test whether PRC2 was critical for the chromocenter clustering (Fig. 7C). Neither EZH2 nor H3K27me3 were detectable by immunostaining in two independent *Dnmt1/Ezh2*-DKO ESC clones (Fig. 7D). However, the pattern of chromocenter clustering was quite different between

these two clones; one clone showed no significant increase, whereas the other clone showed a slight but significant increase (Fig.7E). Even if there are some, PRC2 would have only a limited contribution to the chromocenter clustering under the DNA hypomethylated state.

H2AK119ub, which was catalyzed by PRC1, was enriched in PCH of the *Dnmt1*-KO ESCs as previously reported (Fig.8A and 8B) (Cooper et al., 2014). RING1B, the central component of PRC1, was also increased in the chromocenter of the *Dnmt1*-KO ESCs (Fig.8C). In contrast, the signal intensity of RING1B was decreased and H2AK119ub was undetectable in PCH of the *Dnmt1/Tet1*-DKO ESCs (Fig.8A and 8C). These data showed that *Tet1* plays a critical role in the recruitment of both PRC1 and PRC2 to the chromocenter and regulated the epigenetic state of PCH under DNA hypomethylated condition.

To test whether PRC1 was involved in chromocenter clustering, I targeted *Ring1A* and *Ring1B*, which are core components of PRC1 (Kang & Chun, 2020). It was almost impossible to establish stable *Ring1A/1B*-DKO ESC lines because of the proliferation defect and differentiated phenotype of the *Ring1a/b*-depleted ESCs (Endoh et al., 2008). Then, I analyzed the *Dnmt1/Ring1A/1B*-TKO ESCs shortly after the introduction of the CRISPR cassette encoding Cas9 and sgRNAs (Fig. 8D).

ESCs with depleted *Ring1A/1B* should have been detectable as H2AK119ub-negative cells since H2AK119ub synthesis completely depends on RING1A/1B (Endoh et al., 2008). Although H2AK119ub-negative cells were not detectable in the control wild-type or *Dnmt1*-

KO cells, such cells appeared 4 d after the transfection of the CRISPR cassette (Fig. 8E). These H2AK119ub-negative cells showed a dispersed pattern of chromocenter, and its number was comparable to that of the wild-type ESCs (Fig. 3C and 3D). Notably, HP1 γ enrichment at PCH did not change in the *Dnmt1/Ring1A/1B*-TKO ESCs, suggesting that HP1 γ was dispensable for chromocenter clustering under DNA hypomethylated condition. These results demonstrate that *Tet1*-dependent localization of PRC1 to PCH is one of the molecular bases of chromocenter clustering in DNA hypomethylated ESCs.

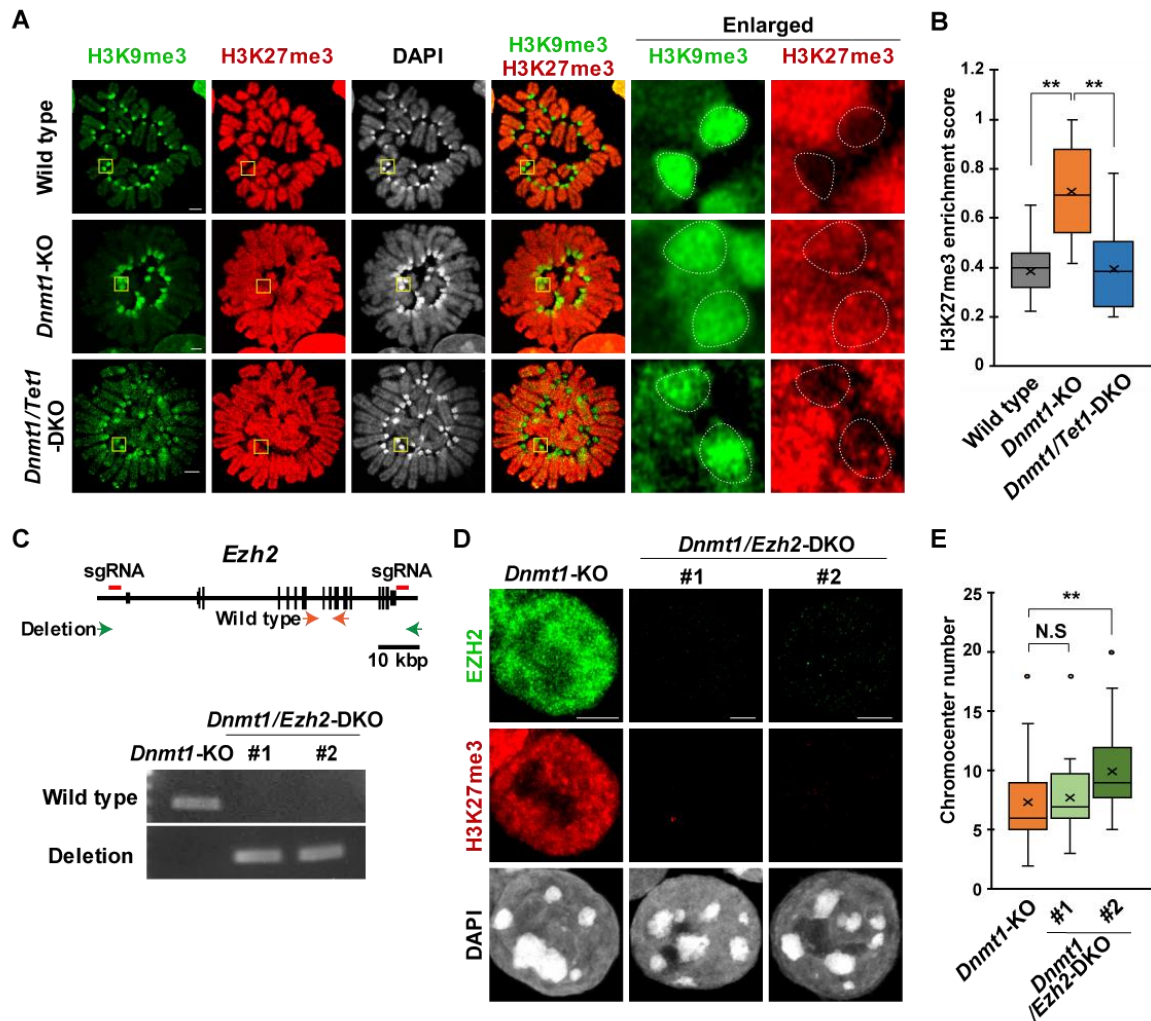
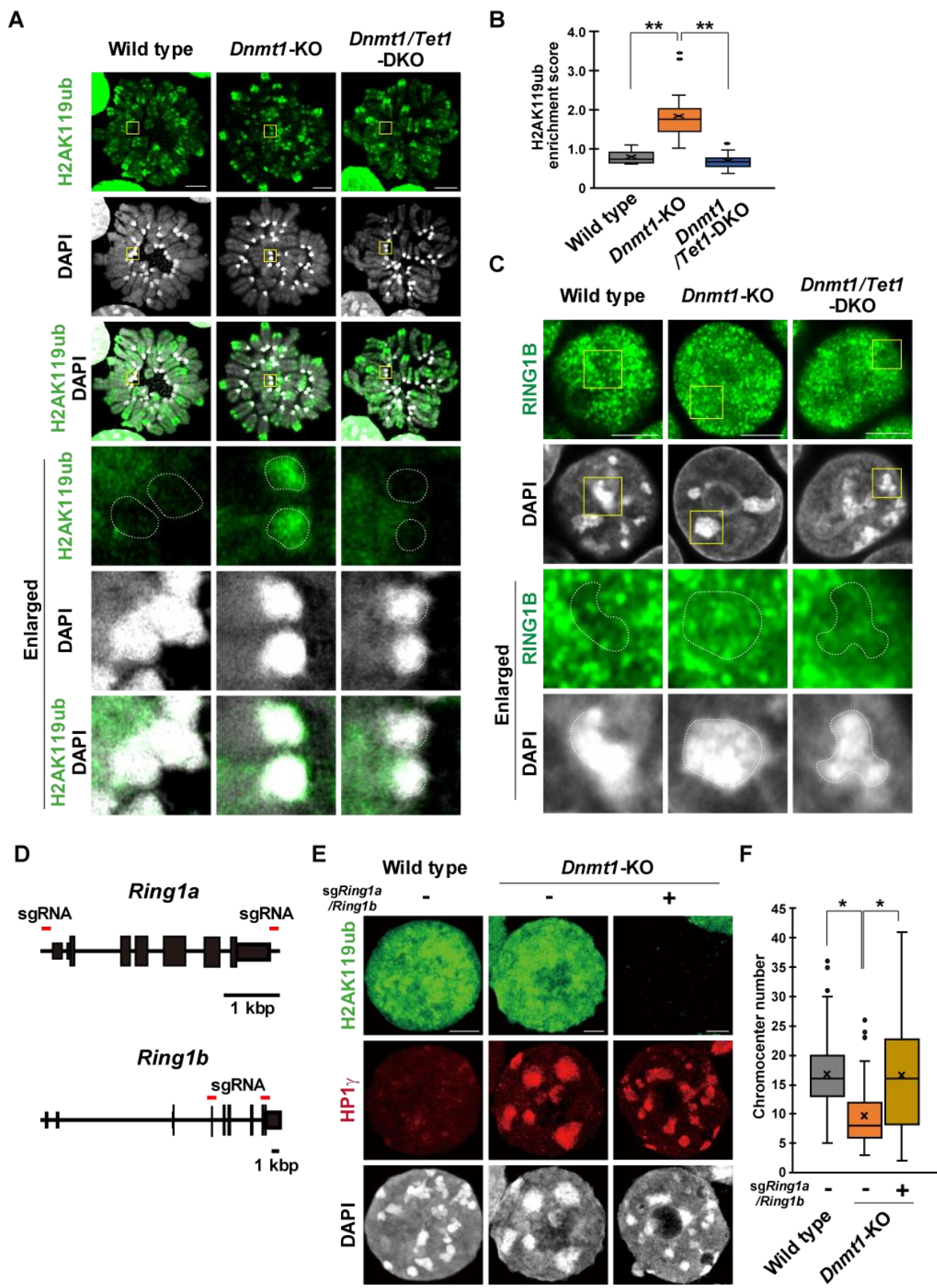


Figure 7. Minor effect of depletion of PRC2 on chromocenter clustering

(A): Representative immunostaining images of surface-spread nuclei of the wild-type, *Dnmt1*-KO and *Dnmt1/Tet1*-DKO ESCs. The yellow squares indicate the enlarged areas shown on the right. The white dashed circles indicate the PCH. (B): Quantification of immunostaining of H3K27me3, related to (A). $n = 20$ per cell line. (C): Schematic illustration showing the design of sgRNA and genotyping PCR for the deletion of *Ezh2* (top). Results of genotyping PCR confirming the successful deletion of whole *Ezh2* gene locus. (D): Representative immunostaining images of surface-spread nuclei of the wild-type, *Dnmt1*-KO and *Dnmt1/Ezh2*-DKO ESCs. (E): Boxplot showing the number of distinct chromocenter in the nuclei of ESCs, related to (D). *Dnmt1*-KO, $n = 55$; *Dnmt1/Ezh2*-DKO #1, $n = 32$; DKO #2, $n = 37$. P values were calculated using the Mann—Whitney U-test. ** $P < 0.01$; N.S, no significance, $P > 0.05$. Scale bar, 5 μm .



(Legend on next page)

Figure 8. *Tet1* controls PRC1 localization to chromocenter and induces clustering through PRC1 in DNA hypomethylated conditions

(A): Representative immunostaining images of surface-spread nuclei of the wild-type, *Dnmt1*-KO and *Dnmt1/Tet1*-DKO ESCs. The yellow squares indicate the enlarged areas shown on the bottom. The white dashed circles indicate the PCH. (B): Quantification of immunostaining of H2AK119ub, related to (A). $n = 20$ per cell line. (C): Representative immunostaining images of the wild-type, *Dnmt1*-KO and *Dnmt1/Tet1*-DKO ESCs. The yellow squares indicate the enlarged areas shown on the bottom. The white dashed circles indicate the chromocenter. The ESCs were plated in a culture slide and cultured for 1 d before immunostaining. (D): Schematic illustration showing the design of sgRNA for the depletion of *Ring1A* (top) and *Ring1B* (bottom) (E): Representative immunostaining images of surface-spread nuclei of the wild-type, *Dnmt1*-KO and *Dnmt1/Ring1A/1B*-TKO ESCs. Cells were analyzed at 4 d after the transfection of the CRISPR cassette. (F): Boxplot showing the number of distinct chromocenter in the nuclei of ESCs, related to (E). Wild-type, $n = 97$; *Dnmt1*-KO, $n = 75$; *Dnmt1/Ring1a/1b*-TKO, $n = 64$. P values were calculated using the Mann—Whitney U-test. * $P < 0.001$, ** $P < 0.01$. Scale bar, 5 μm .

3.5 Liquid-liquid Phase separation (LLPS) contributes to chromocenter clustering in the DNA hypomethylated ESCs

Based on the hypothesis that LLPS drives Tet1-dependent large chromocenter clustering in DNA hypomethylated ESCs, we carried out real-time monitoring of the dynamic behavior of the chromocenters. For this purpose, we established ESC lines expressing hKO1-tagged HP1 γ (hKO1-HP1 γ), which was specifically localized to PCH (Cheutin et al., 2003). Time-lapse analysis of the *Dnmt1*-KO ESCs with the hKO1-HP1 γ reporter revealed that their chromocenters underwent fusion and round up and produced larger chromocenters (Fig.9A). This dynamic change in the nucleus indicated that the chromocenter of DNA hypomethylated cells possessed the property of liquid-like condensates.

LLPS-driven condensation can be disrupted by 1,6-hexanediol (HD) treatment *in vivo* (Tatavosian et al. 2019; Han et al. 2020). I treated ESCs with 1,6-HD and monitored the acute response of the chromocenter clustering. The number and shape of chromocenter of the wild-type ESCs were not affected by 1-min treatment of 1,6-HD, when compared to 2,5-HD, which is an isomer of 1,6-HD and less effective on disrupting LLPS (Fig.9B and 9C). This observation agreed with the previous study that 1,6-HD showed limited effects on the established heterochromatin (Strom et al., 2017). In contrast, the number of chromocenter was significantly increased by 1,6-HD in the *Dnmt1*-KO ESCs (Fig.9D and 9E). Furthermore, the number of chromocenters was reduced to a level comparable to that of the untreated or 2,5-HD-treated *Dnmt1*-KO ESCs after removal of 1,6-HD (Fig.9D and 9E). Taken together, I conclude that *Tet1*-dependent chromocenter clustering in the hypomethylated ESCs was regulated by LLPS. However, the chromocenter number of the *Dnmt1*-KO ESCs was still less than that of the wild-type ESCs even with 1,6-HD treatment (Fig.9C and 9E). It is conceivable that some other physiological interactions, which are resistant to 1,6-HD play a role in chromocenter clustering in the *Dnmt1*-KO ESCs.

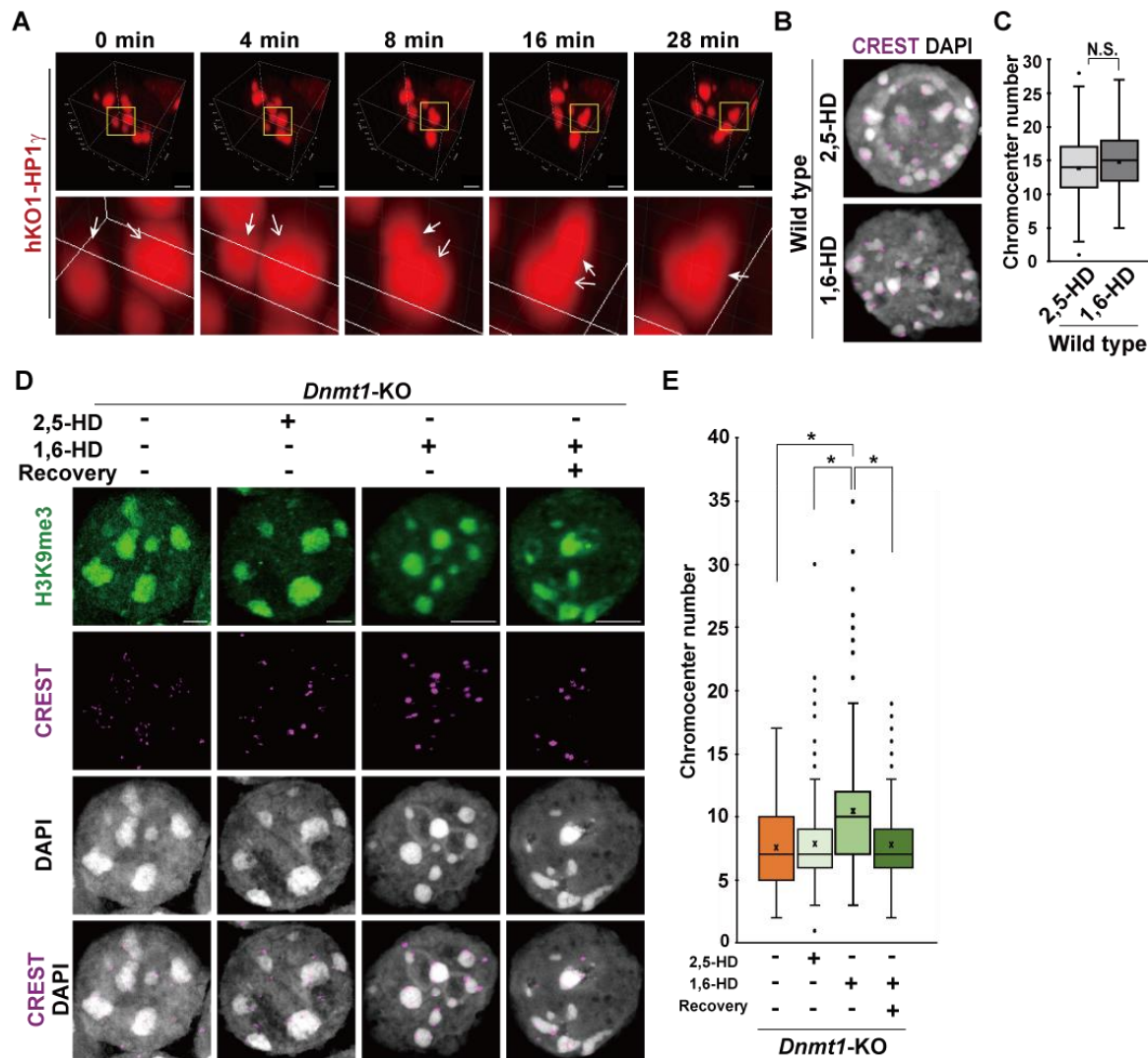


Figure 9. Liquid-liquid phase separation (LLPS) drives chromocenter clustering in DNA hypomethylated cells

(A): Series of time-lapse images of *Dnmt1*-KO ESCs expressing HP1 γ -hKO1. The yellow squares indicate the region highlighted at the bottom panel. HP1 γ puncta fusion event was observed as highlighted. Scale bar, 3 μ m. (B): Representative immunostaining images of surface-spread nuclei of the wild-type ESCs with 2,5- or 1,6-hexanediol (HD) treatment. 10% of 2,5- or 1,6-HD were treated for 1 min. Scale bar, 5 μ m. (C): Boxplot showing the number of distinct chromocenter in the nuclei of wild-type ESCs with 2,5- or 1,6-HD treatment, related to (B). 2,5-HD treatment, n = 179; 1,6-HD treatment, n = 105. (D): Representative immunostaining images of surface-spread nuclei of the *Dnmt1*-KO ESCs with or without 1,6-HD treatment. 10% of 2,5- or 1,6-HD were treated for 1 min. For the recovery experiment, cells were incubated in ESC culture medium for 1 h. Scale bar, 5 μ m. (E): Boxplot showing the number of distinct chromocenter in the nuclei of *Dnmt1*-KO ESCs in the individual condition, related to (D). No treatment, n = 71; 2,5-HD treatment, n = 222; 1,6-HD treatment, n = 217; 1,6-HD treatment with recovery culture, n = 199. P values were calculated using the Mann–Whitney U-test. N.S.: no significant, P > 0.05. *P < 0.001.

3.6. Chromocenter clustering abnormality in *Tet1*-KO meiotic germ cells

Next, I asked whether *Tet1* contributed to chromocenter clustering in the meiotic germ cells of female, which is in hypomethylated state similar to the *Dnmt1*-KO ESCs. The number of chromocenter changed dramatically during meiotic prophase, specifically between early-leptotene and late-leptotene stages (Fig.10A and 10B). Gradual accumulation of chromocenters to form larger chromocenter was commonly observed in the wild-type and *Tet1*-KO germ cells. However, it was incomplete in the *Tet1*-KO germ cells and the number of chromocenter was significantly increased compared to wild-type germ cells at both late-leptotene and zygotene stages (Fig.10A and 10B). Therefore, *Tet1* was suggested to be important for the chromocenter clustering in female germ cells.

I then asked whether *Tet1* controls chromocenter clustering via PRC1 and PRC2 as seen in DNA hypomethylated ESCs, I analyzed H2AK119ub and H3K27me3 enrichment in the germ cells. Unlikely to ESCs, H2AK119ub was enriched in PCH of the *Tet1*-KO in female germ cells (Fig.11A and 11B). In contrary, H3K27me3 was depleted in PCH of the *Tet1*-KO in female germ cell (Fig.11C and 11D). These results suggested that *Tet1* is involved in the chromocenter clustering in both DNA hypomethylated ESCs and female meiotic germ cells, but their underlying molecular mechanisms are different.

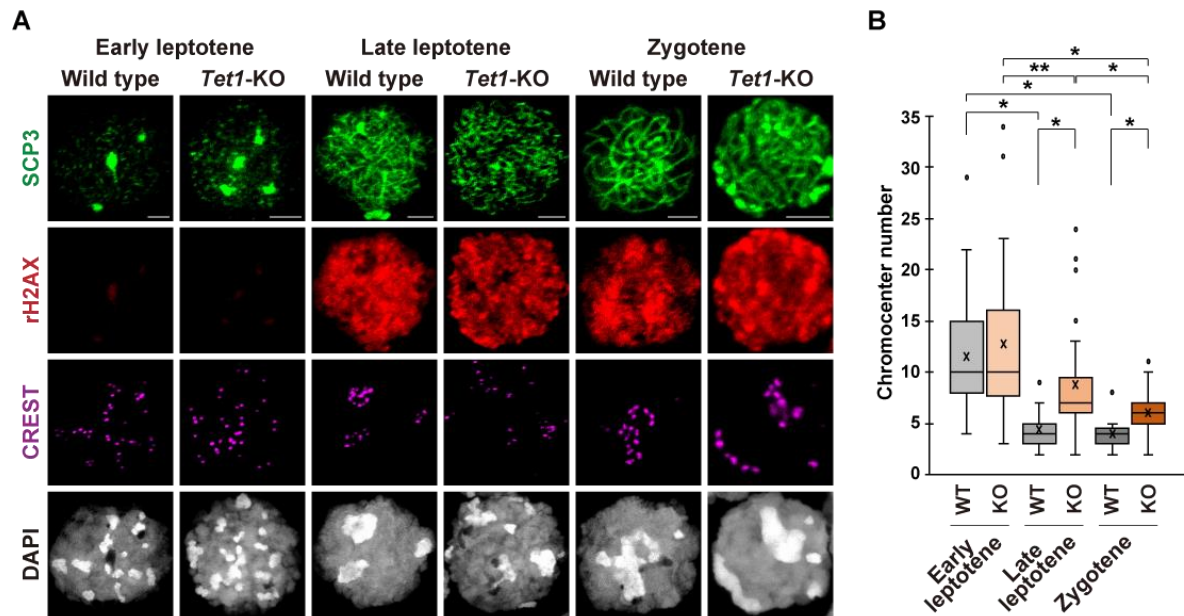


Figure 10. *Tet1*-defect female mice show chromocenter clustering abnormality at meiotic prophase

(A): Representative immunostaining images of surface-spread nuclei at individual stages of the first meiotic prophase. Meiotic germ cells were classified by Female germ cells were harvested from E15.5-E16.5 embryos. Scale bar, 5 μ m. (B): Boxplot showing the number of distinct chromocenter in the nuclei of the wild-type and *Tet1*-KO meiotic germ cells at individual stages, related to (B). Early leptotene, WT n = 27, KO n = 34; Late leptotene, WT n = 23, KO n = 37; Zygotene, WT n = 21, KO n = 33. P values were calculated using the Mann—Whitney U-test. *P < 0.001, **P < 0.01. WT and KO indicate wild-type and *Tet1*-KO, respectively.

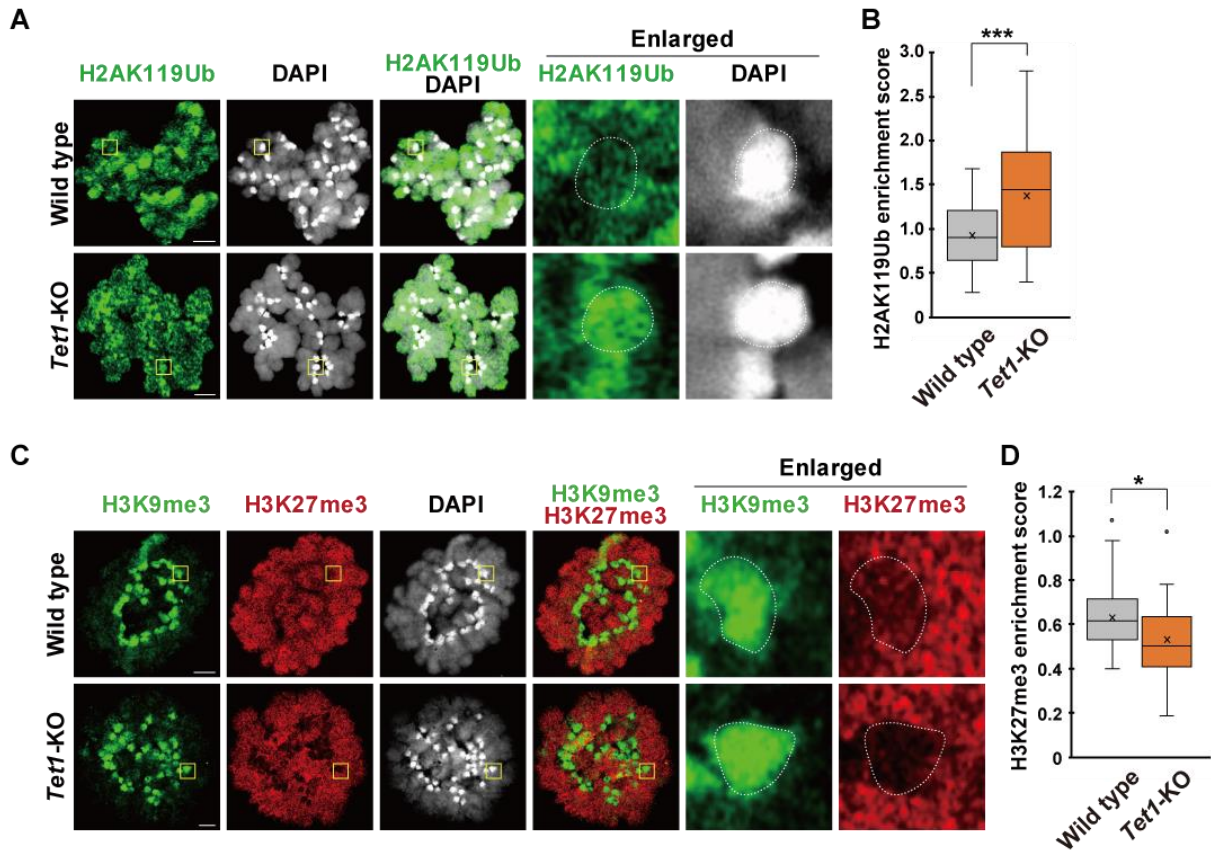


Figure 11. *Tet1* is important for deposition of H3K27me3 but not H2AK119ub at PCH of female germ cells

(A): Representative immunostaining images of surface-spread nuclei of the wild-type and *Tet1*-KO germ cells at E14.5. The yellow squares indicate the enlarged areas shown on the right. The white dashed circles indicate the PCH. (B): Quantification of immunostaining of H2AK119ub, related to (A). Wild-type $n = 18$; *Tet1*-KO $n = 25$. (C): Representative immunostaining images of surface-spread nuclei of the wild-type and *Tet1*-KO germ cells at E14.5. The yellow squares indicate the enlarged areas shown on the right. The white dashed circles indicate the PCH. (D): Quantification of immunostaining of H3K27me3, related to (C). $n = 39$ per samples. P values were calculated using the Mann—Whitney U-test. * $P < 0.001$. *** $P < 0.05$. Scale bar, 5 μm .

DISCUSSION

DNA at PCH region is usually highly methylated and is presumably not targeted by TET proteins, as evidenced by undetectable 5hmC (Ficz et al., 2011). On the contrary, enrichment of 5hmC in PCH was obvious in the *Dnmt1*-KO ESCs and wild-type PGCs during epigenetic reprogramming, both of which possessed globally DNA hypomethylated states (Fig.4D) (Yamaguchi et al., 2013). Although *Tet1* and *Tet2* are highly expressed in ESCs and PGCs, the depletion of *Tet1* resulted in complete abrogation of PCH-5hmC in the *Dnmt1*-KO ESCs and PGCs (Fig.4D) (Yamaguchi et al., 2012). The CXXC domain, which can bind to unmethylated DNA, is present in TET1 but not in TET2, and this difference may account for the difference in PCH targeting. On the other hand, since chromocenter clustering occurred in the *Dnmt1/3a/3b*-TKO ESCs and forced recruitment of TET1-CD-mut to PCH induced its clustering, PCH-5hmC was suggested to be just a byproduct and not crucial for the chromocenter clustering.

A gain of facultative heterochromatin marks, such as H3K27me3 and H2AK119ub, at PCH indicates epigenetic heterochromatin remodeling occurred under the DNA hypomethylated state. I demonstrated that *Tet1* is critical for this DNA hypomethylation-linked heterochromatin remodeling. A total of 97.2% of PRC2-binding sites overlapped with TET1 binding sites and 72.2% of PRC2 binding was affected by the depletion of *Tet1* (H. Wu et al., 2011). Consistent

with these studies, I found that H3K27me3 level at the PCH was decreased in the *Dnmt1/Tet1*-DKO ESCs and female germ cells (Fig.7B and 11D).

I also found that PRC1 recruitment to PCH in the hypomethylated ESCs depends on *Tet1* (Fig.8A-C). Given PRC1 binds to H3K27me3 through CBX7 in ESCs, localization of PRC1 to PCH would be mediated by PRC2 (Morey et al., 2012). However, enrichment of H2AK119ub in PCH was more intense than that of H3K27me3 and the phenotype on the chromocenter clustering in the *Ring1A/1B*-DKO ESCs was more obvious than that of the *Ezh2*-KO ESCs. Thus, I would like to propose the model that PRC1 is recruited to PCH by *Tet1* first, then PRC2 binds to PRC1-mediated H2AK119ub. This model was supported by the study that forced recruitment of MBD-EZH2 to the chromocenter did not lead to H2AK119ub enrichment but the recruitment of MBD-RING1B induced H3K27me3 enrichment (Cooper et al., 2014). It has been also reported that 90% of RING1B targets in ESCs correspond to bivalent promoters, which are the main targets of TET1 (Ku et al., 2008; X. Wu & Zhang, 2017). It remains open whether TET1 and PRC1 interact directly and how they regulate each other.

The PcG body is associated with the enrichment of the facultative heterochromatin marks, H2AK119ub and H3K27me3 (Pachano et al. 2019). The puncta containing RING1B shows a liquid-like feature of fusion and fission (Isono et al., 2013). CBX2, the canonical PRC1 components, mediates PcG body formation in an LLPS-dependent manner (Plys et al. 2019; Tatavosian et al. 2019). Both clustered chromocenters in the hypomethylated ESCs and PcG

body exhibit PRC1 inclusion and LLPS characteristics (Fig.8C, 9D, and 9E). Therefore, it is reasonable to consider that chromosome clustering in DNA hypomethylated cells relies on the biochemical characteristics of PcG proteins, which have the potential to aggregate into large foci.

Chromocenter clustering is believed to lead to spatial reorganization of the nucleus and affect the microenvironment for gene silencing. Pericentromeric regions are reported to associate with other genomic regions and form a large domain called the pericentromere-associate domain (PAD) (Wijchers et al., 2015). PAD patterns vary in different cell types and dynamically change during differentiation, unlike topologically associating domains (TADs), which are less variable among different cell types (Dixon et al., 2012; Wijchers et al., 2015). Forced recruitment of specific genes to PCH using chromodomain of HP1 β resulted in H3K9me3 deposition and transcriptional silencing of that gene. Therefore, chromocenter reorganization, accompanied by chromocenter clustering and its enlargement, may change PAD and provide more space to repress other genomic loci

Heterochromatin remodeling accompanied by an ectopic PRC1 localization to PCH reduces H2AK119ub enrichment in the other genomic loci (Hall et al., 2017). The high-copy number of satellite repeat was proposed to act as a molecular “sponge” which can harbor epigenetic factors abundantly and affect the enrichment of the proteins in genome (Hall et al., 2017). Considering that ectopic localization of both PRC1 and PRC2 to chromocenter was observed,

re-localization of PRC1 and PRC2 and de-repression in other genomic regions might happen in the DNA hypomethylated ESCs.

I demonstrated that *Tet1* is critical for chromocenter clustering not only in the hypomethylated ESCs but also in female meiotic germ cells (Fig.4A and 10A). Since the DNA methylation level of female meiotic germ cells is comparable to that of the *Dnmt1*-KO ESCs (Arand et al., 2015; Tsumura et al., 2006), *Tet1* is suggested to be commonly important for the chromocenter organization under hypomethylated conditions. *Tet1*-deficient mice showed a meiotic defects, and it was reported to be caused by the misregulation of meiotic genes (Yamaguchi et al., 2012). But, since chromocenter clustering is important for subsequent meiosis progression such as centromere clustering and synapsis formation in male, the abnormality in chromocenter clustering would be the cause of the phenotype (Takada et al., 2011). Although H2AK119ub enrichment in PCH was significantly decreased in *Dnmt1/Tet1*-DKO ESCs, it was not changed in *Tet1*-KO germ cells (Fig.8B and 11B). These results suggested TET1-PRC1 axis was not important for the chromocenter clustering of female meiotic germ cells. On contrary, H3K27me3 enrichment was affected in *Tet1*-KO germ cells, similar to *Dnmt1/Tet1*-DKO ESCs (Fig.11D). PRC2 components were highly expressed and H3K27me3 was enriched in the nuclear periphery of PGCs (Prokopuk et al., 2017). But physiological function of PRC2 in meiotic germ cell has not been revealed yet. Further analyses,

such as PRC2-conditional KO mouse study, are required to investigate the molecular mechanism of chromocenter clustering during meiosis.

CONCLUSION

In this study, I identified the novel role of *Tet1* in the chromocenter clustering in the hypomethylated ESCs as well as in female meiotic germ cells. *Tet1*-mediated chromocenter clustering would be the basis of female chromocenter re-organization during meiosis and important for normal meiotic progression. The underline mechanism would be different in these cells, I found *Tet1* controlled chromocenter clustering through PRC1 recruitment in hypomethylated ESCs. Previous studies on PCH were mainly focused on the factors related to constitutive heterochromatin, such as SUV39H1/2 or HP1 (Fioriniello et al., 2020; Lehnertz et al., 2003). My findings, however, demonstrated that TET1 and PRC1, both of which are the facultative heterochromatin factors, control the spatial organization of chromocenter in hypomethylated ESCs. Global hypomethylation and PRC1 localization to the chromocenter have been reported in pre-implantation embryo and cancer cells (Hall et al., 2017; Tardat et al., 2015). TET family proteins would also regulate the epigenetic remodeling of the PCH of these cells. Thus, this research shed a light on the field of chromocenter organization.

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