Disruption of *Th2a* and *Th2b* genes causes defects in spermatogenesis

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**ABSTRACT**

The variant histones TH2A and TH2B are abundant in the testis, but their roles in spermatogenesis remain elusive. Here, we show that male mutant mice lacking both *Th2a* and *Th2b* genes were sterile, with few sperm in the epididymis. In the mutant testis, the lack of TH2B was compensated for by overexpression of H2B, whereas overexpression of H2A was not observed, indicating a decrease in the total histone level. Mutant mice exhibited two defects: incomplete release of cohesin at interkinesis after meiosis I and histone replacement during spermiogenesis. In the mutant testis, secondary spermatocytes at interkinesis accumulated and cohesin was not released normally, suggesting that the retained cohesion of sister chromatids delayed the subsequent entry into meiosis II. In addition, impaired chromatin incorporation of TNP2 and degenerated spermatids were observed in the mutant testis. These results suggest that a loss of TH2A and TH2B function in chromatin dynamics or a decrease in the total histone levels causes defects in both cohesin release and histone replacement during spermatogenesis.

**KEY WORDS:** Cohesin, Histone replacement, Histone variants, Spermatogenesis, Mouse

**INTRODUCTION**

Two histone variants, TH2A and TH2B, are highly expressed in the testis (Trostle-Weige et al., 1982; Shires et al., 1976). *Th2a* and *Th2b* genes (*Hist1h2aa* and *Hist1h2ab*—Mouse Genome Informatics) localize adjacent on chromosome 17, and their expression is controlled by a shared promoter that is located between them (Huh et al., 1991), suggesting that TH2A and TH2B function together. Biochemical data suggest that TH2A and TH2B induce nucleosome instability (Li et al., 2005; Rao and Rao, 1987).

Recently, we have demonstrated high expression of TH2A and TH2B in oocytes and fertilized eggs, with a gradual decrease in their levels as zygotes differentiate into blastocysts (Shinagawa et al., 2014). Female mutant mice lacking both TH2A and TH2B develop normally and exhibit normal oocyte development. However, the number of pups from TH2A/TH2B-deficient females is ∼40% of that of wild type. TH2A and TH2B are maternal-effect proteins, and they contribute to the activation of the paternal genome after fertilization.

During spermatogenesis, primary spermatocytes progress through the following stages: leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase I and metaphase II (Hecht, 1998). Chromosomes begin to condense at leptotene, line up to form the homologous pairs that undergo recombination at zygotene and complete synopsis at pachytene. At metaphase I and II, homologous chromosomes and sister chromatids, respectively, are separated. Cohesin has an essential role in the connection of sister chromosomes. The mitotic cohesion complex consists of four subunits: Smc1, Smc3, Rad21 and SA1/SA2 (Nasmyth, 2011). The meiotic cohesion complex contains several specific subunits, such as Rec8 and Rad21L (Herrán et al., 2011; Ishiguro et al., 2011; Lee and Hirano, 2011). During the metaphase I to anaphase I transition, cohesin complexes are cleaved by separase, which allows homologous chromosomes to segregate. At this stage, the cohesin complexes are retained at the centromere; during meiosis II, the cohesin complexes are cleaved, resulting in chromatid separation (Lee et al., 2008; Llano et al., 2008). During spermiogenesis, chromatin in round spermatids condenses as most of the histones are replaced by transition proteins, and then by protamines. In this study, we show that disruption of *Th2a* and *Th2b* causes defects in spermatogenesis, particularly during the two steps that involve the normal release of cohesin between meiosis I and II, and histone replacement during spermiogenesis.

**RESULTS AND DISCUSSION**

**Defects in spermatogenesis in Th2a⁻/⁻ Th2b⁻/⁻ mice**

Male homozygous mutant mice lacking both *Th2a* and *Th2b* genes are sterile (Shinagawa et al., 2014). The testis and epididymis of 3-month-old mutant mice showed a 28% and 17% reduction in weight, respectively, compared with the controls (Fig. 1A). The epididymis of mutant mice contained few sperm (Fig. 1B). Spermatogonia, primary spermatocytes at meiotic prophase and spermatocytes at metaphase I were found in the mutant testis at frequencies similar to those in the wild-type testis (Fig. 1C), indicating that the proliferation of spermatogonia and the development of spermatocytes from meiotic prophase to metaphase I was not affected by the loss of *Th2a* and *Th2b* genes. Secondary spermatocytes at interkinesis (the interphase between meiosis I and II) were more abundant in the mutant testis than in the control (Fig. 1C), suggesting extended interkinesis in mutant mice.

The results of western blotting, which used samples that spanned 1287-1292 days post-partum (days PP) compared with those at 16 days PP. However, the levels of canonical histones H2A and H2B were similar at 16 and 30 days PP (Fig. 1D). Thus, the ratio of variant and canonical histones was higher in spermatids than that in spermatocytes.
Abnormal retention of cohesin on chromosomes at interkinesis in Th2a−/−Th2b−/− testes

The synaptonemal complex and cohesin are released from chromosomes before metaphase II. Immunostaining of spread cells indicated that Rec8, a meiosis-specific subunit of the cohesin complex, and SCP3, still remained and colocalized in a significant number of mutant spermatocytes, but not in wild-type spermatocytes at interkinesis (Fig. 2A; supplementary material Fig. S2). Furthermore, a significant number of mutant spermatid contained SCP3/cohesin complex, whereas SCP1 was absent in wild-type and mutant cells (Fig. 2B,C). The cells with a 2C DNA content from the mutant testis, but not from the wild-type testis, contained Rad21 signals (Fig. 2D), suggesting that the increase in the population of cells with a 2C DNA content was due to a retention of cohesin. Thus, SCP1 was released normally after the first meiotic division in mutant spermatocytes, but SCP3/cohesin was not, which may have delayed the subsequent entry of these cells into meiosis II. The cleavage of the cohesin subunit, Rec8, by separase is the primary mechanism that removes cohesin from the chromosome, although other mechanisms may also be involved (Nasmuth, 2011; Shintomi and Hirano, 2010). The loss of Th2a and Th2b genes may have induced a conformational change in chromosome-bound cohesin, which resulted in insufficient cleavage by separase.

Defects in the deposition of TNP2 on chromatin in Th2a−/− Th2b−/− spermatids

Morphologically normal spermatids were rare in mutant mice, and many degenerated step 10-16 spermatids, which showed an abnormal nuclear morphology, were observed (supplementary material Fig. S3A). These results suggest that disruption of Th2a and Th2b causes abnormalities in the development of spermatids into spermatooza. The number of CENP-B foci was similar between wild-type and mutant spermatids (supplementary material Fig. S3B), suggesting that homologous chromosomes and sister chromatids had separated normally. During spermiogenesis,
Histones are replaced by transition nuclear proteins (TNP1 and TNP2), which are then replaced by protamines (Gaucher et al., 2010). The TNP2 immunostaining signal in mutant spermatids decreased substantially compared with that of the control (Fig. 3A,B), although the level of Tnp2 mRNA in mutant and wild-type testes was similar (supplementary material Fig. S3C). An abnormal nuclear morphology was evident in the mutant spermatids (Fig. 3B). The levels of TNP2 and protamine 2 in mutant spermatid extracts were lower than those in the controls (Fig. 3C). When spermatid whole extracts were separated into chromatin and non-chromatin fractions, TNP2 and protamine 2 were predominantly recovered in the chromatin fraction, and their levels in the mutant chromatin fraction were lower than those in the wild-type chromatin fraction, suggesting a defect in the chromatin incorporation of TNP2. Because there were no differences in the mRNA levels of several genes \[\text{Prm1}, \text{Foxj1}, \text{Ube2a}, \text{Rnf12 (Rlim)}\] and \[\text{Tpap (Papolb)}\] between wild-type and mutant spermatids with haploid genomes (supplementary material Fig. S3C), the defect in histone replacement may have been due to an abnormality in chromatin structure rather than to a decrease in haploid
genome expression. In addition, we observed moderate differential expression in a few dozen genes in spermatocytes of mutant and wild-type testes (data not shown), suggesting that it was unlikely that the loss of Th2a and Th2b genes in spermatogonial stem cells caused these defects in spermatogenesis.

The results of western blot analysis of round and elongating spermatid extracts indicated that the H2A level was similar between the spermatids of wild-type and mutant mice, whereas the H2B level was higher in mutant spermatids than in the control spermatids (supplementary material Fig. S3D), suggesting the presence of a feedback mechanism. The results of histone liquid chromatography analysis showed that the H2B levels in mutant spermatocytes and spermatids were 2.4-fold and 1.7-fold higher than those in the wild-type spermatocytes and spermatids, respectively (Fig. 4; supplementary material Table S1). This is consistent with the report that TH2B levels in spermatocytes and spermatids are approximately twofold higher than the H2B level (Lu et al., 2009). However, the H2A levels were not significantly different in the mutant cells.

We speculate that the loss of TH2A/TH2B delays differentiation at interkinesis and blocks the replacement of histones during spermiogenesis. Although abnormal spermatids with a 4C DNA content are known to be generated by the inhibition of caspase-induced Rec8 cleavage (Kudo et al., 2009), incomplete release of cohesin in TH2A/TH2B-deficient spermatocytes did not result in the accumulation of abnormal spermatids with a 4C DNA content. Recently, Montellier et al. (2013) reported that mutant mice lacking TH2B expression exhibit no defect during spermiogenesis, indicating that a loss of TH2B alone is not sufficient to disturb histone replacement. Thus, loss of both TH2A and TH2B may be necessary to impair histone replacement and the normal release of cohesion at interkinesis. Alternatively, it is also possible that such defects are due to a decrease in the total histone level. In the mutant testis, the lack of

Fig. 4. Analysis of histones in spermatocytes and spermatids. (A) Typical UV chromatograms of histones from wild-type (+/+) and mutant (−/−) spermatocytes and spermatids. Histones were analyzed using liquid chromatography. The retention times of recombinant TH2B, TH2A and other histones indicated that H2A peak 1 contains H2A1 and TH2B, whereas H2A peak 2 contains H2A2 and TH2A. The results of quantitative analysis of each peak are shown in supplementary material Table S1. (B) Deconvoluted electrospray mass spectra of H2A peak 1 (H2A1 +TH2B) obtained in A were obtained by LC-MS. Ion peaks surrounded with red rectangles are TH2B.
TH2B was compensated for by overexpression of H2B, whereas overexpression of H2A was not observed. Thus, the total amount of histones in the mutant cells was lower than that in the wild-type cells.

Multiple mechanisms may act in combination to destabilize the nucleosome and replace the histones. Hyperacetylation of canonical histones during spermatogenesis, especially H4, and multiple histone variants are thought to weaken DNA-histone octamers and nucleosome-nucleosome interactions (Govin et al., 2004; Govin et al., 2007; Gaucher et al., 2010). Nucleosomes containing hyperacetylated histones are thought to be more prone to displacement by protamines (Oliva et al., 1987). These previous reports are consistent with the observations that TH2A and TH2B, which induce an open chromatin structure (Shinagawa et al., 2014), are required for the replacement of histones by TNP s. However, we cannot exclude the possibility that such defects are due to a decrease in the total histone level, because canonical histone H2A and H2B underdosage leads to defective meiosis in yeast (Hanlon et al., 2003).

Conclusions
We have demonstrated that loss of two genes encoding the histone variants TH2A and TH2B causes defects in spermatogenesis by disturbing cohesin release at interkinesis and histone replacement during spermiogenesis.

MATERIALS AND METHODS

Animals
TH2a/Th2b-deficient mice were generated as described previously (Shinagawa et al., 2014). Mutant mice were backcrossed with BALB/c mice at least five times before use in the experiments.

Histological and immunohistochemical examination of testes
Testes and caput epididymides were fixed in Bouin’s solution overnight, embedded in paraffin and sectioned. Sections were used for Hematoxylin and Eosin staining, periodic acid Schiff (PAS) staining and immunofluorescence. For immunostaining, sections were subjected to antigen retrieval and stained with the antibodies described in the methods in the supplementary material.

Separation of spermatids and western blot analysis
Round spermatids and elongating spermatids were isolated from a crude germ cell suspension from testes of two adult mice using the STAPUT method (Bellvé, 1993; Pivot-Pajot et al., 2003). Whole-cell extracts, and chromatin and non-chromatin fractions were used for western blotting. For further details, see methods in the supplementary material.

FACS analysis
A suspension of single germ cells was prepared as described by Bastos et al. (2005). Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson). For further details, see methods in the supplementary material.

Immunostaining of testicular cells
For immunostaining of spermatocytes and spermatids, seminiferous tubules were spread as described by Peters et al. (1997), except that 80% ethanol was used for fixation. The antibodies used are described in the methods in the supplementary material.

qRT-PCR
Total RNA was extracted from spermatids and used for qRT-PCR. For further details, see methods in the supplementary material. Primers used are listed in Table S2 in the supplementary material.

Liquid chromatography mass spectrometry (LC-MS)
Histones were extracted from spermatocytes and spermatids by acid extraction and analyzed using LC-MS. For further details, see methods in the supplementary material.

Acknowledgements
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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi=10.1242/dev.121830/DC1

References


Supplementary Materials and Methods

Histological examination of testes

Testes and caput epididymides were fixed in Bouin's solution overnight, embedded in paraffin, and sectioned at a thickness of 4 μm. Sections were used for hematoxylin and eosin staining, periodic acid Schiff (PAS) staining, and immunofluorescence. For immunostaining, sections were subjected to antigen retrieval by boiling in 10 mM sodium citrate buffer (pH 6.0) for 20 min, and allowing them to gradually cool to room temperature. Slides were incubated with PBS containing 0.5% Triton X-100 and 0.5% BSA for 1 h, and then stained with an anti-TNP2 antibody (1:300; Santa Cruz Biotechnology) or an anti-γH2AX antibody (1:1000; Millipore). The secondary antibodies were Alexa Fluor 488- or Alexa Fluor 568-conjugated anti-goat or anti-mouse IgGs (Invitrogen).

Separation of round and elongating spermatids

Spermatids were isolated using the STAPUT method (Bellve, 1993; Pivot-Pajot et al., 2003). The testes of two adult mice (8–12 weeks old) were dissected, and the seminiferous tubules were released during a 15-min incubation at 37°C in 1 ml of RPMI medium containing 1 mg/ml of collagenase (Sigma). The tubules were then allowed to sediment, the medium was replaced with 1 ml of RPMI without collagenase, and the tubules were washed three times with 1 ml of RPMI. To obtain tubule fragments, tubules were incubated for 15 min at 37°C in 1 ml of trypsin solution (50 μl/ml in RPMI; Invitrogen). After adding fetal bovine serum (FBS) to a final concentration of
10%, germ and Sertoli cells were released by pipetting and filtered through a 70-µm pore filter (Becton Dickinson). The cells were pelleted by centrifugation, resuspended in 10 ml of sedimentation solution (RPMI containing 0.5% BSA), and layered on top of a 580 ml BSA gradient (2–4%) in an airtight sedimentation chamber. The cells were allowed to sediment at room temperature for 180 min. Ten-milliliter fractions were then collected and centrifuged, and the cells in each fraction were assessed by observation under a phase-contrast microscope. The fractions enriched in round or elongating spermatids were collected.

**Western blot analysis**

Whole cell extracts and chromatin or non-chromatin fractions from testicular cells or purified spermatids were used for western blotting. To prepare whole cell lysates, cells were extracted in five volumes of NETN buffer (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 2 mM EDTA, and 0.5% NP-40) for 15 min. The pellet (chromatin fraction) was separated from the supernatant (non-chromatin fraction) by centrifugation of whole cell lysates and adjusted to the original volume with sample buffer. After electrophoresis and transfer of the proteins to membranes, the membranes were incubated with the indicated antibodies and washed in PBS or PBS containing 0.5% Triton X-100 (only for experiments that involved the anti-TH2B antibody). The signals were detected using ECL western blotting reagents (GE Healthcare). The primary antibodies used were as follows: anti-H2A (1:1500; Abcam), anti-H2B (1:2000, Millipore) anti-TH2A (1:9000), anti-TH2B (1:5000), anti-H3 (1:10000; Abcam), anti-H4 (1:1250; Millipore),
anti-protamine 2 (1:300, Santa Cruz Biotechnology), and anti-tubulin (1:5000, Santa Cruz Biotechnology).

**qRT-PCR**
Total RNA was isolated using Isogen (Nippon Gene). qRT-PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen). Primer sequences are shown in Supplementary Table S2.

**FACS analysis**
FACS analysis was performed as described by Bastos et al. (2005). For DNA content analysis using propidium iodide (PI), seminiferous tubules from testes of 2- to 3-month-old mice were dissociated in DMEM supplemented with 1 mg/ml of collagenase type IV, 1 mM CaCl$_2$, and 7.5% FBS for 15 min at room temperature. Single-cell suspensions were obtained by passing dissociated seminiferous tubules through a 40-μm nylon mesh. The cells were washed with PBS and then fixed with 70% ethanol overnight at 4°C. Fixed cells were washed, stained with an anti-MSY2 antibody (1:500, Santa Cruz Biotechnology), and suspended in PBS containing 10 μg/ml of PI and 10 μg/ml of RNase A. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson). Cell sorting was performed using FACSARia III cell sorter (BD Biosciences).

**Immunostaining of testicular cells**
For immunostaining of spermatocytes and spermatids, seminiferous tubules were spread as described by Peters et al. (1997), except that 80% ethanol was used for fixation. For
staining with anti-H2B antibody, cells were subjected to antigen retrieval by boiling in 10 mM sodium citrate buffer (pH 6.0). The antibodies used were as follows: anti-SCP1 (1:1000; Abcam), anti-SCP3 (1:1250; ab97672, Abcam), anti-SCP3 (1:500, ab15092, Abcam), anti-γH2AX (1:1000), anti-Rad21 (1:1000; Millipore), anti-Rec8 (1:300), and anti-CENP-B (1:100, Santa Cruz Biotechnology).

Liquid chromatography mass spectrometry

Histones were extracted from nuclei of spermatocytes or spermatids as described by Lu et al. (2009) and dialyzed against 0.2 M acetic acid. Recombinant TH2A, TH2B, H2A and H2B proteins were expressed in *E. coli* as described by Tachiwana et al. (2010), purified, and used as controls. Aliquots of histones were injected into the liquid chromatography-mass spectrometer (LC-MS). LC was performed on an Agilent 1100 series system (Agilent Technologies) consisting of a binary pump (Cap pump), autosampler (Micro-WPS) and a UV-detector (MWD). A wavelength of 215 nm was selected for qualitative and quantitative analysis using ChemStation Software (Agilent Technologies). Chromatography was performed on a Inertsil WP 300 C4 column (150 mm×1.0 mm, 3 μm) using a gradient of solvent A (0.1% trifluoroacetic acid) and solvent B (80% acetonitrile, 0.1% trifluoroacetic acid) elution at 20 μL/min according to the following program: 12–58% B over 0–10 min; 58–58% B over 10–35 min; and 58–100% B over 35–38 min. The HPLC elute was electrosprayed directly into a Q-Exactive mass spectrometer (Thermo Fisher Scientific) using a electrospray ion
source. The Q-Exactive mass spectrometer was operated in positive mode with a resolution $R = 35,000$ at m/z 400. Full scan MS spectra from m/z 150–2000 were acquired. The full mass spectra obtained were deconvoluted using Protein Deconvolution software (Thermo Fisher Scientific).

References

Supplementary Figures

Supplementary Figure S1. Accumulation of spermatocytes at interkinesis in mutant testes. Testicular cells prepared at the indicated time after birth were stained with PI and an anti-MSY2 antibody, and analyzed by FACS. The average percentage of the population in each fraction is shown (± SEM (n = 3 or 4)) on the right. *, P < 0.05; **, P < 0.01; ***, P < 0.001; N.S., not significant.
Supplementary Figure S2. Normal synapsis formation in wild-type and mutant spermatocytes at different stages of differentiation. Spermatocyte chromosomes were spread and immunostained with anti-γH2AX (green) and anti-SCP3 (red) antibodies. DNA was counterstained with TO-PRO-3 (blue). The localization of SCP3 within mutant spermatocytes indicates normal formation of the synaptonemal complex. The anti-γH2AX antibody labeled only unpaired sex chromosome regions in pachytene and diplotene cells.
Supplementary Figure S3. Characterization of spermatids from Th2a<sup>−/−</sup>Th2b<sup>−/−</sup> mice. (A) Hematoxylin and eosin staining of testis and epididymis sections from Th2a<sup>−/−</sup>Th2b<sup>−/−</sup> mice. Abnormal cells were observed in the seminiferous epithelium in
mutant mice (left panels). Typical germ cell types are depicted (1–4). No mature sperms were detected in the epididymis (right panel). (B) Testicular germ cells were stained with antibodies against SCP3 (red) and the centromeric protein CENP-B (green). DNA was counterstained with TO-PRO-3 (blue). The number of CENP-B foci per nucleus is shown in the graph (n = 21). (C) Comparison of mRNA levels of genes that are typically transcribed in spermatids in wild-type (+/+) and mutant (-/-) testes. The mRNA levels are shown as an average of three measurements (± SD (n = 3)). N.S., not significant. (D) Levels of canonical and variant histones of H2A and H2B in spermatids. Round and elongating spermatids were isolated from wild-type and mutant testes, and whole cell lysates were used for western blotting. A three-fold serial dilution of proteins was used for H2A, while a two-fold serial dilution of proteins was used for the others.
**Supplementary Table S1.** Quantification of histone H2A and H2B peaks.

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<th>H2A peak 2/H4 Mean ± SD</th>
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<td></td>
<td></td>
<td>Relative (%)</td>
<td>Relative (%)</td>
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To estimate the ratio of canonical to variant histones, we measured the height of the UV-peak normalized by the height of the H4 peak. The results are shown as averages with SD (n =3). As shown Fig. S3A, H2A peak 1 contains H2A1 and TH2B, while H2A peak 2 contains H2A2 and TH2A. The H2B level was higher in mutant spermatocytes and spermatids.
**Supplementary Table S2.** Sequences of primers used for qRT-PCR

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