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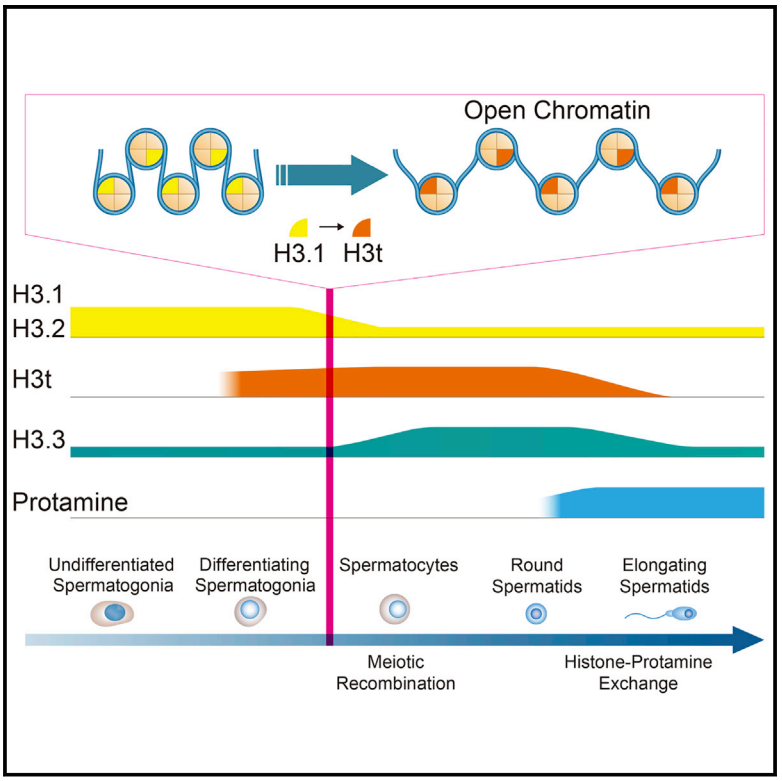
Report

Testis-Specific Histone Variant *H3t* Gene Is Essential for Entry into Spermatogenesis

Graphical Abstract



Highlights

- H3t is essential for spermatogenesis, and loss leads to azoospermia
- H3t is expressed in differentiating spermatogonia but lost from spermatozoa
- H3t is required for spermatogonial differentiation and ensures entry into meiosis
- H3t-containing nucleosomes form an open chromatin structure

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In Brief

When undifferentiated spermatogonia enter differentiation, they go through meiotic recombination followed by histone-protamine transition, eventually to become highly specialized haploid cells called spermatozoa. Ueda et al. reveal a testis-specific histone variant H3t that enables nucleosomes to form an open chromatin structure and is essential for the initial step of spermatogenesis. *H3t* deficiency leads to azoospermia.

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Testis-Specific Histone Variant *H3t* Gene Is Essential for Entry into Spermatogenesis

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SUMMARY

Cellular differentiation is associated with dynamic chromatin remodeling in establishing a cell-type-specific epigenomic landscape. Here, we find that mouse testis-specific and replication-dependent histone H3 variant H3t is essential for very early stages of spermatogenesis. *H3t* gene deficiency leads to azoospermia because of the loss of haploid germ cells. When differentiating spermatogonia emerge in normal spermatogenesis, H3t appears and replaces the canonical H3 proteins. Structural and biochemical analyses reveal that H3t-containing nucleosomes are more flexible than the canonical nucleosomes. Thus, by incorporating H3t into the genome during spermatogonial differentiation, male germ cells are able to enter meiosis and beyond.

INTRODUCTION

When stem cells are committed to enter certain cell lineages, they undergo epigenetic and chromatin remodeling to acquire unique genomic structures that ensure the stability of their fate. The fundamental unit of chromatin is the nucleosome, consisting of ~150 bp DNA and two copies each of histones H2A, H2B, H3, and H4 (Luger et al., 1997). Modifications of histones, especially those of H3, affect the nucleosome structure and consequently play pivotal epigenetic roles in gene regulation (Strahl and Allis, 2000; Goldberg et al., 2007). In addition, the incorporation of nonallelic H3 variants that harbor a few to several amino acid differences into specific genomic loci are thought to play an important role in changing the chromatin structure and gene regulation during differentiation (Maze et al., 2014; Talbert and Henikoff, 2010). It has been proposed that these might be linked to specific genomic loci, where they act like barcodes with distinct functions (Hake and Allis, 2006). Recently, histone H3 variants of human and mouse have been identified by in silico hybridization screening (Maehara et al., 2015). Some of these variants showed tissue-specific expression, but their significance in cellular and tissue functions remained elusive. Whereas most of the newly identified mouse variants do not have human orthologs and are likely to be derived from H3.3, H3t has a human counterpart, H3T (H3.4) (Witt et al., 1996) and shares a common chaperon recognition motif with H3.1 and H3.2 (Figure 1A), which was then shown to be incorporated into the genome in a replication-coupled manner (Maehara et al., 2015).

RESULTS AND DISCUSSION

H3t Deficiency Leads to Male Infertility in Mice

We first analyzed the expression pattern of H3t in various tissues on the FANTOM5 mouse promoterome database deposited in ZENBU (http://fantom.gsc.riken.jp/zenbu) (Table S1), then confirmed its expression by qRT-PCR amplified with primer sets designed from 5′ cap analysis of gene expression (CAGE) and 3′-sequence (seq) data (Figures S1A and S1B) (Maehara et al., 2015). Consistent with our previous result (Maehara et al., 2015), *H3t* mRNA was specifically expressed in the testis (Figures S1A and S1B; Table S1).

To establish the significance of H3t, we used the CRISPR/Cas9 gene-editing method to generate knockout mice (Mashiko et al., 2013). This gene was located near histone cluster 3 of chromosome 11 and within another gene called *Trim17* (Figure S1C), which made it difficult to disrupt by conventional

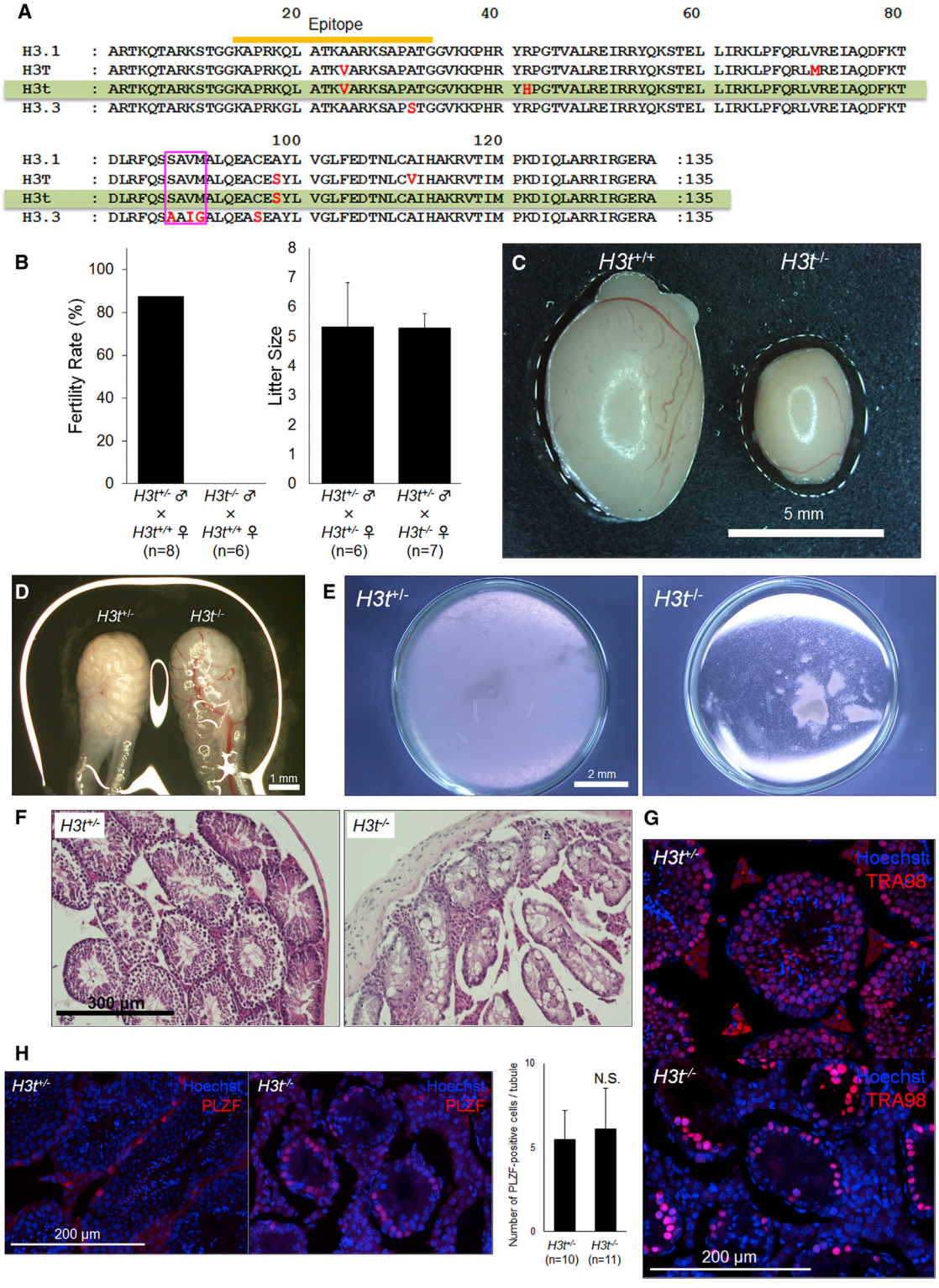

Figure 1. H3t Functions in Spermatogenesis and Is Essential for Male Fertility

(A) Amino acid sequence alignment of histone variants H3.1, H3T, H3t, and H3.3. Amino acids unique to each variant in comparison with H3.1 are highlighted in red. The chaperon recognition motif is boxed in pink, and the epitope used to generate an anti-H3t antibody is indicated (orange bar).

(B) Fertility rate (left) and litter size (right) of *H3t* null mice. *H3t*-homozygous knockout male mice were infertile, whereas the female mice had fertility similar to heterozygous ones.

(legend continued on next page)

gene-targeting methods. As shown in Figures S1D–S1F, we succeeded in generating an *H3t* null mouse line, which lacked 16 nt including the start codon. Although both male and female knockout mice were viable and healthy, the male mice turned out to be sterile (Figures 1B, S1G, and S1H). In line with this phenotype, *H3t* null mice had strikingly smaller testes compared with wild-type and heterozygous knockout mice (Figures 1C and S1I). Moreover, the caudae epididymides of *H3t* null mice were transparent and lacked spermatozoa (Figures 1D and 1E). There were no indels at the top-scoring potential off-target sites (Figure S1J). Additionally, small testes and infertility were also observed in a different *H3t* knockout founder mouse, generated using the CRISPR/Cas9 method (Figure S1K). These data suggest that these phenotypes were not caused by off-target effects.

In the testis, stem cells are located at the periphery of the seminiferous tubules, and when these cells enter differentiation, they migrate toward the center (Yoshida, 2012). At the onset of differentiation, undifferentiated spermatogonia yield differentiating spermatogonia via reversible, flexible, and stochastic mechanisms (Yoshida, 2012). After premeiotic replication, these differentiating spermatogonia undergo dynamic chromatin remodeling along with meiotic recombination and histone-to-protamine transitions to become highly specialized haploid cells called spermatids (Maze et al., 2014; Talbert and Henikoff, 2010; Bowles and Koopman, 2007).

Next, we analyzed *H3t* null testes in detail to investigate the possible cause of infertility. Intriguingly, although germ cells were retained in these organs, elongating and acrosome-positive spermatids were absent in the *H3t*-deficient testes (Figures 1F, 1G, and S2A), and most of the seminiferous tubules lacked synaptonemal complex protein 3 (SCP3)-positive cells (Figure S2A). These observations suggest that the defect possibly occurs before meiosis. Then we stained seminiferous tubules with stage-specific markers to reveal to what extent germ cells are restored in *H3t* null testes. As shown in Figures 1H and S2B, *H3t* null testes retained promyelocytic leukemia zinc finger (PLZF)-positive undifferentiated spermatogonia comparable with that of control, but the numbers of c-Kit-positive differentiating spermatogonia and γH2A.X histone variant-positive spermatocytes were decreased significantly in the *H3t*-deficient testis. Thus, H3t is likely to function at an early stage of spermatogenesis, probably at the premeiotic differentiation and/or replication stage. From these data, we conclude that *H3t* deficiency leads to azoospermia stemming from spermatogenic failure, but not from other male reproductive tract abnormalities.

To determine the timing of the deficiency more precisely, we investigated earlier stages of spermatogenesis. In testes from 4-week-old mice, the first wave of spermatogenesis was already impaired in the *H3t*-deficient mice (Figures S2C and S2D), implying that H3t is essential for the initial step of spermatogenesis, and that the loss of germ cells is not caused by aging. Although gonocytes were present in the *H3t* null testes (Figures S2E and S2F), immunostaining for TRA98 (a germ-cell-specific marker) in germ cells started to decline from postnatal day (P) 10 testes, around the time at which spermatocytes emerge and begin to expand (Meikar et al., 2011); this defect became more severe at later stages, and germ cells eventually died via apoptosis (Figures S2G–S2I). These results suggest that H3t is required for spermatogonia (i.e., spermatogenic stem cells) to enter differentiation.

H3t Is Expressed from Differentiating Spermatogonia but Lost from Spermatozoa

To understand the tissue distribution and expression pattern of the H3t protein responsible for the knockout phenotype, we developed an H3t-specific monoclonal antibody targeted against the Val 24 amino acid, which is specific to both H3t and H3T (Figures 1A and S3A–S3E). Consistent with the gene expression data, immunoblotting showed the existence of H3t protein exclusively in the testis among various tissues examined including the ovary (Figures 2A and S3D). However, unlike the H3.3 variant, the H3t protein was absent from mature spermatozoa (Figure 2B), implying that H3t is replaced with the H3.3 variant (van der Heijden et al., 2005; Santenard et al., 2010) and/or protamines during spermatogenesis, so it might not act as a sperm-borne paternal epigenetic memory to offspring (Talbert and Henikoff, 2010). Next, testes from mice showing the first wave of spermatogenesis were examined by immunoblotting. We found that H3t became apparent from P8 to P10, implying that H3t is induced when differentiating spermatogonia emerge, and slightly earlier than the expression of TH2B, a testis-specific histone H2B (Figures 2C, S3F, and S3G) (Shinagawa et al., 2015). Moreover, in testicular sections, canonical H3.1/3.2-type histones were expressed from PLZF-positive undifferentiated spermatogonia (Figure S3H), and these became stronger in c-Kit-positive differentiating spermatogonia, but rapidly lost in spermatocytes and later stages (Figure 2D). In contrast, H3t expression was not observed in PLZF-positive undifferentiated spermatogonia (Figure 2E), but was in c-Kit-positive differentiating spermatogonia. Although *H3t*-deficient germ cells showed defects in an earlier stage, the H3t protein level peaked at the spermatocyte stage, and eventually diminished at the elongating spermatid stage (Figure 2D), indicating that it might also have functions during meiosis and spermiogenesis. Finally, we found that H3t is distributed throughout the genome but is excluded from the X-Y body (Figures 2F and S3I), which is consistent with the replacement of H3.1/3.2-type variants with H3.3 during meiotic sex chromosome inactivation (van der Heijden et al., 2007). Because the X-Y body contains less unsynapsed regions

(C–E) Abnormalities of the reproductive tracts of *H3t* null male mice, showing the testis (C), cauda epididymidis (D), and cells dispersed from the cauda epididymidis (E). Note that the cauda epididymidis of $H3^{-/-}$ mice was relatively transparent compared with that of $H3t^{+/-}$ mice (D), because there were no spermatozoa present in the lumen.

(F) H&E staining of testis sections.

(G) Germ cells were retained in *H3t* null testes. Testes were stained with an anti-TRA98 antibody (a pan-germ cell marker), counterstained with Hoechst 33342.

(H) Undifferentiated spermatogonia were unaffected in *H3t* null testes. Testes were stained with an anti-PLZF antibody, counterstained with Hoechst 33342 (left panel). The number of PLZF-positive cells was counted and indicated as the bar chart (right panel). NS, not significant.

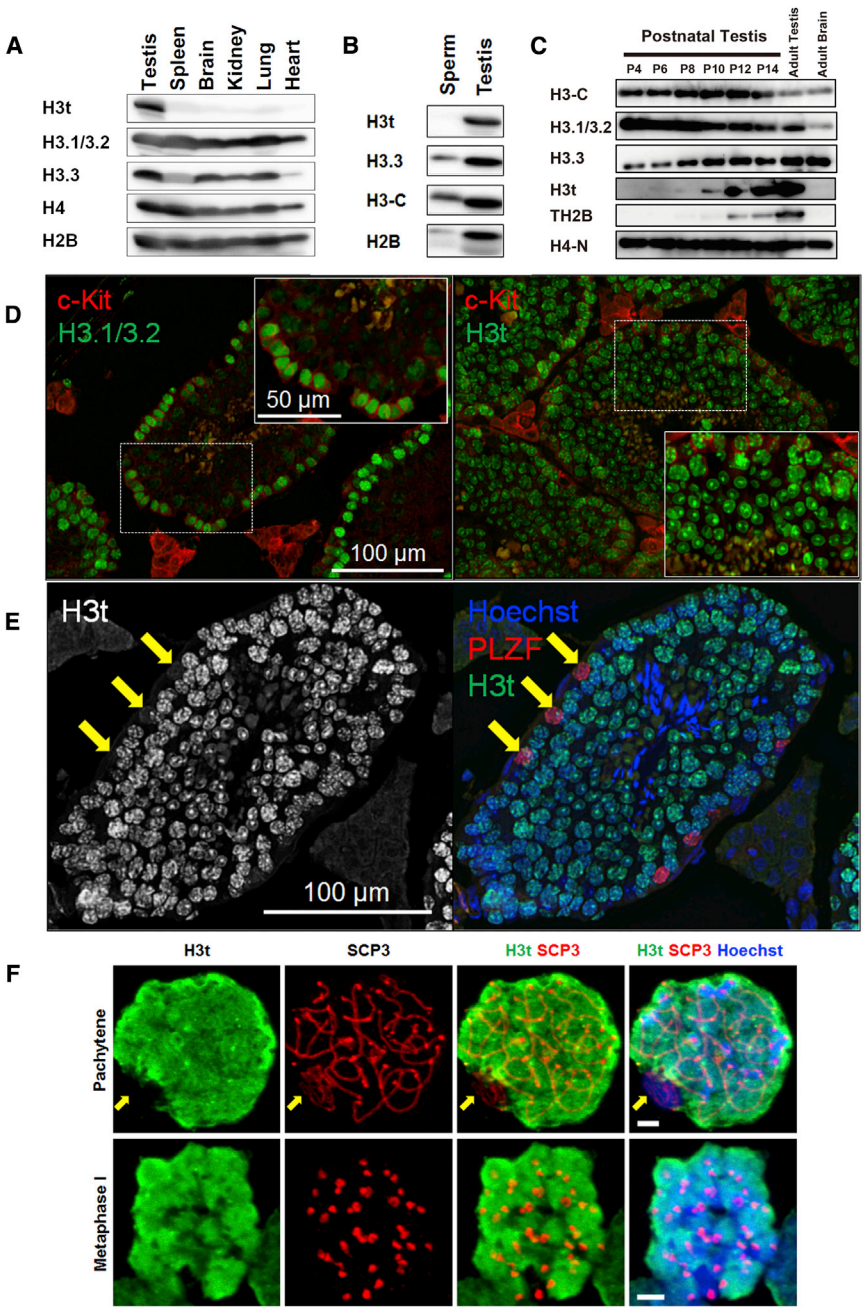

Figure 2. Expression and Functional Timing of H3t Protein Expression in Spermatogenesis

(A–C) Western blot analysis of histone variants in mouse tissues (A), sperm (B), and testes from postnatal mice (P4–P14) (C). H3-C and H4-N represent antibodies against the C-terminal region of H3 and the N-terminal region of H4, respectively, and these recognize all of the H3 and H4 variants.

(D) Immunostaining of testis sections by antibodies against H3.1/3.2 (left) and H3t (right) (green). Both sections were co-stained with an anti-c-Kit antibody (red). Magnified images of boxed areas are shown in each panel.

(E) H3t is expressed from differentiating spermatogonia. Note that H3t is not expressed in PLZF-positive undifferentiated spermatogonia (arrows).

(F) H3t is distributed throughout the genome, but excluded from the X-Y body. Meiotic chromosome spreads were stained with anti-H3t and -SCP3 antibodies. Arrows indicate the X-Y body in pachytene chromosomes. Scale bars, 2 μm.

compared with autosomes, it is interesting to speculate that H3t is involved in chromosome pairing.

H3t Is Required for Spermatogonial Differentiation and Ensures Entry into Meiosis

Germline stem (GS) cells are known to maintain the characteristics of undifferentiated spermatogonia in media containing glial cell line-derived neurotrophic factor (GDNF), but when treated with retinoic acid they undergo differentiation (Dann et al., 2008; Kanatsu-Shinohara et al., 2004; Bowles and Koopman, 2007). To recapitulate this spermatogonial differentiation defect in vitro, we used a GS cell differentiation system. As expected from the nature of undifferentiated spermatogonia, H3t was absent from undifferentiated wild-type GS cells, but was induced by retinoic acid upon differentiation (Figures 3C, 3D, S3D, and S3J). Confirming that H3t is dispensable for GS cell self-renewal and maintenance of undifferentiated spermatogonia, we successfully established multiple *H3t* null GS cell lines (Figures 3A and 3B). Once the cells were induced to undergo differentiation, "linked-together" morphology appeared in wild-type GS cells, whereas this was hardly observed in *H3t*-deficient cells (Figures 3C–3E) (Dann et al., 2008). This suggests that H3t is also required for GS cell differentiation in vitro. Furthermore, the total H3 protein level in differentiated *H3t*-deficient GS cells was not significantly attenuated compared with wild-type cells (Figure 3D, shown by H3-C), indicating that the differentiation defect observed is not caused by under-dosage of H3 protein level within the nucleus.

The differentiation defect in *H3t*-deficient testes was further validated by mRNA-seq analysis with P10 testes, the stage when germ cells started to show abnormalities (Figure S2G). Although most gene expression was not affected (Figure S4A), the expression levels of genes associated with meiosis as well as other germ-cell-specific histone variants were found to be significantly dysregulated in the *H3t*-deficient testis, and these results were further confirmed in GS cells (Figures 3F–3H and S4B–S4D). Hence, loss of H3t seemed to impair entry into

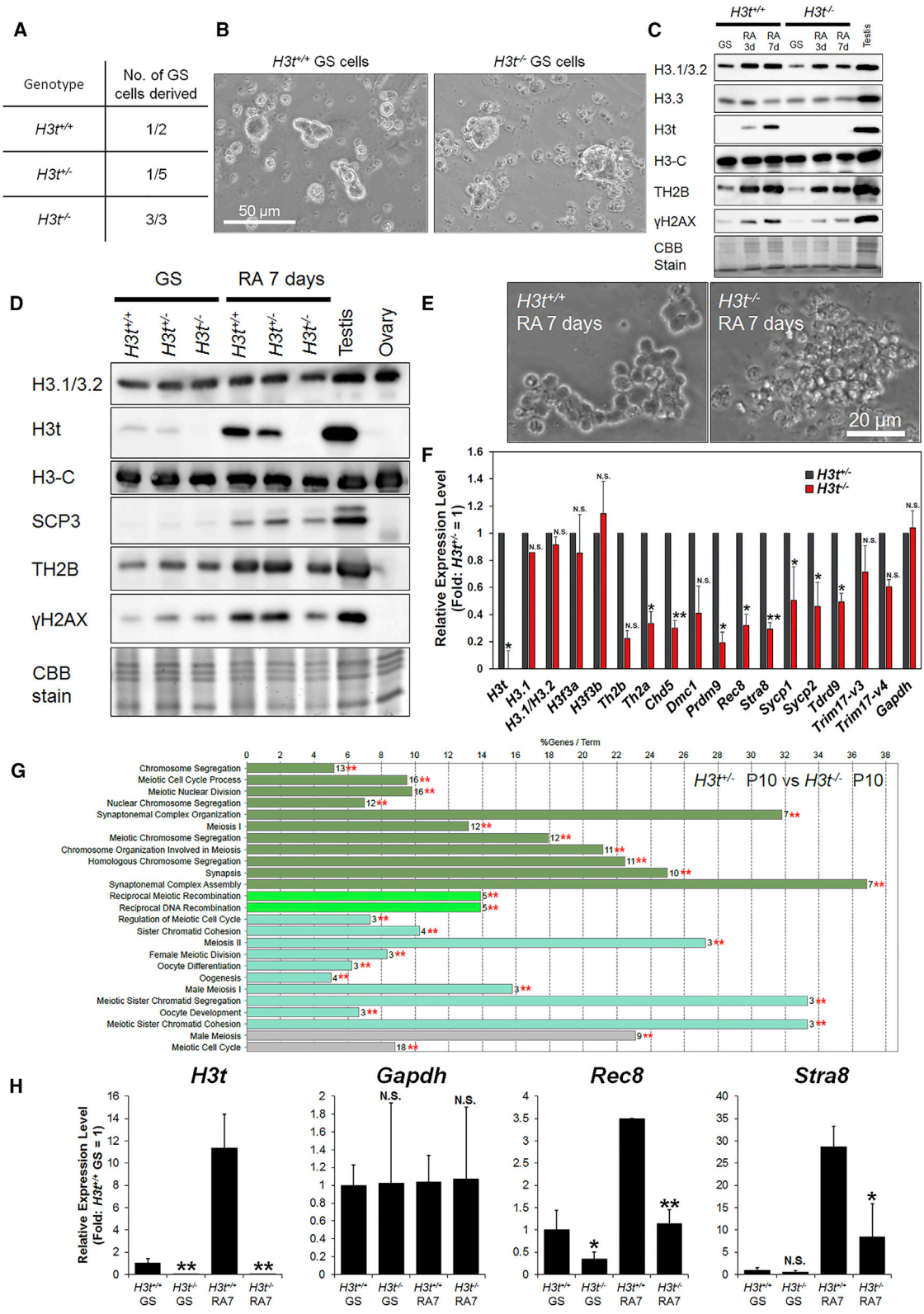

Figure 3. H3t Is Expressed upon Spermatogonial Differentiation and Ensures Entry into Meiosis

(A and B) Derivation of GS cell lines from *H3t* null testes. Note that these lines were established regardless of their genotypes (A), and there was no difference in their morphologies at the light microscopic level (B).

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meiosis. Moreover, we noted that *Trim17* was not expressed in neonate testes, but highly expressed in adult testes, and its protein expression level was affected in *H3t* heterozygous knockout adult testes (Figures 3F, S4B, and S4C). These results suggest that, although *H3t* and *Trim17* genes are expressed at different time points, early expressed *H3t* gene seems to have a positive effect on the following *Trim17* gene expression. Taken together with the GS cell data, H3t appears to be induced upon spermatogonial differentiation and possibly replaces canonical H3 proteins, which have been utilized during stem cell replication, ensuring that differentiating spermatogonia can progress into meiosis and beyond.

H3t-Containing Nucleosomes Form Open Chromatin Structure

Our results suggest that the other canonical H3 variants cannot compensate for the functions of H3t in the testis, even though there are only three amino acid differences between H3t and H3.1 (Figure 1A). To gain more insights into its molecular properties, we performed structural and biochemical analyses of the H3t nucleosome. Tachiwana et al. (2010) previously reported that human H3T-containing nucleosomes formed less stable structures than those containing canonical H3.1, by analyzing their crystal structures and biochemical properties. However, the human H3T M71 and V111 residues, which are responsible for instability of the H3T-containing nucleosome, are not conserved in H3t (Figure 1A). Salt and thermal stability assays for the H3t nucleosome revealed that the H3t nucleosome was not substantially unstable compared with the H3.1 nucleosome (Figures S4E and S4F). Therefore, we focused on the H3t-specific H42 residue, which is located near the DNA entry-exit region of the nucleosome. In the H3.1 nucleosome, the basic R42 side chain, corresponding to the H3t H42 residue, forms electrostatic interactions with DNA backbones located at the entry-exit and the nucleosomal dyad regions (Figure 4B, right panel). In contrast, the H42 side chain of H3t is less basic, and this weakens the electrostatic interactions between H3t and DNA around the entry-exit DNA regions of the nucleosome (Figure 4B, left panel). This finding led us to speculate that the DNA around the entry-exit regions in the H3t nucleosome is more flexible than that of the H3.1 nucleosome. To test this possibility, we performed micrococcal nuclease (MNase) and exonuclease III (ExoIII) treatment assays, because these enzymes preferentially degrade DNA that is detached flexibly from histone surfaces. As shown in Figures 4C and 4D, the H3t nucleosome was more sensitive to both MNase and ExoIII, and these were further confirmed by reciprocal amino acid substitutions. That is, the H3.1 R42H nucleosome became sensitive to nuclease digestions, whereas the H3t H42R nucleosome became resistant. Therefore, we conclude that the H42 residue of H3t endows DNA with flexibility at the entry-exit regions of the H3t nucleosome. Last, we tested whether the DNA end flexibility of the H3t nucleosome might result in an open configuration of the chromatin. To this end, we reconstituted polynucleosomes and performed analytical ultracentrifugation assays (Figures S4G–S4J). In good agreement with the above findings, the H3t-containing polynucleosome formed a more open structure than that for H3.1, in the presence and absence of $MgCl_2$ (Figure 4E). Therefore, this unique biochemical property of H3t arising from a single amino acid substitution might give instability between DNA and nucleosomes, and consequently provide an open chromatin structure that is necessary for germ cells to progress into spermatogenesis.

Amino acid sequence implied that H3t is incorporated into chromatin during DNA replication; our results also indicated that H3t is induced upon spermatogonial differentiation, eventually distributed throughout the genome, except for the X-Y body. Because spermatogonia are the only cell types that undergo mitotic replication during spermatogenesis, it is likely that canonical H3 histones are exchanged with H3t while the differentiating spermatogonia are replicating. Consequently, this dynamic exchange of H3 variants might ensure entry into meiosis. Although we did not observe any clear decrease in the total H3 protein in in vitro-differentiated *H3t*-deficient cells (Figure 3D), and no global gene expression alterations were observed in *H3t*-deficient P10 testis (Figure S4A), our present study cannot fully exclude the possibility that the defects observed in vivo might be a mere effect of H3 protein under-dosage. To this end, it will be important to generate knock-in mice, in which H3t is replaced with the H42R point mutant and/or H3.1, and investigate their effects on spermatogenesis. Nevertheless, how the H3t-specific open chromatin structure affects spermatogenesis is of particular interest. Because the phenotype of *H3t*-deficient mice is rather severe compared with known knockout mice of histone modification-related genes (Sasaki and Matsui, 2008), we speculate that H3t, together with other histone variants (Shinagawa et al., 2015; Montellier et al., 2013;

(C) H3t is induced upon GS cell differentiation and protein level increases during this process. GS cells from $H3t^{+/+}$ or $H3t^{-/-}$ mice were treated with retinoic acid (RA) for indicated days; total proteins were extracted, electrophoresed, and probed with the indicated antibodies.

(D) Expression level of histone variants in GS cells, before and after the induction of differentiation. $H3t^{+/+}$, $H3t^{+/-}$, or $H3t^{-/-}$ GS cells were treated with RA for 7 days; total proteins were extracted, electrophoresed, and probed with the indicated antibodies. CBB stain data are indicated as a loading control. Note that there was no drastic reduction in the total H3 protein level in $H3t^{-/-}$ GS cells after the induction of differentiation (indicated by H3-C antibody).

(E) Morphologies of GS cells 7 days after the induction of differentiation with RA. The $H3t^{-/-}$ GS cells seldom showed "linked-together" morphology.

(F) Meiosis-related genes were less strongly expressed in the testes of $H3t^{-/-}$ mice than in $H3t^{+/-}$ mice at P10. The expression levels of the indicated mRNAs were analyzed by RT-qPCR. The values in the $H3t^{+/-}$ mouse were set to 1 following normalization against the *Eef1a1* expression level. Data represent the mean of three independent experiments ± SD. *$p < 0.05$; **$p < 0.01$. NS, not significant.

(G) Meiosis-related phenotypes were preferentially extracted in gene ontology (GO) analysis. GO pathway terms were specified using downregulated genes in the $H3t^{-/-}$ mouse at P10. The bars represent the numbers of genes associated with the terms. The percentages of genes for each GO term are shown on the horizontal axis.

(H) Expression level of meiosis-related genes in GS cells, before and after the induction of differentiation, were analyzed by RT-qPCR. $H3t^{+/+}$ or $H3t^{-/-}$ GS cells were treated with RA for 7 days. The values in the $H3t^{+/+}$ GS cells were set to 1 following normalization against the *Eef1a1* expression level. Data represent the mean of three independent experiments ± SD.

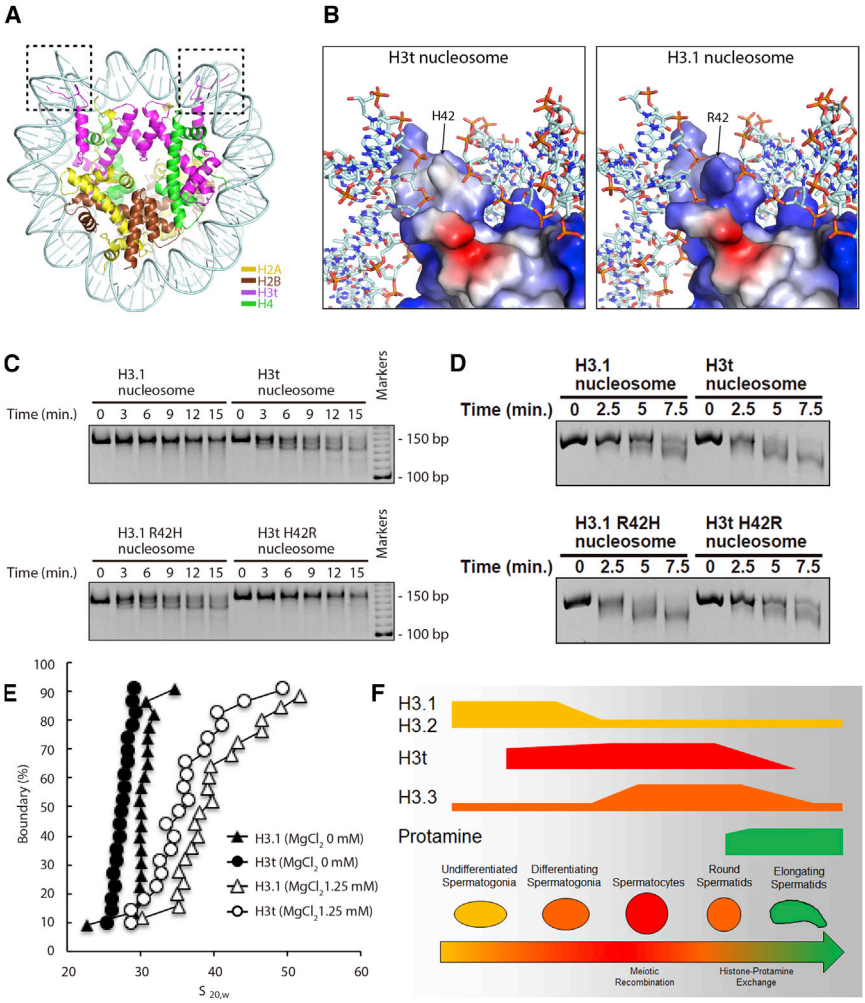

Figure 4. The H3t Variant Forms an Open Chromatin Structure In Vitro

(A) Overall structure of the H3t nucleosome. Dotted squares indicate the enlarged regions around the H3t His42 residue presented in (B). H2A, yellow; H2B, brown; H3t, magenta; H4, green; and DNA, gray.

(B) Close-up view around the H3t His42 region in the H3t nucleosome (left) and the corresponding H3.1 Arg42 region in the H3.1 nucleosome (right). The H3t and H3.1 molecules are shown as an electrostatic surface, and DNAs are shown as a stick representation.

(C) MNase assay. H3t, H3.1, the H3t H42R, and H3.1 R42H nucleosomes were treated with MNase for 0, 3, 6, 9, 12, and 15 min and then deproteinized. The resulting DNA fragments were analyzed by nondenaturing 10% PAGE with ethidium bromide (EtBr) staining. The gel image shown is a representative of three or more independent experiments with similar results.

(D) ExoIII assay. Each nucleosome was treated with ExoIII for 0, 2.5, 5, and 7.5 min and then deproteinized. The resulting DNA fragments were extracted and analyzed by denaturing 14% PAGE with EtBr staining. The gel image shown is a representative of two independent experiments with similar results.

(E) Sedimentation velocity analyses by analytical ultracentrifugation. The experiments with the H3t and H3.1 polynucleosomes are represented as circles and triangles, respectively. Open and filled symbols indicate experiments in the presence and absence of $MgCl_2$, respectively. The sedimentation coefficient ($S_{20,w}$) distributions were determined by the method of van Holde and Weischet (Demeler and van Holde, 2004).

(F) Schematic diagram of how histone variants and protamines appear to be exchanged during spermatogenesis.

Yuen et al., 2014), lays the structural foundations needed for spermatogenesis-specific events, such as meiotic recombination and histone-protamine exchange (Figure 4F).

In summary, we have demonstrated that a tissue-specific H3 variant has a direct impact on cellular differentiation. We previously found 13 H3 variants in addition to H3t with characteristic tissue distributions (Maehara et al., 2015), and our present study demonstrates the intriguing idea that these variants might also have distinctive roles in tissue formation and cell differentiation. Hence, not only histone modifications, but also a histone variant by itself might dictate the fate of cell lineages during their differentiation.

EXPERIMENTAL PROCEDURES

Animals

All animal care was in accordance with institutional guidelines and was approved by the Institutional Animal Care and Use Committees of both Chubu and Osaka universities.

Statistical Methods

The data are presented as the mean ± SD of three independent experiments. Statistical significance of each pair was calculated with Student's t test, and p values <0.05 were considered statistically significant and are indicated as follows: *$p < 0.05$; **$p < 0.01$.

mRNA-Seq and Analysis

Total RNAs were extracted from whole testes of *H3t* homozygous and heterozygous knockout mice at P10. The mRNA-seq libraries were prepared and sequenced according to the Illumina protocol. Differentially expressed genes were extracted using Cufflinks (version 2.2.1, with options "cuffdiff –u –b") with reads mapped onto the mouse genome (mm9) using Tophat (version 2.0.12, with default options). The gene ontology (GO) analysis was performed using ClueGO (Bindea et al., 2009).

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is DNA Data Bank of Japan: DRA004195. The accession numbers for the atomic coordinates of the H3t and H3.1 nucleosomes reported in this paper are RCSB PDB: 5B1L and 5B1M, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.12.065.

AUTHOR CONTRIBUTIONS

J.U., A.H., T.U., H. Kimura, Y. Ohkawa, H. Kurumizaka, and K.Y. designed the study. J.U., A.H., T.U., S.M., K.M., M.H., Y.M., J.N., N.H., and K.Y. performed experiments with assistance from A.O., H. Taguchi, H. Tanaka, H. Tachiwana, T.Y., M.Y., T.I., A.I., M.I., T.T., and Y. Okada. All authors contributed to data analysis and discussion. J.U., A.H., T.U., H. Kimura, Y. Ohkawa, H. Kurumizaka, and K.Y. wrote the paper, and all authors contributed to editing.

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