Title: The histone methyltransferase Setd2 is required for expression of acrosin-binding protein 1 and protamines and essential for spermiogenesis in mice

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Abstract

Spermatogenesis is precisely controlled by complex gene expression programs and involves epigenetic reprogramming including histone modification and DNA methylation. SET domain–containing 2 (Setd2) is the predominant histone methyltransferase catalyzing the trimethylation of histone H3 Lysine 36 (H3K36me3) and plays key roles in embryonic stem cell differentiation and somatic cell development. However, its role in male germ cell development remains elusive. Here we demonstrate an essential role of Setd2 for spermiogenesis, the final stage of spermatogenesis. Using RNA-Seq, we found that in postnatal mouse testes, Setd2 mRNA levels dramatically increase in 14-days-old mice. Using a germ cell–specific Setd2 knockout mouse model, we also found that targeted Setd2 knockout in germ cells causes aberrant spermiogenesis with acrosomal malformation before step eight of the round-spermatid stage, resulting in complete infertility. Furthermore, we noted that the Setd2 deficiency results in complete loss of H3K36me3 and significantly decreases expression of thousands of genes, including those encoding acrosin-binding protein 1 (Acrbp1) and protamines, required for spermatogenesis. Our findings thus reveal a previously unappreciated role of the Setd2-dependent H3K36me3 modification in spermiogenesis, and provide clues to the molecular mechanisms in epigenetic disorders underlying male infertility.
INTRODUCTION

Mammalian spermatogenesis is a highly specialized differentiation process that can be typically divided into a mitotic phase, a meiotic phase, and a phase of spermiogenesis (1,2). During the mitotic phase, spermatogonial stem cells (SSCs) either undergo self-renewal to maintain the stem cell pool or enter the differentiation pathway to generate differentiating spermatogonia, which are committed to produce primary spermatocytes (3). In meiosis, spermatocytes then undergo one round of replication followed by meiotic homologous recombination and two rounds of chromosome segregation to give rise to haploid round spermatids (4). During spermiogenesis, round spermatids undergo dramatic morphological and molecular changes, including high chromatin condensation, acrosome formation, flagellum development, and cytoplasm elimination, to transform into elongated mature spermatozoa (5-9). According to morphological characteristics, mouse spermiogenesis is subdivided into 16 steps. Steps 1 to 8 include round spermatids, whereas steps 9 to 16 include elongating spermatids (5-9). These processes are characterized by the dynamic changes to gene expression patterns and the dramatic changes of chromatin structure, which have been shown to involve epigenetic regulatory mechanisms (e.g. histone modification, DNA methylation, RNA methylation, noncoding RNAs, etc.) (10-15).

Emerging evidence has emphasized a crucial role of histone posttranslational modifications (PTMs) in mammalian spermatogenesis. Knockout mice carrying mutations in genes encoding histone modification enzymes display male sterility with a broad range of defects of spermatogenesis, revealing a crucial role of histone modifications in mammalian spermatogenesis (11,16-28). The tri-methylation of lysine 36 of histone 3 (H3K36me3) is a well-characterized feature of transcriptionally active genes and usually enriched in the gene body regions. H3K36me3 is also implicated in pre-mRNA splicing, DNA mismatch repair, and recruitment of Dnmt3b. SET-domain-containing 2 (SETD2, also known as HYPB and KMT3A) is the major methyltransferase responsible for H3K36me3 in mammals (29-34). Ablation of Setd2 ablation in mice leads to global loss of H3K36me3 and embryonic vascular remodeling defects, indicating a critical role of SETD2-mediated H3K36me3 during development (32,34-36). Although
the functions of Setd2 and its associated H3K36me3 have been studied in diverse biological processes, the requirement for Setd2 and H3K36me3 during spermatogenesis remains elusive.

In this report, we show the spatiotemporal distribution of Setd2 and its associated H3K36me3 in mouse testes. We demonstrate that loss of H3K36me3 by germ cell-specific disruption of Setd2 leads to impaired spermiogenesis due to significant downregulation of its direct target genes, which include many critical master regulators for spermiogenesis. This study thus reveals a crucial role of Setd2 and its associated H3K36me3 in spermiogenesis and uncovers Acrbp1 and protamine genes as direct targets of Setd2-dependent H3K36me3 during this process.

RESULTS
Spatial and Temporal Patterns of Setd2 and H3K36me3 Distribution during Spermatogenesis.

To explore the role of Setd2 and the associated H3K36me3 during spermatogenesis, we first examined the expression pattern of Sedt2 in mouse testes. In postnatal mouse testes, Setd2 mRNA level dramatically increases at 14-day-old, a stage that corresponds to the appearance of pachytene spermatocytes in the seminiferous epithelium (Fig. S1A), and remained at high level afterwards, suggesting that Setd2 is abundantly expressed in pachytene spermatocytes and spermatids. We then performed immunofluorescence staining to further investigate the subcellular localization of the Setd2 protein in the testis. Consistent with the mRNA expression, we found that Setd2 protein is mainly detected in the nucleus of pachytene spermatocytes and round spermatids, which are confirmed by the counterstaining of peanut lectin (PNA), a marker for acrosome, and γH2AX, a maker for XY body, respectively (Fig.1A, and Fig. S1B)(37). Moreover, Setd2 is also present in Sertoli cells as confirmed by counterstaining of GATA4, a marker for Sertoli cells (and round spermatids)(38) (Fig. S1C). Collectively, Setd2 is predominantly expressed in pachytene spermatocytes and post-meiotic germ cells in the adult mouse testes.

As Setd2 is considered as the main histone H3K36me3 methyltransferse in mammals, we next analyzed the distribution of H3K36me3 in mouse testis. Interestingly, we found that H3K36me3 distribution did not show identical
pattern as Setd2 in the testes. As shown in Fig. 1B, H3K36me3 exhibited strong staining in the nucleus of all stages of spermatocytes and the nucleus of round spermatids while weak H3K36me3 signal was detected in spermatogonia and elongated spermatids. Taken together, these observations indicated that Setd2 might be responsible for cellular H3K36me3 in late-stage spermatocytes (pachytene/diplotene spermatocytes) and spermatids. However, whether it is predominantly responsible for H3K36me3 in the early-stage spermatocytes, such as preleptotene spermatocytes, remains to be determined in the future.

**Setd2 is Essential for Murine Spermiogenesis.**

To determine the role of Setd2 during mammalian spermatogenesis, we used a conditional knockout approach that generated a Setd2 floxed line (Setd2\(^{\text{fl/fl}}\)), in which exons 6 and 7 of the Setd2 allele is flanked by loxP sites (Fig. S2A). To generate germline-specific deletion, mice bearing loxP flanked alleles of Setd2 were crossed with Stra8-GFPCre knockin mice (Setd2\(^{\text{fl/del}}\), Stra8-GFPCre) (Fig. S2A). The Stra8-GFPCre knockin mice express Cre from A1 spermatogonia onwards, in which both alleles of Setd2 could be excised at A1 spermatogonia (Setd2\(^{\text{fl/del}}\), Stra8-GFPCre)(10). Thus, we designate the two different genotypes of mice as: control (Setd2\(^{\text{fl/del}}\)), Setd2 cKO (Setd2\(^{\text{fl/del}}\), Stra8-GFPCre). Immunostaining verified that Setd2 protein were absent in the mutant germ cells (Fig. S2B). Notably, in Setd2 cKO mice, H3K36me3 signals were also completely absent in pachytene spermatocytes and round spermatids, whereas it still remained in preleptotene spermatocytes. These results further confirmed Setd2 as the major H3K36me3 methyltransferase in late-stage spermatocytes and round spermatids but not in early stage spermatocytes (Fig. S2C). We also found that the levels of H3K9me2, H3K9me3, H3K27me3, H3K79me3 and H3K27Ac were not affected in the mutants, indicating Setd2 loss did not cause global changes of other histone modifications (Fig. S2D).

Although the Setd2 cKO males copulate normally, they were completely sterile. Testes from adult Setd2 cKO mice were significantly smaller than those of the littermates as controls [Fig. 2A and 2B, age of 8 wk, control = 0.3867 ± 0.0746, Setd2 cKO mice = 0.2425 ± 0.0416; P<0.001, n = 8].
Histological examinations of adult germ cell mutant testes show that the seminiferous tubules are devoid of mature spermatozoa and contain exclusively round-headed spermatids. Moreover, round spermatids in Setd2 cKO mice did not undergo cellular elongation, indicating a developmental arrest before step 8 spermatids (Fig. 2C). Consistent with this, no sperm was found in the epididymis of germ cell mutants (Fig. 2C). These results suggest that Setd2 plays an essential role in spermatogenesis and its loss leads to developmental arrests at round spermatid stage.

Close examination of the mutant seminiferous tubules showed a high frequency of degenerated germ cells in the spermatocyte and spermatid layers. TUNEL staining further identified a dramatic increase of apoptotic spermatocytes and spermatids in the mutant testes (Fig. 2D and 2E). To distinguish whether the defects in spermatocytes resulted from an intrinsic requirement of Sedt2 function or secondary effects of impaired spermiogenesis, we examined the first occurrence of the apoptotic germ cells in Setd2 cKO mouse testes. At 2-week-old, while the pachytene spermatocytes start to occur in the testis, we found that there is no significant difference between Setd2 cKO and control mouse testes (Fig. S3A and S3C). In 4-week-old mice, when the first population of spermatids reaches the elongated spermatid in the control testes, elevated apoptosis in germ cells are observed in Setd2 cKO mouse testes (Fig. S3B and S3C). Similar apoptotic spermatocyte defects are also observed in Crem, Trf2, Miwi, Tdrd5, and Mrg15 mutants, while these mutants show impaired spermatid differentiation (39-45). Taken together, these findings suggest that, in Setd2 cKO mouse testes, spermatocyte defects resulted from the spermiogenic arrest.

To verify the requirement for Setd2 in spermiogenesis, but not in the early stages of spermatogenesis, we conditionally deleted Setd2 allele in germ cells as early as embryonic day 15 using the Vasa-Cre transgenic line (Setd2<sup>fl/del</sup>, Vasa-Cre). Inactivation of the Setd2 in gonocytes resulted in a similar phenotype and the loss of H3K36me3 was only observed in pachytene spermatocytes and spermatids, as shown in Setd2<sup>fl/del</sup>, Stra8-GFPCre mice (Fig. S4A-S4C). Thus, these results further demonstrated an essential role for Setd2 and its associated H3K36me3 in spermiogenesis.

Loss of Setd2 Results in Malformation of Acrosome.
To elucidate the nature of the defects in spermiogenesis of Setd2 cKO mice, we performed immunostaining using FITC-conjugated peanut agglutinin (PNA) capable of binding the outer acrosomal membrane, and anti-Golgi 58K antibody, a marker for proacrosome in spermatids(46). Compared to a single acrosomal vesicle in the Golgi phase at steps 2 and 3 of spermiogenesis in controls (Fig. 3A), we found several acrosomal vesicle-like structures in Golgi phase spermatids of Setd2 cKO mice (Fig. 3B), indicating that disruption of Setd2 could lead to acrosomal malformation as early as in the Golgi phase during spermiogenesis. In the cap phase at steps 4-7 of spermiogenesis, the acrosome expanded to form cap-like structure covering the nucleus of the round spermatids (Fig. 3A). However, in Setd2 cKO mouse spermatids, the cap-like acrosome failed to form, resulting in a discontinuous acrosomal structure in the anterior region of the nucleus (Fig. 3B).

To characterize the defective acrosome biogenesis in spermiogenesis of Setd2 cKO mice in further detail, we performed a transmission electron microscopy (TEM) study (Fig. 4). Consistent with immunofluorescence analyses, the first acrosomal defects were detected in the Golgi phase spermatids, in which several acrosomal structures appeared in Setd2 cKO mouse round spermatids while a single uniformed acrosomal structure was present in control spermatids (Fig. 4A and 4B). Compared with control spermatids, acrosomal cap structures displayed discontinues and abnormally fragmented in Setd2 cKO mouse spermatids at the cap phase (Fig. 4C and 4D). It is of note that the fragmented acrosome persisted in all subsequent stages of spermiogenesis in Setd2 cKO mutants. Taken together, these observations reveal that Setd2 plays crucial roles in the acrosome biogenesis and is required for spermiogenesis.

Setd2 Inactivation Led to Complete Loss of H3K36me3 in Pachytene Spermatocytes and Round Spermatids

To better gain the mechanistic insights of the spermiogenesis defects caused by Setd2 loss, we set out to determine the genome-wide distribution of H3K36me3 by ChIP-seq in the pachytene/diplotene spermatocytes (PD) and round spermatids (RS) from the control and mutant mice. Our H3K36me3 ChIP-seq analyses identified 28,270 and 26,109 peaks, which cover 10,027 and 10,164 genes in PD and RS cells, respectively (Fig. 5A). In both PD and
RS cells, we found that H3K36me3 is mainly enriched in the gene bodies, especially, the exonic, intronic and 3'UTR regions, agreeing with the canonical understanding of this histone mark (Fig. 5B). Consistent with our hypothesis of Setd2 being the major H3K36me3 methyltransferase in the late stages of spermatogenesis, we found the mutant cells showed drastic loss of H3K36me3 (Fig. 5C, normalized by Spike-In approach, see methods). The ChIP-seq results were also confirmed by ChIP-quantitative PCR (ChIP-qPCR) over a few select targets (Fig. 5D).

**Setd2 Loss Led to Misregulation of Critical Regulators in Spermiogenesis**

To further investigate the expression changes caused by Setd2 loss, we profiled the transcriptomes of the PD and RS from control and Setd2 cKO mouse testes, respectively. We found that Setd2 loss led to a total of 2,48 and 1,546 transcripts, and 1,088 and 1,145 transcripts, significantly (FDR<0.05, >2 fold difference) down- and up-regulated in PD and RS cells, respectively (Fig. 6A and Fig. S5A). Further analyses found that the down-regulated genes are generally marked with higher levels of H3K36me3 in contrast to the up-regulated genes, suggesting the down-regulated expression is likely the direct effect of Setd2 loss (Fig. 6B). Consistent with higher H3K36me3 level, the down-regulated genes also express at higher levels than the up-regulated genes in the wide-type cells (Fig. 6C). Gene ontology (GO) analysis of the top-ranked differential genes indicated enrichment in genes associated with system development and motility for up-regulated genes, and cell-cell signaling and cytoskeleton for down-regulated genes in RS, respectively (Fig. S5B), suggesting that the developmental defects caused by Setd2 loss might be due the compromised functions of these pathways in RS.

It is of note that the expressions of many critical regulators for spermiogenesis, such as Acrbp1, Pick1, Odf1, and Spem1, are also significantly impaired upon Setd2 loss (Fig. 6D, and Fig. S5C), which is also confirmed by RT-qPCR (Fig. 6E) (47-50). Among these genes, both Acrbp1 and Pick1 are known to be required for acrosome development, and knockout of either Acrbp1 or Pick1 led to fragmentation of the acrosome, which is similar to that seen in Setd2 mutants. Moreover, loss of Spem1 resulted in sperm deformation. Thus the malformation of acrosome caused by Setd2 loss
could be due to the misregulation of these genes, which are likely the Setd2 direct targets.

**Impaired Activation of Transition Nuclear Protein and Protamine Genes in Setd2 cKO Mutants.**

Among the deregulated genes identified in Setd2 cKO mice, we also noticed aberrant expressions of many replication dependent histone genes (Fig. 6F). It is well known that, during late stages of sperm differentiation, the majority of the core histones are subsequently replaced by transition nuclear proteins (TNPs) and protamines (PRMs), leading to the formation of highly condensed chromatin in elongated spermatids(51). Either Tnps or Prms deficient mice are infertile because of disruption of chromatin condensation in elongating spermatids(52,53). Such processes are also accompanied with a coordinated and gradual transcriptional silencing of the canonical replication dependent histone genes and activation of the germ line specific histone and Tnp genes. Consistent with the spermatid developmental defects of the Setd2 cKO mice, we identified significant down-regulation of Tnps, Prm1/2/3 and several testis specific histone variant genes, such as H1fnt, H2afb1, H2afv, accompanied with up-regulation of many replication dependent histone genes (Fig. 6F), in both isolated pachytene spermatocyte and purified round spermatids. These observations were further validated by RT-qPCR (Fig 6E). These findings indicate that the transition from histone-to-protamine replacement could also be compromised upon Setd2 loss, thereby contributing to the defective spermiogenesis.

**DISCUSSION**

Setd2 is the major methyltransferase of histone H3K36me3, a mark associated with active transcription. Here, we show that Setd2 is the primary H3K36me3 methyltransferase in PD and RS, and is essential for spermiogenesis. Loss of Setd2 led to complete loss of H3K36me3 in PD and RS cells, stalled development at round spermatid stage, thereby resulting in sterility. Mechanistically, we found that the activation of several critical master regulators for spermiogenesis is impaired in Setd2 cKO germ cells, companied with compromised expression of motility related genes. Interestingly, we also found that the expressions of many histone genes and Tnp as well as protamine genes were significantly affected, indicating an
impaired histone-to-protamine transition during spermiogenesis. Taken together, we revealed an essential role of Setd2 during spermiogenesis but not early stages of spermatogenesis.

Setd2-mediated H3K36me3 is a transcriptionally coupled histone methylation mark, enriched over gene bodies undergoing active transcription(31). Although the enzymology and molecular function of Setd2 have been well documented, the biology consequences of Setd2 inactivation in animal and organism levels have not been well explored. To understand its biological significance during spermatogenesis, we generated a Setd2 cKO model in this study. Consistent with previously reports, we found that H3K36me3 patterns in PD and RS are also enriched in gene bodies in PD and RS cells. Setd2 loss leads to expression changes of hundreds to thousands of genes in PD and RS cells (Figure 6a, FDR<0.05, >2 fold difference). However, since H3K36me3 is a mark associated with active transcription, we are surprised to find less down-regulated genes than up-regulated genes in both PD and RS cells from Setd2 cKO mice. Further analyses found that the down-regulated genes are generally marked by higher levels of H3K36me3 in the wild-type germ cells (Fig. 6B and 6C) and also expressing at higher levels. These observations not only suggest that the down-regulated expression is likely the direct effect caused by Setd2 loss, but also indicate that Setd2 and H3K36me3 are required for transcriptional activities of higher expressing genes, which may play important roles in spermiogenesis. In contrast, the up-regulated genes are generally low expressers and the elevated expression may be due to the compromised distribution of transcriptional machineries upon global loss of H3K36me3. Indeed, we observed significant impaired expression of critical spermiogenesis regulators, including Acrbp1, Pick1, Odf1, Spem1, Tnp and Prm genes and mis-expression of histone genes, which are normally undergoing transcriptional silencing at these stages. For example, loss of either Acrbp1 or Pick1 resulted in similar acrosomal fragmentation that observed in Setd2 mutants. This suggests that malformation of acrosome in Setd2 mutants result from decreased expression of Acrbp1 and Pick1. Moreover, downregulation of Tnp and Prm genes could disrupt histone-to-protamine transition, thereby causing the spermatid chromatin condensation...
defects. However, we cannot directly observe the spermatid chromatin condensation phenotype because arrest of spermiogenesis in Setd2 mutants occurred long before the spermatid chromatin condensation. Thus, deregulation of multiple pathways might together contribute to the spermatid developmental defects in Setd2 cKO mice.

Notably, at global levels, we found that Setd2 and H3K36me3 patterns displayed dissimilarities during spermatogones. Highest level of Setd2 was observed in pachytene spermatocytes and post-meiotic germ cells in the adult mouse testes, while strong H3K36me3 level are maintained throughout all stages of spermatocytes and round spermatids with spermatagonia and elongated spermatids showing weaker H3K36me3. Importantly, we found that H3K36me3 level was largely unchanged in preleptotene spermatocytes of the Setd2 cKO mice when compared to control littermates. Such findings suggested that H3K36me3 might be subjected to the regulation of other methyltransferases in the early stages of spermatogenesis. Consistently, Prdm9 was recently reported to mediate non-canonical H3K36me3 at the hot spots of recombination sites during meiosis(54). As the epigenome is undergoing rapid programming during spermatogenesis, whether there are other unknown methyltransferases responsible for H3K36me3, especially in the early stages of spermatogenesis, will need further investigation.

Experimental procedures

Mice. Setd2 conditional knockout mouse line was produced by Shanghai Biomodel Organism Co. as shown in Supplementary Fig. S2a. The germline-specific deletion of Setd2 was generated by crossing with Stra8-GFPCre mice or Vasa-Cre mice as previously described(10). All mice were maintained on the C57BL/6J background. All animal experiments were conducted in accordance with the guidelines in the Animal Care and Use Committee at Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science.

Histology, Immunofluorescence, and TUNEL Staining. For testis histology, Bouin’s fixed sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). For immunofluorescence analysis, sections were retrieved in 10 mM sodium citrate buffer (pH 6.0) by boiled in microwave, and further washed in PBS with 0.1% Triton X-100. The sections
were blocked with blocking buffer (10% donkey serum and 0.1% Triton X-100 in PBS), and then incubated with the primary antibodies overnight at 4 °C followed by Alexa Fluor 488- and Alexa Fluor 594-conjugated donkey secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The fluorescent sections were mounted with Prolong Gold Antifade medium containing DAPI (Molecular Probes), and then analyzed by fluorescence microscopy (Olympus) or confocal microscopy (Olympus). Apoptotic cells were analyzed by the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science) according to the manufacturer’s instructions. The following primary antibodies were used in this study: rabbit anti-Setd2 (LS-B12260, and LS-C332416; LSBio), rabbit anti-H3K36me3 (4909S; Cell signaling), mouse anti-γH2AX (05-636; Millipore), goat anti-GATA4 (SC-1237; Santa Cruz), and FITC-conjugated PNA (L7381, Sigma).

**Isolation of Pachytene/diplotene Spermatocytes and Round Spermatids.**
Spermatocytes and round spermatids were enriched using Hoechst-33342 (Hoechst)/PI-based fluorescence-activated cell sorting (FACS) as previously described with minor modification (55). In brief, testes from adults were collected in PBS and placed on ice. After removal of the tunica albuginea and interstitial testicular cells, the seminiferous tubules were digested with PBS containing 120 U/ml of collagenase type I (Worthington), further digested with 0.25% trypsin (Gibco), and DNAse I (5mg/ml, Gibco), and then terminated by adding fetal bovine serum (FBS). The dissociated testicular cell suspension was collected and resuspended at a concentration of 1x10^6 cells/ml in DMEM (Hyclone) with 0.1mg/ml of DNAse I. The testicular cell suspension was then stained with Hoechst at the final concentration of 6µg/ml for 30min, and stained with PI prior to sorting immediately. Sorting of the spermatocytes and round spermatids was performed using flow cytometric analysis (Becton Dickinson). The collected spermatocytes contained ~85% and ~10% of pachytene and diplotene spermatocytes, respectively, based on nuclear spreading analysis. About 90% of the sorting spermatids were round spermatids as determined by PNA staining.

**Western Bolt.** The sorted pachytene spermatocytes and round spermatids were lysed in the 2xSDS loading buffer, and a total of 10 µg of whole cell
extracts were loaded to each lane for Western blot analyses. Antibodies used in this study are H3K9me2 (ABclonal A2359), H3K9me3 (ABclonal A2360), H3K27me3 (Active Motif 39158), H3K27ac (Active Motif 39133), H3K36me3 (Cell Signaling 4909S), H3K79me3 (Abcam ab2621) and H3 (Proteintech 7168-1-AP).

**Transmission Electron Microscopy.** Fresh testes were fixed in 2.5% (Vol/Vol) glutaraldehyde in 0.1M phosphate buffer (PB), pH 7.4, for 2h at 4°C, washed with PB, postfixed in 2% OsO4 for 1.5h, dehydrated in a graded ethanol series before transferring them to acetone, and embedded in Poly/Bed 812. Ultrathin sections were taken with a Leica EM UC7 ultramicrotome (Leica, Inc.), doubly stained with uranyl acetate and Reynold’s lead citrate, and then imaged on a FEI Tecnai G2 Spirit TEM (FEI Company) at 120-kV accelerating voltage.

**Chromatin Immunoprecipitation (ChIP) and ChIP-seq.** ChIP assay was carried out as previously described (Lan 2007). The sorted pachytene spermatocytes and round spermatids were crosslinked with 1% formaldehyde for 10 mins and then stopped by adding 125 mM Glycine. Chromatin samples were lysed with lysis buffer (20 mM Tris-HCl pH8.0, 500 mM NaCl, 1 mM EDTA, 1% TritonX-100 and 0.1% SDS) and sonicated by Qsonica R2. A total of 2 µg of H3K36me3 antibody (CST 4909S) was incubated with each chromatin sample overnight at 4 °C, together with 0.5 ug spike-in antibody (Active motif 61686) and 20ng spike-in chromatin (Active motif 53083) for normalization purpose. The protein-DNA complexes were immobilized on 15 µl protein A/G beads (SA032005, Smart Lifesciences Inc., Changzhou) and then washed 4 times with lysis buffer, twice with low salt buffer (10 mM Tris-HCl, 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Na-deoxylcholate) and once with 10 mM Tris-HCl pH8.0. Decrosslink was carried out in elution buffer (50 mM Tris-HCl pH8.0, 10 mM EDTA and 1% SDS) at 65°C for 5 hours. Proteinase K and RNase A digestion at 55°C for 1 hour. DNA samples were then purified with PCR extraction kit (QIAGEN #28006), and analyzed by real time PCR or prepared for deep sequencing according to the manufacture’s guidelines (KAPA Biosystems KK8503).
RNA-seq and qRT-PCR. RNA samples were prepared by RNeasy minikit (Qiagen). mRNA pools were enriched from total RNA by poly A beads. Barcoded RNA-seq libraries were constructed using NEBNex Ultra Directional RNA library prep kit for Illumina according to the manufacturer’s instructions (New England Biolabs). Non-strand-specific pair end 150 bp sequencing was by X-Ten (Illumina, run by WuXi App Tec). qRT-PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen).

ChIP-seq Peak Calling and Distribution Analysis. ChIP-seq reads were mapped with Bowtie (v0.12.7) (parameters: -m 1). Duplicate reads were removed by Samtools. The mapped unique and monoclonal reads were extended to 350 bp based on the average sonicated chromatin DNA length. H3K36me3 peaks were called using MACS1.4 with default settings. For H3K36me3 distribution analysis, the ChIP-seq signals over gene bodies of the up- and down-regulated genes in Setd2KO PD and RS cells were analyzed by a home-made script called Signalplot based on the normalized ChIP-seq tag density, all the gene bodies were aligned as the same length.

Heatmap analysis. The RNA-seq heatmap were generated based on the expression fold of changes in WT and Setd2 KO cells using Pheatmap, which is a R package. The H3K36me3 heatmap over gene bodies were generated using Deeptools2.0.

RNA-Seq Related Bioinformatics Analysis. Low-quality bases and adapter-containing reads were trimmed from raw data by Trim-galore with default parameters. Then remaining trimmed sequence were mapped against the reference genome (mm9) with Bowtie/TopHat version 2, which allowed mapping across splicing junctions by read segmentation. All programs were performed with default settings unless otherwise specified. Unique mapped reads were subsequently assembled into transcripts guided by reference Refseq annotation with Cufflinks. The expression level of each gene was quantified with normalized FPKM with FPKM_count in RSeQC software. Differentially expressed genes were identified by asking for a Log2 (Fold change) >=1 or <=-1 with FPKM > 0.3 in at least one sample.

GO analysis. GO analysis for differentially expressed genes were performed on the annotation, visualization and integrated discovery (DAVID) website.
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Conflict of interest

The authors declare no competing financial interests.

Author contributions

M.-H.T. conceived the project, and with F.L. designed the project and data analysis. M.-H. T. and F.L. wrote the manuscript with contributions from all authors. X.Z. conducted phenotype analysis, and isolated spermatogonial cells for all experiments in the project. B.R. conducted ChIP-seq experiments and analysis. L.L. established the Setd2 floxed mouse model. R.L. assisted in data analysis.

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Figure 1. Setd2 expression and H3K36me3 distribution during spermatogenesis. (A) Immunofluorescent staining for Setd2 (Green), rhodamine-labeled peanut lectin (PNA, a marker for acrosome; red), and DAPI (Blue) in sections of adult mouse testes. Setd2 immunoreactivity was mostly seen in the nucleus of pachytene spermatocytes and round spermatids. (B) Immunofluorescent detection for H3K36me3 (Green), rhodamine-labeled PNA (Red), and DAPI (Blue) in sections of adult mouse testes. H3K36me3 staining was detected in the nucleus of all stage of spermatocytes and round as well as elongating spermatids. (Scale bar: 40µm)
Figure 2. Impaired spermiogenesis in Setd2 cKO mice. (A) Gross morphology of representative testes from an 8-week-old control and age-matched Setd2 cKO mice. (B) Comparison of testis weight from 8-week-old controls and Setd2 cKO mice. Data are presented as mean ± SD (n = 8 for each genotype). (C) H&E staining of testis and epididymis from control and Setd2 cKO adult mice. Red arrows indicate apoptotic spermatocytes and spermatids, whereas black arrows represent multinucleated giant cells (formed by arrested spermatids). (D) TUNEL staining of representative testes from adult control and age-matched Setd2 cKO mice. Apoptotic cells were labeled by TUNEL staining (Green). The stars (*) indicate the spermatocyte and spermatid layer. (E) Comparison of TUNEL positive seminiferous tubules from adult controls and Setd2 cKO mice. Data are presented as mean ± SD (n = 3 for each genotype). Error bars represent SD. **P < 0.01, t-test. (Scale bars: 40 µm)
Figure 3. Abnormal acrosome biogenesis in Setd2 cKO mouse spermatids. Histochemical staining of FITC-conjugated PNA (Green) and DAPI (Blue) in sections of adult control (A) and Setd2 cKO (B) mouse testes. The Golgi, cap, and acrosome phase of spermiogenesis are shown in order. (A) PNA staining of representative testes from adult controls. Acrosomes are labeled by PNA (Green). Arrow indicates the representative acrosome. In the Golgi phase, acrosome displays a single granule close to the nucleus. In the cap phase, acrosome grows to form the cap structure covering the nucleus. In the acrosome phase, acrosome forms the moon-shaped structure covering the nucleus. (B) PNA staining of representative testes from adult Setd2 cKO mice. There are multiple PNA-positive structures (as indicated by the arrows) in Setd2 cKO mouse spermatids throughout spermiogenesis. Right panels show the higher-magnification views of the framed areas in the left panels. (Scale bars: 40 µm)
Figure 4. Abnormal acrosomal morphology of Setd2 cKO mouse spermatids. TEM analyses in ultrathin sections of spermatids from adult control (A and C) and Setd2 cKO (B and D) mice. (A) In the Golgi phase of control spermatids, a single acrosomal granule is observed attached to the nuclear envelope. (B) In the Golgi phase of Setd2 cKO mouse spermatids, as indicated by the arrows, multiple acrosomal vesicular structures are seen. (C) In the cap phase, acrosome flattens and forms a cap covering one pole of the nucleus. (D) In the cap phase of Setd2 cKO mouse spermatids, multiple acrosomal vesicular structures are observed.
Figure 5. Setd2 inactivation led to global loss of H3K36me3 in pachytene spermatocytes and round spermatids. (A) Table summarizes the numbers of H3K36me3 ChIP-seq peaks and H3K36me3 covered genes in PD and RS cells. (B) Genomic distribution of H3K36me3 ChIP-seq peaks in PD and RS cells, compared to genome background. **** $P < 0.0001$, One-sided binomial test. (C) Global loss of H3K36me3 in both PD and RS cells from Setd2 cKO mice. Left, UCSC track showing H3K36me3 ChIP-seq signals at a select genomic region in control and Setd2 cKO mice. Right, numbers of H3K36me3 ChIP-seq peaks in PD and RS cells from control and Setd2 cKO. (D) Global loss of H3K36me3 in Setd2 cKO mice was confirmed by ChIP-qPCR. q-PCR Data are represented as mean ± SD from two biological replicates. * $P < 0.05$; ** $P < 0.01$, t-test.
Figure 6. Compromised expression of key spermiogenesis regulators and genes involved in histone-to-protamine replacement in PD and RS upon Setd2 loss. (A) Hierarchical clustering analyses of H3K36me3 ChIP-seq and RNA-seq of the differentially expressed genes in PD and RS cells from the control and Setd2 cKO. (B) Signal plot showing the H3K36me3 distribution over the up- and down-regulated genes in PD ($P = 1.54e^{-92}$, ANOVA-test) and RS ($P = 1.23e^{-34}$, ANOVA-test) cells upon Setd2 loss. (C) Box plot analyses of the expression levels of the up- and down-regulated genes in PD and RS cells upon Setd2 loss. **** $P < 0.0001$ by Unpaired t test. (D and E) Expression analyses of the indicate genes in PD and RS cells upon Setd2 loss. (D) Snapshots showing RNA-seq results, and (E) RT-qPCR confirmation, data are represented as mean ± SD from two biological replicates. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ by t-test. (F) Gene set enrichment analyses (GSEA) of histone gene expression in PD and RS cells from Setd2 cKO versus control. Normalized enrichment score (NES) and nominal p-value were provided according to GSEA.
The histone methyltransferase Setd2 is required for expression of acrosin-binding protein 1 and protamines and essential for spermiogenesis in mice
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