Histone methyltransferase DOT1L is essential for self-renewal of germline stem cells

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Self-renewal of spermatogonial stem cells is vital to lifelong production of male gametes and thus fertility. However, the underlying mechanisms remain enigmatic. Here, we show that DOT1L, the sole H3K79 methyltransferase, is required for spermatogonial stem cell self-renewal. Mice lacking DOT1L fail to maintain spermatogonial stem cells, characterized by a sequential loss of germ cells from spermatogonia to spermatids and ultimately a Sertoli cell only syndrome. Inhibition of DOT1L reduces the stem cell activity after transplantation. DOT1L promotes expression of the fate-determining HoxC transcription factors in spermatogonial stem cells. Furthermore, H3K79me2 accumulates at HoxC9 and HoxC10 genes. Our findings identify an essential function for DOT1L in adult stem cells and provide an epigenetic paradigm for regulation of spermatogonial stem cells.

[Keywords: DOT1L, epigenetics, adult stem cell, self-renewal, spermatogonial stem cell, transplantation, histone methyltransferase]

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renewal have been identified through genetic studies. PLZF (also known as ZBTB16), a transcription factor, was the first identified germ cell-intrinsic SSC self-renewal factor [Buaas et al. 2004; Costoya et al. 2004]. Historically, a spontaneous mutation in Plzf called luxoid was first reported in 1955 and exhibited pleiotropic defects in fertility, limb development, and skeleton development [Green 1955]. Subsequent studies revealed that PLZF is expressed in spermatogonial progenitor cells and that Plzf mutant males exhibit a progressive loss of germ cells with age, demonstrating its role in SSC self-renewal. Mechanistically, PLZF binds to the promoters of thousands of genes and promotes SSC self-renewal through opposing the activity of mTORC1, a critical mediator of cell growth [Hobbs et al. 2010; Lovelace et al. 2016]. NANOS2, an RNA-binding protein, and RB, the retinoblastoma tumor suppressor protein, are expressed in spermatogonia. Conditional deletion studies have shown that NANOS2 and RB are required for self-renewal of spermatogonial stem cells [Sada et al. 2009; Hu et al. 2013].

GDNF, ETV5, and CARF are expressed in Sertoli cells, the supporting somatic cells in the testis, and thus constitute SSC niche factors [Meng et al. 2000; Chen et al. 2005; Cui et al. 2020]. GDNF, a cytokine, was identified as a key SSC niche factor, since mice heterozygous for the Gdnf deletion allele showed a depletion of spermatogonia, and mice overexpressing Gdnf had increased accumulation of undifferentiated spermatogonia in testis [Meng et al. 2000]. GDNF stimulates SSC self-renewal through the cell surface coreceptors RET and GFRα1. Notably, the identification of GDNF as a Sertoli cell-secreted niche factor has enabled the in vitro culture of SSCs. When combined with bFGF, GDNF preserves stem cell activity and allows SSC expansion in vitro [Kanatsu-Shinohara et al. 2004; Kubota et al. 2004; Seandel et al. 2007]. Bcl6b is a GDNF-responsive gene that functions in SSC renewal [Oatley et al. 2006]. Mice deficient for the transcription factor ETV5 (previously known as ERM), which is expressed in Sertoli cells, exhibit an age-dependent depletion of undifferentiated spermatogonia [Chen et al. 2005]. Interestingly, ETV5 is also expressed in SSCs in response to GDNF [Morrow et al. 2007; Oatley et al. 2007; Schmidt et al. 2009]. Finally, loss of CARF [also known as CDKN2AIP] in Sertoli cells or spermatogonia leads to defects in SSC proliferation, which can be rescued by reactivation of the Wnt signaling pathway [Cui et al. 2020]. Despite the identification of these key SSC self-renewal factors, the mechanistic basis of SSC self-renewal remains unclear. Moreover, PLZF does not appear to be regulated by GDNF, suggesting that additional unidentified factors remain and that their discovery will be critical for unraveling the mechanisms of SSC self-renewal.

DOT1L [disruptor of telomere silencing 1-like] is an evolutionarily conserved H3K79 methyltransferase that catalyzes H3K79 monomethylation, dimethylation, and trimethylation using SAM [S-adenosylmethionine] as the methyl donor [van Leeuwen et al. 2002]. In general, H3K79 methylations are associated with active transcription [Vlaming and van Leeuwen 2016; Wood et al. 2018]. Dot1, the yeast ortholog of DOT1L, is essential for telomere silencing, meiotic checkpoint control, and the DNA damage response [San-Segundo and Roeder 2000]. Mouse DOT1L is essential for embryonic development, since Dot1l-null embryos die by E11.5 [Jones et al. 2008; Feng et al. 2010; Liao and Szabó 2020]. Lineage-specific deletion studies show that DOT1L regulates neurogenesis and hematopoiesis by promoting proliferation of cerebral and hematopoietic progenitor cells [Jo et al. 2011; Franz et al. 2019]. In MLL-rearranged leukemias, MLL1 translo
cation partners such as AF10 recruit DOT1L to cause aberrant H3K79 methylation and leukemogenesis [Okada et al. 2005; Deshpande et al. 2014]. EPZ5676 is a potent DOT1L inhibitor and shows promising results in clinical trials for leukemia treatment [Sarno et al. 2020]. Therefore, most mammalian DOT1L studies have focused on its role in leukemia. Due to the early embryonic lethality of the global Dot1l deletion, its role in male germ cell development has not been examined. However, maternal DOT1L is dispensable for embryonic development in mice [Liao and Szabó 2020]. Here we unexpectedly discovered that DOT1L is a regulator of mouse SSC self-renewal. Mechanistically, DOT1L activates the transcription of the HoxC homeobox gene cluster in SSCs.

Results

DOT1L-dependent self-renewal of spermatogonial stem cells

DOT1L is broadly expressed and its global loss leads to embryonic lethality [Jones et al. 2008; Feng et al. 2010]. To investigate its role in mammalian spermatogenesis, we generated Dot1l<sup>fl/fl</sup>- Ddx4-Cre mice (referred to as Dot1l<sup>cko</sup>) [Bernt et al. 2011]. Ddx4-Cre is specifically expressed in germ cells beginning at embryonic day 15 and continuing after birth [Gallardo et al. 2007]. The testis from 6-mo-old Dot1l<sup>cko</sup> mice was dramatically smaller [Fig. 1A]. However, the testis/body weight ratio of Dot1l<sup>cko</sup> males was comparable with that of control males at postnatal day 8 [P8], P18, and P25, but sharply decreased at day 40 and beyond [Fig. 1B]. Dot1l<sup>cko</sup> males failed to sire offspring, as the sperm count of adult Dot1l<sup>cko</sup> males was extremely low or zero [Fig. 1C]. Together, these results show that DOT1L is essential for germ cell development in adult males.

To investigate the cause of sperm production failure, we examined the first wave of spermatogenesis in juvenile testes. Embryonic gonocytes become spermatogonia after birth, a subset of which were thought to become spermatogonial stem cells [SSCs] [de Rooij 1998; Nakagawa et al. 2007; Law et al. 2019]. The remaining spermatogonial proliferate, differentiate, enter meiosis, and initiate production of the first wave of sperm by day 35 in mice. Therefore, the first [juvenile] wave of spermatogenesis is SSC-independent. Notably, the testis weight was comparable between Dot1l<sup>cko</sup> and control pups up to P25 [Fig. 1B]. Histological analysis of Dot1l<sup>cko</sup> testes showed the presence of spermatogonia [meiotic] at P8, spermatocytes [meiotic] at P18, and elongated spermatids [haploid] at P40 [Fig. 1D], at a time line expected in wild type. H3K79me2
was present in both germ cells and somatic cells in the control (Dot1l^fl/+; Ddx4-Cre) testis (Supplemental Fig. S1). As expected, H3K79me2 was lost in germ cells but still present in somatic cells, including Sertoli cells, in all tubules from the Dot1l^fKO testis (Supplemental Fig. S1). Together, these results demonstrate that the first wave of spermatogenesis occurs normally in Dot1l^fKO males and that DOT1L is not required for survival of spermatogonia or for progression of meiosis.

We next closely examined 40-d-old testes, which contain adult waves of spermatogenesis (Fig. 1E). Adult waves of spermatogenesis are SSC-dependent: SSCs divide to preserve the stem cell population (self-renewal) and also produce spermatogonia that differentiate to initiate spermatogenesis. Scale bar, 10 µm. [Asterisk] Loss of germ cells, [SG] spermatogonia, [SC] spermatocyte, [RS] round spermatid, [ES] elongated spermatid.
overlapping waves of spermatogenesis. The control seminiferous tubule consists of a complete cohort of spermatogonia, spermatocytes, round spermatids, and/or elongated spermatids [Fig. 1E, left panel]. However, Dot1lKO testes showed a variety of abnormal tubules with a loss of one or more types of germ cells. Dot1lKO tubules showed a sequential loss of spermatogonia, spermatocytes, round spermatids, and finally elongated spermatids [Fig. 1E,F]. The progressive germ cell loss phenotype beginning with spermatogonia is characteristic of a failure in SSC self-renewal, as observed in Gdnf, ETV5, Zbtb16, Rb, and Nanos2 mutant testes [Meng et al. 2000; Buaas et al. 2004; Costoya et al. 2004; Chen et al. 2005; Sada et al. 2005]. Absence of SALL4+ (a marker of spermatogonia) cells in P40 Dot1lKO testes, resulting in a Sertoli cell only syndrome (Fig. 1D). At 6 mo of age, all germ cells were lost in Dot1lKO testes, adult mutant testes, which still contain a significant fraction of tubules with germ cells even at 8 mo [Buaas et al. 2004]. Absence of SALL4+ (a marker of spermatogonia) cells in P40 Dot1lKO testes revealed a loss of spermatogonia in all tubules [Supplemental Fig. S2A,B; Hobbs et al. 2012, Gassei and Orwig 2013]. Moreover, the number of SALL4+ spermatogonia was comparable between control and Dot1lKO testes at P8, but significantly lower in Dot1lKO testes at P18 and P25, providing further support for a defect in the SSC renewal [Supplemental Fig. S2C,D].

Requirement of DOT1L in SSC self-renewal in adulthood

We next investigated the requirement of DOT1L in adult SSC self-renewal through tamoxifen-induced inactivation of Dot1l in 8-wk-old Dot1lfl/fl Ddx4-CreERT2 mice (referred to as Dot1lKO) [Fig. 2A]. At 35 d posttamoxifen treatment (35 dpt), Dot1lKO males displayed a reduction in testis size and sperm count (Fig. 2B–D). Similar to P40 Dot1lKO testes, adult Dot1lKO testes at 35 dpt exhibited a sequential loss of germ cells, starting from early germ cells [spermatogonia] to more differentiated germ cells [elongated spermatids] [Fig. 2E–G]. The number of SALL4+ spermatogonia was sharply reduced in Dot1lKO testes at 35 dpt [Supplemental Fig. S3A,B,D].

We looked earlier to determine how quickly the phenotype was observable. While Dot1lKO testes at 9 dpt showed no obvious histological defects, they already displayed a decrease in the number of SALL4+ spermatogonia [Supplemental Fig. S3B,C]. The proliferation rate of SALL4+ spermatogonia was lower in 35-dpt Dot1lKO testes but was not statistically significant [Supplemental Fig. S3E,F]. The number of TUNEL-positive germ cells was increased in 35-dpt Dot1lKO testes [Supplemental Fig. S3G,H]. These results further support a requirement for DOT1L in SSC self-renewal in adult mice.

We examined the distribution of DOT1L and H3K79me2 in control and Dot1lKO testes at 35 dpt by immunofluorescence. In the control testis, the DOT1L signal was high in round spermatids but was present at low levels in other germ cells and somatic cells. In the Dot1lKO testis, DOT1L was depleted in germ cells in ~75% of tubules at 35 dpt [Supplemental Fig. S4A]. As expected, H3K79me2 was absent in germ cells but still present in somatic cells, including Sertoli cells, in 50% of the tubules from Dot1lKO testis [Supplemental Fig. S4B]. The reduction of DOT1L and H3K79me2 in the Dot1lKO testis was confirmed by Western blotting analyses [Supplemental Fig. S4C–E].

DOT1L inhibition reduces SSC stemness after transplantation

To further examine the role of DOT1L in SSC self-renewal, we treated SSCs with EPZ5676, a potent inhibitor of the DOT1L methyltransferase [Sarno et al. 2020], followed by SSC transplantation into testis. Mouse SSCs were derived from ROSA mice that express a LacZ reporter gene. SSCs, which form clumps in culture, were treated with EPZ5676 or DMSO as a vehicle control. The number of spermatogonia was comparable between the two groups at day 7 of culture, but at day 14 the number of spermatogonia in EPZ5676-treated cultures was 27% less than in DMSO cultures [Fig. 3A], suggesting that EPZ5676 reduces SSC proliferation and/or self-renewal. As expected, EPZ5676 treatment of SSCs reduced the abundance of H3K79me2 [Fig. 3B]. After culturing with EPZ5676 or DMSO for 2 wk, clump-forming cells were transplanted into infertile mice to determine the stem cell activity [Brinster and Zimmermann 1994; Kubota and Brinster 2008]. Two months after transplantation, the testes were stained with X-gal to quantify the number of LacZ-expressing SSCs in donor cell suspensions [Fig. 3C]. EPZ5676 treatment of SSCs reduced the stem cell activity by 50% [Fig. 3D]. These transplantation results further support a critical role for DOT1L in SSC self-renewal.

DOT1L promotes the expression of the HoxC and Dlx3/4 transcription factors in SSCs

DOT1L catalyzes H3K79 methylation, and inactivation of DOT1L leads to a global loss of H3K79 methylation [Jones et al. 2008]. H3K79 methylation marks are associated with active transcription [Vlaming and van Leeuwen 2016; Wood et al. 2018], suggesting that DOT1L may control SSC self-renewal through modulating transcription. To identify DOT1L-dependent genes in SSCs, we established SSC lines from DBA2 mice [Supplemental Fig. S5A; Kubota and Brinster 2008; Kanatsu-Shinohara et al. 2014]. As expected, the SSCs expressed SALL4 and LIN28A, markers of undifferentiated spermatogonia [Supplemental Fig. S5B; West et al. 2009; Zheng et al. 2009; Hobbs et al. 2012]. RNA-seq analysis was performed on SSCs collected at day 14 of inhibitor or mock treatment. With the FDR (false discovery rate) cutoff of 5%, we identified 38 differentially expressed (DE) genes in EPZ5676-treated SSCs: 22 up-regulated and 16 down-regulated [Fig. 4A; Supplemental Table S1]. Gene ontology (GO) analysis of up-regulated DE genes did not reveal enrichment in any biological process or molecular function [Supplemental Fig. S5C]. Strikingly, GO analysis of down-
Figure 2. Inducible deletion of Dot1l causes a failure in SSC self-renewal in adult mice. (A) Scheme of tamoxifen treatment in adult male mice. (B) Image of adult testes at 35 dpt. (Dot1l^{fl/fl}{Ddx4-CreERT2}) (C) Testis/body weight ratio of control and Dot1l^{iKO} males. (**) P < 0.01, Student’s t-test. (D) Epididymal sperm count of control and Dot1l^{iKO} males. (*) P < 0.05, Student’s t-test. Control mice: Dot1l^{fl/+}, Dot1l^{fl/fl}, or Dot1l^{fl/+}{Ddx4-CreERT2}. (E) Histology of control and Dot1l^{iKO} testes at 35 dpt. Scale bar, 50 µm. Types of tubules in Dot1l^{iKO} testes: type 1, apparently normal; type 2, presence of Sertoli cells and round and elongated spermatids; and type 3, presence of Sertoli cells and elongated spermatids. (F) Progressive depletion of germ cells begins with spermatogonia in Dot1l^{iKO} testes at 35 dpt. The cartoon below depicts the germ cell composition of each tubule shown above. Scale bar, 50 µm. (G) Percentage of each type of seminiferous tubules in control and Dot1l^{iKO} testes at 35 dpt. Statistics: (*) P < 0.05, (**) P < 0.001, Student’s t-test or Mann–Whitney U-test.
regulated DE genes revealed enrichment in “anterior/posterior pattern specification” and “sequence-specific DNA binding” [Fig. 4B], because nine out of 16 down-regulated DE genes encoded homeobox (Hox) transcription factors [Fig. 4C]. Seven down-regulated genes belonged to the Hoxc gene cluster on chromosome 15: Hoxc4, Hoxc6, Hoxc8, Hoxc9, Hoxc10, Hoxc11, and Hoxc12, and two were Dlx genes (distal-less homeobox): Dlx3 and Dlx4 [Fig. 4C], which are adjacent genes on chromosome 11. Notably, Hotair, a lncRNA gene located between Hoxc11 and Hoxc12, was also down-regulated in EPZ5676-treated SSCs. The Hox family of transcription factors specifies segmental identity along the body axis in animals ranging from flies to humans [Mallo and Alonso 2013]. As such, mutations in a particular Hox gene lead to homeotic transformation—fate switch of specific body segments. In mice, there are four Hox gene clusters located on four separate chromosomes: Hoxa, Hoxb, Hoxc, and Hoxd [Supplemental Fig. S5D]. Notably, none of the members in the other three clusters [Hoxa, Hoxb, and Hoxd] were significantly down-regulated in EPZ5676-treated SSCs, indicating that DOT1L is specifically required for expression of the Hoxc cluster in SSCs [Supplemental Fig. S5D]. Strikingly, Hoxc4 was identified as a GDNF-responsive gene in rat SSCs, and RNAi knockdown of Hoxc4 in vitro reduced the stem cell activity of rat SSCs in culture, as shown by transplantation [Schmidt et al. 2009]. Differential expression of the Hox genes in EPZ5676-treated SSCs was confirmed by qRT-PCR [Supplemental Fig. S5E].

We derived Dot1l−/− and Dot1l−/− SSC lines [Supplemental Fig. S6A]. Because Dot1l is essential for SSC self-renewal, the ability of Dot1l−/− spermatogonia to proliferate in culture seemed to be counterintuitive. As a precedent, Plzf−/− SSCs were successfully derived and maintained in long-term cultures [Hobbs et al. 2010]. Western blot analyses confirmed the depletion of the DOT1L protein and H3K79me2 in Dot1l−/− SSCs [Supplemental Fig. S6B–D]. By qRT-PCR analysis, we found that five Hoxc genes [Hoxc8, Hoxc9, Hoxc10, Hoxc11, and Hoxc12] were significantly down-regulated in Dot1l−/− SSC lines [Supplemental Fig. S6E], which was consistent with the RNA-seq results from EPZ5676-treated SSCs. Therefore, our RNA-seq analysis shows that DOT1L is required for transcription of the Hoxc genes in SSCs and thus implicates the Hoxc transcription factors in the regulation of SSC self-renewal.

**Genome-wide distribution of H3K79me2 in mouse SSCs**

We determined the distribution of H3K79me2 in SSCs treated with or without EPZ5676 by chromatin immunoprecipitation and next-generation sequencing [ChIP-seq]. Consistent with prior reports [Vlaming and van Leeuwen 2016; Wood et al. 2018], we found that H3K79me2 accumulated significantly in the promoter regions or gene bodies with the highest levels immediately downstream from the transcription start sites (TSSs) [Fig. 4D, Supplemental Fig. S7A]. Consistently, more than half of the H3K79me2 ChIP-seq reads mapped to the promoters, the first exons,
and the first introns (Fig. 4E). As expected, the H3K79me2 signal was reduced substantially in EPZ5676-treated SSCs (Fig. 4D; Supplemental Fig. S7B). Six of 16 down-regulated genes such as Dlx3, Hoxc9, Hoxc10, Icam1, and Slc35f2 were occupied by H3K79me2, showing that they are DOT1L direct target genes (Fig. 4F; Supplemental Table S2). In contrast, all 22 up-regulated genes were devoid of H3K79me2 (Fig. 4F; Supplemental Table S2). While thousands of genes were associated with H3K79me2 in SSCs, the DOT1L inhibition only led to a small number of differentially expressed genes in SSCs. This discrepancy was also observed in other cells such as hematopoietic cells and neuronal progenitor cells (Bernt et al. 2011; Franz et al. 2019) and is in fact a hallmark of most epigenetic marks (Lenstra et al. 2011).

Discussion

DOT1L is the sole methyltransferase for H3K79 and is essential for development and thus viability (Jones et al. 2008). Our genetic and transplantation results demonstrate that DOT1L is essential for SSC self-renewal. Several stem cell-intrinsic factors, including RB, NANOS2, PLZF, ID4, and BCL6B, regulate SSC self-renewal. While depletion of RB or NANOS2 leads to a rapid loss of spermatogonial stem cells [Sada et al. 2009; Hu et al. 2013], mice lacking PLZF, ID4, or BCL6B exhibit milder defects in SSC self-renewal [Buaas et al. 2004; Costoya et al. 2004; Oatley et al. 2006, 2011]. The defining characteristics in SSC self-renewal mouse mutants are a normal first wave of spermatogenesis and a sequential loss of germ cells beginning with spermatogonia in later waves of spermatogenesis. Notably, DMRT1 and PRAMEF12 are essential for the maintenance of spermatogonia (Takashima et al. 2013; Zhang et al. 2016; Wang et al. 2019). For factors essential for the survival of spermatogonia such as DMRT1 and PRAMEF12, it would be challenging to determine whether they also play a role in SSC self-renewal. Here we found that inactivation of DOT1L, like loss of RB, results in a dramatic defect in SSC self-renewal, showing that DOT1L is an essential germ cell-intrinsic regulator of SSC self-renewal. None of the known SSC self-renewal factors were down-regulated in EPZ5676-treated SSCs, suggesting that DOT1L may function in SSC self-renewal in parallel pathways.
DOT1L catalyzes H3K79 methylation, an epigenetic mark associated with actively transcribed genes. DOT1L interacts directly with the phosphorylated C-terminal domain of RNA polymerase II, which leads to its recruitment to transcribed genes [Kim et al. 2012]. Our RNA-seq analysis revealed that seven HoxC cluster genes and two Dlx [homeobox] genes were down-regulated in EPZ5676-treated SSCs: Hoxc4, Hoxc6, Hoxc8, Hoxc9, Hoxc10, Hoxc11, Hoxc12, Dlx3, and Dlx4. However, no genes from the other three Hox clusters (Hoxa, Hoxb, and Hoxd) were down-regulated, suggesting that Hoxc genes are specifically targeted in SSCs by DOT1L. In addition, Hoxc9, Hoxc10, Dlx3, and Dlx4 are marked by H3K79me2 [Fig. 4F; Supplemental Table S2]. Six down-regulated Hox genes have been disrupted in mice [Supplemental Table S3]. These mutants show homeotic transformation phenotypes—skeletal defects, as expected for Hox genes. All of the viable knockout mice were reported to be fertile, but fertility was not rigorously assessed, as the focus of these studies was on the role of Hox genes in skeletal development. Therefore, SSC self-renewal defects may have been missed. However, Hoxc4 was identified as a GDNF-regulated gene in rat SSCs and was shown to be important for rat SSC self-renewal by siRNA knockdown and transplantation [Schmidt et al. 2009]. In addition to the traditional homeotic skeletal function, Hox genes have important nontraditional roles. For example, HoxA9–13 are required for DOT1L-mediated malignant transformation of myeloid progenitor cells [Okada et al. 2005; Bernt et al. 2011; Deshpande et al. 2014]. Therefore, future genetic studies are warranted to examine the role of the HoxC gene cluster and Dlx3/4 genes individually or collectively in SSC self-renewal.

Somatic cells can be reprogrammed into induced pluripotent stem [iPS] cells by defined factors [Takahashi and Yamanaka 2006]. DOT1L functions as a barrier to somatic reprogramming into iPS cells. Specifically, inhibition of DOT1L enhances generation of iPS cells from somatic cells [Onder et al. 2012]. This is also consistent with the dispensable role of DOT1L in ES cell self-renewal (Cao et al. 2020). In contrast, here we found that DOT1L is essential for self-renewal in SSCs, raising the possibility that DOT1L could also play a critical role in self-renewal of other adult stem cells. ES cells and iPS cells can be induced into primordial germ cell-like cells [PGCLCs] [Hayashi et al. 2011]. To date, direct reprogramming of somatic cells into SSCs has not been achieved. Given its essential role in SSC self-renewal, DOT1L is expected to be required for reprogramming of somatic cells into SSCs in mammals, including humans.

**Materials and methods**

*Generation of Dot1l conditional knockout mice*

Dot1l floxed [Dot1fl] mice were generated previously [Bernt et al. 2011]. To inactivate Dot1l specifically in germ cells, Dot1l floxed mice were crossed with two Cre mouse strains: Ddx4-Cre [Jackson Laboratory 006954] and Ddx4-CreERT2 [Jackson Laboratory 024760] [Gallardo et al. 2007; John et al. 2008]. For inducible deletion, tamoxifen [Sigma T5648] dissolved in corn oil [Sigma C8267] at a concentration of 20 mg/mL was intraperitoneally injected into 8- to 9-week-old Dot1flfl Ddx4-CreERT2 males [2 mg/30 g body weight] once per day for five consecutive days. Dot1flfl, Ddx4-CreERT2, Dot1fl+/-, or Dot1flfl males injected with tamoxifen were used as controls. A toxic effect of EGFP-Cre in cultured SSCs was previously reported [Kanatsu-Shinohara et al. 2008]. However, adult Dot1flfl Ddx4-CreERT2 males treated with tamoxifen as described in this study showed no defects in SSC self-renewal or spermatogenesis. Testes were harvested for analysis at 9 or 35 d posttamoxifen treatment [dpt]. PCR genotyping primers were as follows: the Dot1 l floxed allele [418 bp]: p2 [5′-GCCAAAAGGCTTTTCAACA-3′] and p3 [5′-ATGGGAATCTCATGGAACCAA-3′]; the Dot1 l deletion allele [620 bp]: p1 [5′-CTCACAGTCACATCCTCCTGAC-3′] and p3 [5′-ATGGGATTTCCATGGGAACCAA-3′]; Ddx4-Cre [240 bp]: VasaCre-1 [5′-CAGGGCAGCTTTTAAAGCGCGGT-3′] and VasaCre-2 [5′-TTTCATTCTAACAACACCTCAGAA-3′]; and Ddx4-CreERT2 [205 bp]: KI-1 [5′-ATACCCGAGATCATGGAACGCCACG-3′] and KI-2 [5′-GCCACAGCTTGTTCCTTCTTGA-3′]. The University Laboratory Animal Resources (ULAR) at University of Pennsylvania provided veterinary care and husbandry for all of the mice, and the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania approved all procedures.

*SSC culture and transplantation*

Mouse SSC cultures were established as described previously [Kubota et al. 2004] with some modifications [Kubota and Kakiuchi 2020]. In brief, testicular cells were prepared from 7-d-old B6.129S7-Gt(ROSA)26SorJ [Jackson Laboratory] that expressed a LacZ [β-galactosidase] reporter gene in all tissues by enzymatic digestion using trypsin-EDTA [Thermo Fisher Scientific]. Germ cells were cultured on SNL 76/7 feeders (from Allan Bradley, The Welcome Trust Sanger Institute) in a serum-free medium with 10 ng/mL GDNF [R&D Systems] and 0.5 ng/mL FGF2 [Corning] [Kubota and Kakiuchi 2020]. After 2 mo in culture, 1 × 10^5 clump-forming cells were seeded on SNL feeder cells in 24-well plates with 2 µM EPZ5676 [Cayman Chemical] or DMSO [Sigma-Aldrich] as a vehicle control. Medium was changed every 2–3 d, and the cells were subcultured every 7 d. After culturing with EPZ5676 or DMSO for 2 wk, clump-forming cells were harvested using trypsin-EDTA and transplanted into recipient testes. For gene expression analysis, clumps were collected by gentle pipetting, and total RNA was extracted with TRZolol [Thermo Fisher Scientific] followed by RNaseasy column purification [Qiagen]. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cultured SSCs were transplanted into recipient C57BL/6 mouse testes via efferent duct injection as described [Kubota and Kakiuchi 2020]. Recipient mice were treated with 44 mg/kg busulfan [Sigma-Aldrich] at least 6 wk prior to transplantation. Eight microliters of 8.3 × 10^5 cells/mL was transplanted into each testis. Two months after transplantation, the testes were stained with X-gal to quantify the number of SSCs in donor cell suspensions. Spermatogenic colonies consisting of X-gal-stained blue cells were counted and normalized to 1 × 10^5 cells transplanted into the recipient testes.

*SSC culture for RNA-seq*

Gelatin selection was used for enrichment of spermatogonia and removal of testicular somatic cells as described previously [Kanatsu-Shinohara et al. 2014]. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For
Western blotting and mRNA sequencing, SSCs were cultured on γ-irradiated MEFs prepared from E13.5 DR4 mouse embryos. Culture medium was based on IMDM (Sigma I3390) supplemented with 1% 10 μL/mL FBS [HyClone SH30396.03], 5 mg/mL bovine serum albumin [MP Biomedicals 810661], 15 ng/mL rat GDNF [Peprotech 450-51], 10 ng/mL human FGF2 [Peprotech 100-18B], and other nutrients [Kanatsu-Shinohara et al. 2014]. In brief [Supplemental Fig. SSA], one pair of testes from postnatal day 5–8 DBA/2) pups [Jackson Laboratory 000671] was harvested and dissociated with 1 mg/mL collagenase type IV (Sigma C5138) for 10 min, followed by digestion with 0.25% trypsin-EDTA (Gibco 25200-056) for 10 min. Single-cell suspension was plated on one 60-mm cell culture dish precoated with 0.1% gelatin and incubated overnight. Nonattached spermatogonia in the media were then transferred to MEF-coated plates. Medium was changed every 2–3 d. After 7–14 d of culture, SSCs formed clumps [Supplemental Fig. SSBl and were subcultured at a ratio of 1:3 to 1:6 every 5–7 d. To collect cells for RNA-seq and Western blotting, cell suspensions after digestion were plated on the 0.1% gelatin-coated cell culture dishes for 2 h to remove the adherent feeder cells.

RNA-seq analysis of SSCs

SSCs from two pups were established in culture and treated with EPZ5676 or solvent (triplicates per treatment) for 14 d as described above. Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific 15596026). Four micrograms of total RNA from each sample was used. Poly[A]⁺ mRNA was isolated with oligo(dT) magnetic beads and converted into cDNA to generate RNA-seq libraries using TruSeq stranded mRNA library preparation kit set A (Illumina RS-122-2101) by following the manufacturer’s instructions. Six individual libraries [three EPZ5676-treated and three control samples] were pooled in equal amounts. Libraries were first test-sequenced on an Illumina MiSeq system (paired-end sequencing, 150 cycles, ~1 million reads per library) and then on an Illumina NovaSeq 6000 system [single-end sequencing, 100 cycles, ~25 million reads per sample] at the University of Pennsylvania Next-Generation Sequencing Core.

We mapped sequenced reads to the mouse [mm10] reference genome in the unique mapping mode. Uniquely mapped reads were used to evaluate expression of protein-coding genes. STAR was used for alignment with the default parameters. The annotation of genes was acquired from the UCSC ReSeq annotation. FeatureCount was used to determine the number of reads mapping to genes. Differential gene expression was called by using EdgeR with the FDR cutoff of 5%. The bulk RNA-seq data are available in GEO under accession number GSE181590.

qRT–PCR validation

The expression of nine down-regulated, two up-regulated, and nine nondifferentially expressed genes determined by RNA-seq was analyzed by quantitative real-time reverse transcription PCR (qRT–PCR). The same RNA samples for RNA sequencing and different RNA samples extracted from another four samples [two EPZ5676-treated and two ethanol-treated] were used. qRT–PCR primers are listed in Supplemental Table S4. Each reaction [in a final volume of 10 μL] consisted of 5 μL of 2× SYBR Green Master mix, 0.25 μL of each primer at 10 μM, 0.5 μL of cDNA, and 4.0 μL of H₂O, except for Hoxc genes. For Hoxc genes, each reaction [in a final volume of 10 μL] consisted of 5 μL of 2× SYBR Green Master mix, 0.1 μL of each primer at 10 μM, 0.5 μL of cDNA, and 4.3 μL of H₂O. Each sample was assayed in triplicates. The expression level was normalized to Gapdh using the 2−ΔΔCT method.

Chromatin immunoprecipitation and sequencing (ChIP-seq)

Mouse SSCs were cultured in the presence or absence of EPZ5676 for 7 d. SSCs were resuspended at 1 million/mL in PBS and cross-linked with formaldehyde [Inser] at a final concentration of 1% for 10 min at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M for 5 min at room temperature. All of the following buffers were supplemented with protease inhibitors [Complete Mini, Roche, Halt, or Thermo Fisher Scientific]. Cells were washed twice in ice-cold PBS and lysed in ChIP lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl at pH 8.1]. Lysate of the equivalent of 1 million cells was transferred into microTUBE AFA fiber preslit snap cap tubes [Covaris PN 520045] and sonicated with a Covaris ME220 using the following settings: 100-sec duration, 75–W peak power, 20% duty factor, and 1000 cycles per burst. The resulting lysate was spun in a tabletop centrifuge at full speed for 10 min at 10°C. One percent of the supernatant was set aside as input control. The supernatant was diluted 1:10 in ChIP dilution buffer [0.01% SDS, 1% Triton X-100, 1 mM EDTA, 16.7 mM Tris-HCl at pH 8.1, 167 mM NaCl]. ChIP-seq libraries were prepared with the NEBNext Ultra II DNA library preparation kit for Illumina [NEB E7645S] by following the manufacturer’s instructions. In brief, 1 ng of DNA from H3K79me2 ChIP was used. End repair, 5’ phosphorylation, and dA-tailing were performed in a thermocycler. The samples were incubated for 90 min at 20°C and subsequently for 30 min at 65°C. Adaptor [NEB E6440S] was diluted at 1:25 and ligated to the DNA sample. Adaptor-ligated DNA was cleaned up without size selection by using 0.8× volume of NENext sample purification beads. Purified adaptor-ligated DNA was labeled and enriched by PCR amplification with unique dual-index primer pairs [A11–H11, NEB E6440S]. PCR reaction was cleaned up by using 0.9× sample volume of NENext sample purification beads. The libraries were analyzed on TapeStation, and the DNA concentration was measured using Qubit 4.0. Eight individual libraries [two vehicle ChIP, two EPZ5676-treated ChIP, two vehicle
input, and two EPZ5676-treated input) were pooled in equal amounts. Libraries were sequenced with the NextSeq 1000/2000 P2 reagents v3 kit (Illumina 20046811) on an Illumina NextSeq 2000 system [single end, 100 cycles] at the University of Pennsylvania School of Veterinary Medicine Center for Host–Microbial Interactions Sequencing Core.

Fastq files of ChIP-seq were decoded by bcl2fastq2 [v2.20.0.422]. The index and barcode in Fastq files were removed by Trimmomatic (v0.32), Rsreadr (v 2.10.0) was used to map reads to the mouse reference genome (UCSC GRCm38/mm10), and uniquely mapped reads were used for downstream analysis. After mapping, BAM files were sorted and indexed by SAMtools (v2.20.0.422). The index and barcode in Fastq files were removed by DeepTools (v2.20.0.422). The index and barcode in Fastq files were removed by ChipSeeker (v1.32.0). ChIPpeakAnno (v3.30.0). ChIP-seq coverage was calculated with DeepTools (v3.4.3). Data were visualized with the IGV browser (v 2.12.3). The H3K79me2 ChIP-seq data are available in GEO under accession number GSE193575.

GO analysis
Gene ontology (GO) analysis of differentially expressed genes was performed using DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov).

Statistical analysis
Student’s t-test or Mann–Whitney U-test were used for statistical analysis. Mean and standard deviation or standard error of mean were used to describe the average and distribution of the data.

Competing interest statement
The authors declare no competing interests.

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