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

Huijuan Lin,[1,2,8] Keren Cheng,[2,8] Hiroshi Kubota,[3] Yemin Lan,[4] Simone S. Riedel,[5,6,7] Kazue Kakiuchi,[3] Kotaro Sasaki,[2] Kathrin M. Bernt,[5,6,7] Marisa S. Bartolomei,[4] Mengcheng Luo,[1] and P. Jeremy Wang[2]

[1]School of Basic Medical Sciences, Wuhan University, Wuhan, Hubei Province 430072, China; [2]Department of Biomedical Sciences, University of Pennsylvania School of Veterinary Medicine, Philadelphia, Pennsylvania 19104, USA; [3]Laboratory of Cell and Molecular Biology, Department of Animal Science, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan; [4]Epigenetics Institute, Department of Cell and Developmental Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA; [5]Division of Pediatric Oncology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA; [6]Department of Pediatrics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA; [7]Abramson Cancer Center, Philadelphia, Pennsylvania 19104, USA

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.

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Males produce sperm continuously through adult life, driven by the presence of spermatogonial stem cells (SSCs) that continue to renew (Oatley and Brinster 2006; Brinster 2007; Law and Oatley 2020). SSCs are a subpopulation of undifferentiated spermatogonia that proliferate, differentiate, and eventually enter meiosis to become spermatocytes. After meiosis, spermatocytes become round spermatids (haploid germ cells), which undergo dramatic differentiation and morphogenesis to become elongated spermatids—spermatozoa with flagella. Failure in SSC self-renewal leads to a lack of sperm production and thus male infertility.

The presence of SSCs in testis was definitively demonstrated by SSC transplantation, in which donor germline progenitor cells were found to restore spermatogenesis when transplanted into germ cell-depleted testis in mice (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). To date, SSC transplantation remains an unequivocal functional assay for SSCs (Oatley and Brinster 2006; Dobrinski 2008; Kubota and Brinster 2008). A second breakthrough in the field was the identification of in vitro culture conditions that permit indefinite expansion of SSCs on feeder cells with growth factors (Kanatsu-Shinohara et al. 2004; Kubota et al. 2004). These two technologies have spurred many functional studies of SSCs and have advanced translational applications of SSCs in animal transgenesis (Ciccarelli et al. 2020).

Adult tissues maintain homeostasis through a balance between adult stem cell renewal and differentiation. Although much is known about spermatogenesis, the regulation of SSC self-renewal remains poorly understood. Stem cell renewal requires both stem cell-intrinsic factors and external niche factors. To date, a number of SSC factors have been identified: PLZF, NANOS2, RB, GDNF, ETV5, BCL6B, and CARF. Their functions in SSC self-

[8]**These authors contributed equally to this work.**
Corresponding authors: pwang@vet.upenn.edu, luomengcheng@whu.edu.cn
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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 translocation 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*$^{fl/-}$ *Ddx4*-Cre mice (referred to as *Dot1l*^{cKO}) (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*^{cKO} mice was dramatically smaller (Fig. 1A). However, the testis/body weight ratio of *Dot1l*^{cKO} 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*^{cKO} males failed to sire offspring, as the sperm count of adult *Dot1l*^{cKO} 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 spermatogonia 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*^{cKO} and control pups up to P25 (Fig. 1B). Histological analysis of *Dot1l*^{cKO} testes showed the presence of spermatogonia (mitotic) at P8, spermatocytes (meiotic) at P18, and elongated spermatids (haploid) at P40 (Fig. 1D), at a time line expected in wild type. H3K79me2

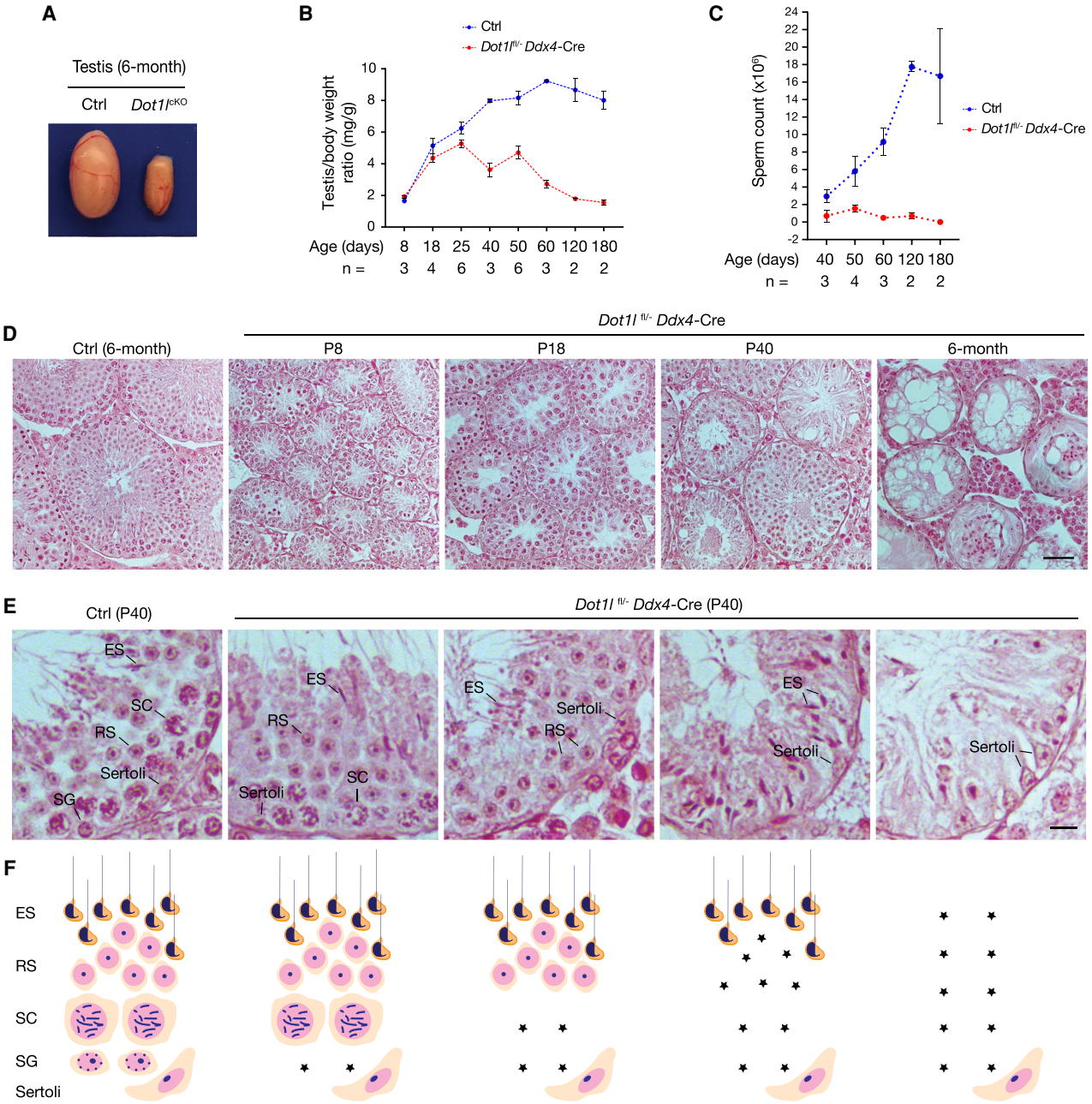

Figure 1. DOT1L is essential for SSC self-renewal in mice. (*A*) Testes from control (*Dot1l*$^{fl/+}$ *Ddx4*-Cre) and *Dot1l*^{cKO} male mice. (*B*,*C*) Testis/body weight ratio (*B*) and sperm count (*C*) of *Dot1l*^{cKO} and control males at different ages. Control males: *Dot1l*$^{fl/+}$, *Dot1l*$^{fl/-}$, or *Dot1l*$^{fl/+}$ *Ddx4*-Cre. (*D*) Histology of mouse testes at P8, P18, P40, and 6 mo. Scale bar, 50 µm. (Ctrl) *Dot1l*$^{fl/+}$ *Ddx4*-Cre. (*E*,*F*) Representative seminiferous tubules showing sequential and progressive loss of germ cells from spermatogonia to elongated spermatids in *Dot1l*^{cKO} testes at P40. (Ctrl) *Dot1l*$^{fl/+}$ *Ddx4*-Cre. The diagram illustrates the presence and absence of different types of germ cells in the tubules *above*. Scale bar, 10 µm. (Asterisk) Loss of germ cells, (SG) spermatogonia, (SC) spermatocyte, (RS) round spermatid, (ES) elongated spermatid.

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*^{cKO} testis (Supplemental Fig. S1). Together, these results demonstrate that the first wave of spermatogenesis occurs normally in *Dot1l*^{cKO} 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

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, *Dot1l*^{cKO} testes showed a variety of abnormal tubules with a loss of one or more types of germ cells. *Dot1l*^{cKO} 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. 2009; Hu et al. 2013). As expected, the *Dot1l*^{cKO} testis weight decreased progressively at day 50 and beyond (Fig. 1B). At 6 mo of age, all germ cells were lost in *Dot1l*^{cKO} testes, resulting in a Sertoli cell only syndrome (Fig. 1D). These data strongly suggest that DOT1L is essential for SSC self-renewal. Furthermore, the SSC self-renewal defect in *Dot1l*^{cKO} testes was much more severe than that in *Zbtb16*$^{-/-}$ 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 *Dot1l*^{cKO} 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 *Dot1l*^{cKO} testes at P8, but significantly lower in *Dot1l*^{cKO} 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 *Dot1l*$^{fl/fl}$ *Ddx4*-Cre^{ERT2} mice (referred to as *Dot1l*^{iKO}) (Fig. 2A). At 35 d posttamoxifen treatment (35 dpt), *Dot1l*^{iKO} males displayed a reduction in testis size and sperm count (Fig. 2B–D). Similar to P40 *Dot1l*^{cKO} testes, adult *Dot1l*^{iKO} 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 *Dot1l*^{iKO} testes at 35 dpt (Supplemental Fig. S3A,B,D). We looked earlier to determine how quickly the phenotype was observable. While *Dot1l*^{iKO} 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 *Dot1l*^{iKO} testes but was not statistically significant (Supplemental Fig. S3E,F). The number of TUNEL-positive germ cells was increased in 35-dpt *Dot1l*^{iKO} 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 *Dot1l*^{iKO} 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 *Dot1l*^{iKO} 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 *Dot1l*^{iKO} testis (Supplemental Fig. S4B). The reduction of DOT1L and H3K79me2 in the *Dot1l*^{iKO} 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-

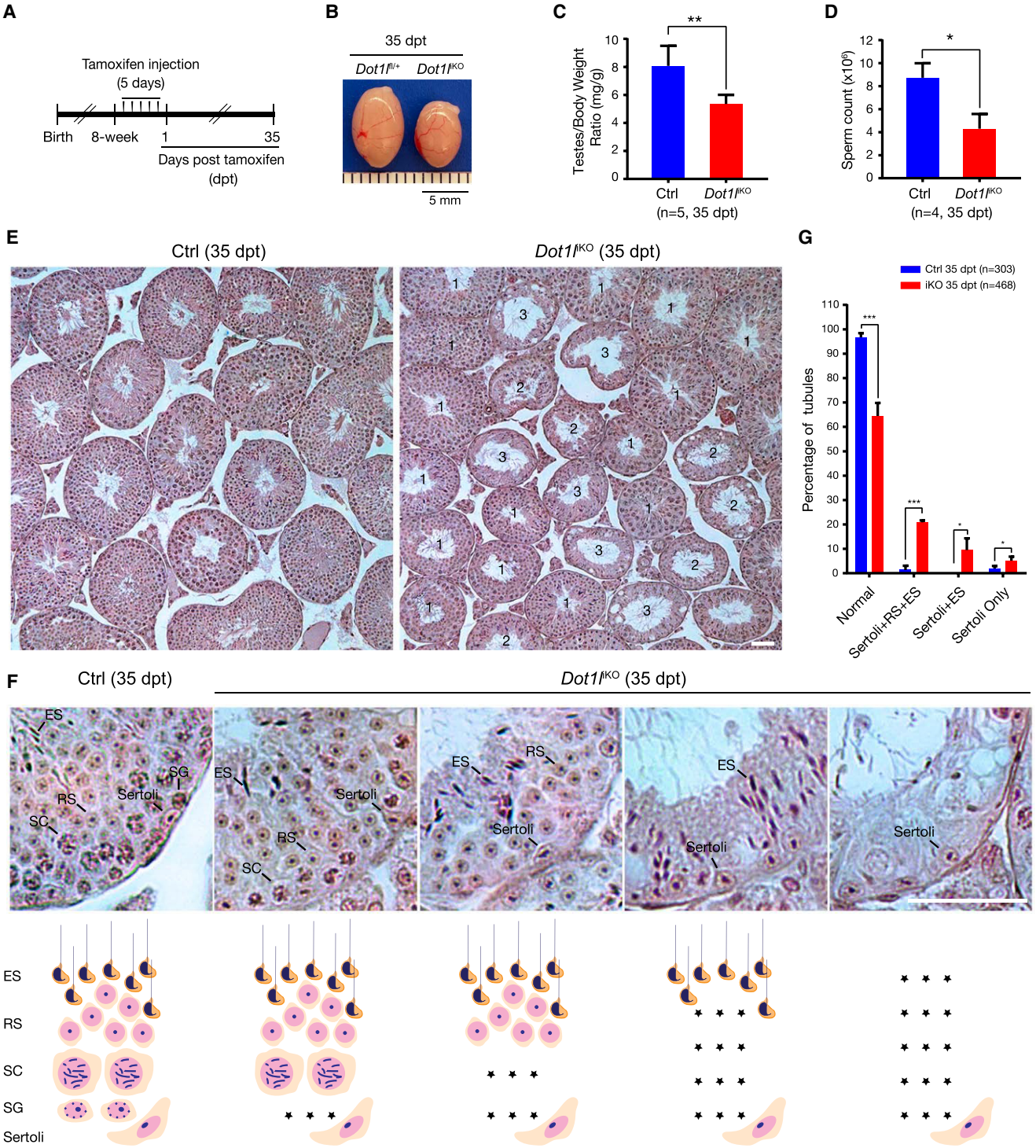

Figure 2. Inducible deletion of *Dot1l* causes a failure in SSC self-renewal in adult mice. (*A*) Scheme of tamoxifen treatment in adult male mice. (*B*) Image of adult testes at 35 dpt. (*Dot1l*^{iKO}) *Dot1l*$^{fl/fl}$ *Ddx4*-Cre^{ERT2}. (*C*) Testis/body weight ratio of control and *Dot1l*^{iKO} males. (**) $P < 0.01$, 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*-Cre^{ERT2}. (*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. (Asterisk) Loss of germ cells, (SG) spermatogonia, (SC) spermatocyte, (RS) round spermatid, (ES) elongated spermatid. (*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.

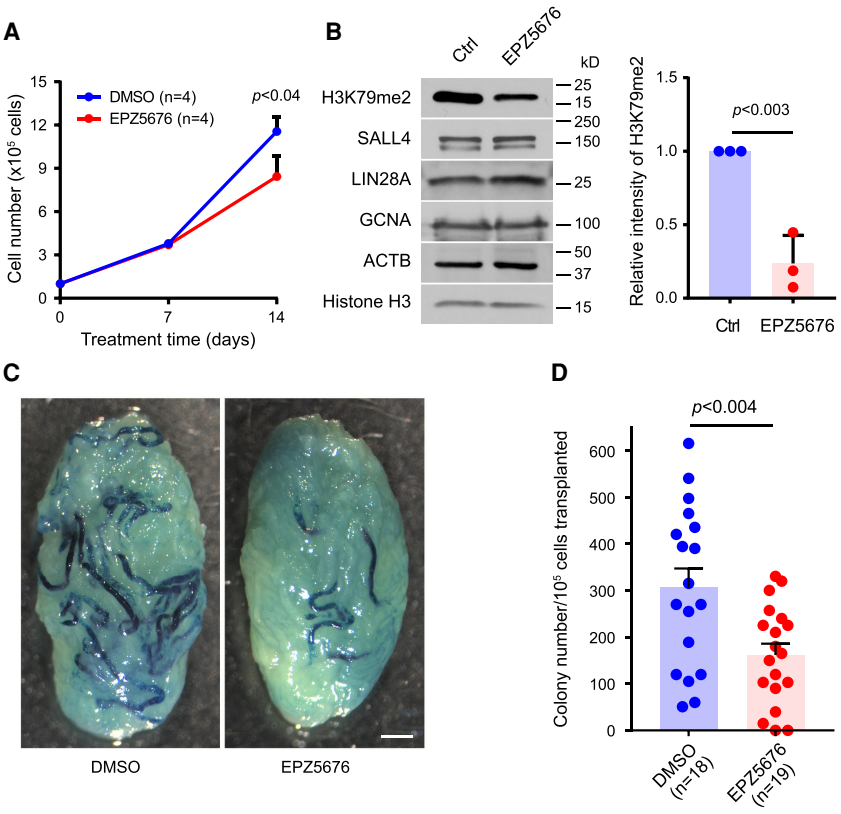

Figure 3. Inhibition of DOT1L in mouse SSCs reduces stem cell activity. (*A*) Total number of spermatogonia in culture. (*n*) Number of experiments. Statistics: paired Student's *t*-test. (*B*) Down-regulation of H3K79me2 in mouse SSCs cultured with or without 2 μM EPZ5676 for 2 wk. The graph shows the relative intensity of H3K79me2 normalized to histone H3 from three experiments. (*C*) β-Gal staining of mouse testes at 2 mo after SSC transplantation. (*D*) Number of colonies derived from EPZ5676- or DMSO-treated SSCs after transplantation. (*n*) Number of recipient testes from four independent experiments. Statistics: unpaired Student's *t*-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 *Hoxc* 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,

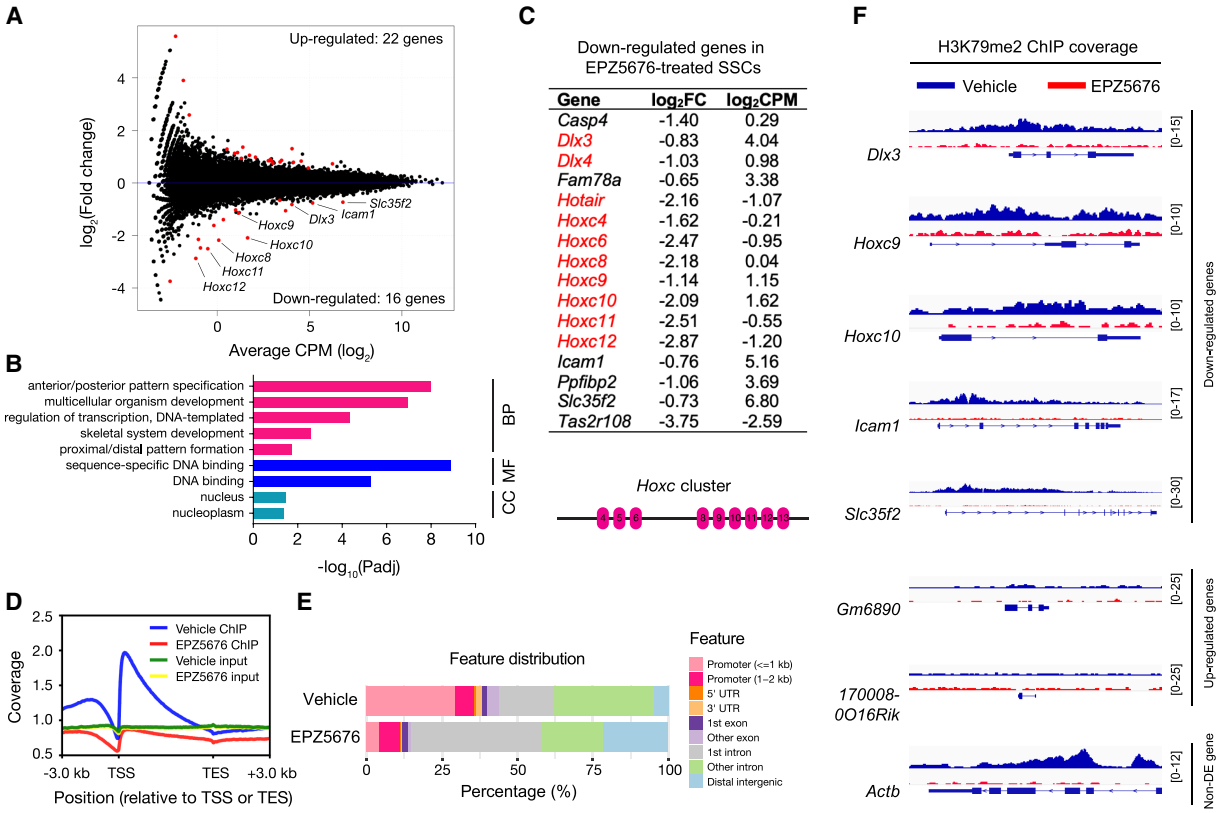

Figure 4. Inhibition of DOT1L down-regulates *Hoxc* genes in SSCs. (*A*) MA plot of expression changes. Mouse SSCs were cultured with or without 2 µM EPZ5676 for 14 d. The DE genes are shown as red dots. (*B*) List of down-regulated DE genes in EPZ5676-treated SSCs. The diagram shows the organization of the *Hoxc* gene cluster on chromosome 15 (Mallo and Alonso 2013). (*C*) GO analysis of down-regulated DE genes in EPZ5676-treated SSCs. (BP) Biological process, (MF) molecular function, (CC) cellular component. (*D*) Position and enrichment of H3K79me2 ChIP-seq signals from vehicle (no inhibitor) and EPZ5676-treated SSCs relative to the TSS (transcription start site) and TES (transcription end site). Inputs are shown for comparison. Genes were binned to the same overall relative length. (*E*) Feature distribution of H3K79me2 ChIP-seq peaks in the mouse genome. (*F*) H3K79me2 profiles of selected DE genes and non-DE genes in vehicle- and EPZ5676-treated SSCs. Note that *Actb* is one of many non-DE genes with abundant H3K79me2. Numbers at the *right* indicate the signal range.

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 (*Dot1l*^{fl}) 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*-Cre^{ERT2} (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-wk-old *Dot1l*$^{fl/fl}$ *Ddx4*-Cre^{ERT2} males (2 mg/30 g body weight) once per day for five consecutive days. *Dot1l*$^{fl/+}$ *Ddx4*-Cre^{ERT2}, *Dot1l*$^{fl/+}$, or *Dot1l*$^{fl/fl}$ 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 *Dot1l*$^{fl/+}$ *Ddx4*-Cre^{ERT2} 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 *Dot1l* floxed allele (418 bp): p2 (5′-CCCAAAAGGGTCTTTTCACA-3′) and p3 (5′-ATGGGATTT CATGGAAGCAA-3′); the *Dot1l* deletion allele (620 bp): p1 (5′-CTCACAGTCACATACTACCTCTGAC-3′) and p3 (5′-ATGG GATTTCATGGAAGCAA-3′); *Ddx4*-Cre (240 bp): VasaCre-1 (5′-CACGTGCAGCCGTTTAAGCCGCGT-3′) and VasaCre-2 (5′-TTCCCATTCTAAACAACACCCTGAA-3′); and *Ddx4*-Cre^{ERT2} (205 bp): KI-1 (5′-ATACCGGAGATCATGCAAGC-3′) and KI-2 (5′-GGCCAGGCTGTTCTTCTTA-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)26Sor/J mice (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 TRIzol (Thermo Fisher Scientific) followed by RNeasy column purification (Qiagen). All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cultured SSC cells 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 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. S5A), one pair of testes from postnatal day 5–8 DBA/2J 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. S5B) 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.

DOT1L inhibitor treatment

EPZ5676 (Cayman Chemical 1380288-87-8) was dissolved in DMSO or 100% ethanol. During treatment, EPZ5676 solution was added to IMDM/FBS medium to achieve a final concentration of 2 μM in the experimental group. The same amount of solvent was added to the medium in the control group. Medium was changed every 2–3 d, and the cells were subcultured every 7 d.

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 RefSeq 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 H_2O, 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 H_2O. Each sample was assayed in triplicates. The expression level was normalized to *Gapdh* using the $2^{-\Delta\Delta Ct}$ 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 (Insert) 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: 130-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, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl at pH 8.1, 167 mM NaCl). H3K79me2 antibody (Abcam ab3594) was added at 2 μg per 1 million cells and incubated overnight at 4°C. Protein A+G-coated magnetic beads (Millipore 16663) were added for 3 h and then washed once with each of the following buffers: low-salt buffer (1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 150 mM NaCl), high-salt buffer (1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% Igepal-CH 630 [NP-40], 1% deoxycholic acid sodium salt, 1 mM EDTA, 10 mM Tris-HCl at pH 8.1), and TE buffer (1 mM EDTA, 10 mM Tris-HCl at pH 8.1). Cross-linking was reversed in elution buffer (1% SDS, 0.1 M $NaHCO_3$, 200 mM NaCl) overnight at 65°C. Beads were eluted by adding 20 μg/mL RNase A (Ambion AM2271) at 37°C and 100 μg/mL proteinase K (Invitrogen 43-337-93) for 1 h each at 55°C. DNA was purified using a PCR purification kit (Qiagen 28104). Eluted DNA was quantified using the Qubit assay (dsDNA HS assay kit, Invitrogen Q32851).

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× sample volume of NEBNext 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 NEBNext 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). Rsubread (v 2.10.0) was used to map reads to the mouse reference genome (USCS GRCm38/*mm10*), and uniquely mapped reads were used for downstream analysis. After mapping, BAM files were sorted and indexed by SAMtools (v1.11). Duplicated reads were flagged by Picard (v1.96) and removed for downstream analysis. Peaks were called by MACS3 with FDR < 1%, $P < 0.01$, and broad parameters. Blacklist regions of ChIP-seq were downloaded from the ENCODE Project. Peaks were annotated by Chipseeker (v1.32.0) and 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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Author contributions: P.J.W., M.L., and H.L. conceptualized the study. H.L. and K.C. carried out experiments and data analysis. H.K. and K.K. performed the SSC transplantation. K.C. and Y.L. performed bioinformatic analyses. S.S.R. and K.M.B. contributed to ChIP-seq. P.J.W., K.S., M.S.B., and M.L. analyzed data. P.J.W., H.L., H.K., K.C., and S.S.R. wrote the manuscript. All of the authors were involved in the discussion and commented on the manuscript.

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