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RESEARCH ARTICLE

Zebrafish *prmt5* arginine methyltransferase is essential for germ cell development

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ABSTRACT

Protein arginine methyltransferase 5 (Prmt5), a type II arginine methyltransferase, symmetrically dimethylates arginine in nuclear and cytoplasmic proteins. Prmt5 is involved in a variety of cellular processes, including ribosome biogenesis, cellular differentiation, germ cell development and tumorigenesis. However, the mechanisms by which *prmt5* influences cellular processes have remained unclear. Here, *prmt5* loss in zebrafish led to the expression of an infertile male phenotype due to a reduction in germ cell number, an increase in germ cell apoptosis and the failure of gonads to differentiate into normal testes or ovaries. Moreover, arginine methylation of the germ cell-specific proteins Zili and Vasa, as well as histones H3 (H3R8me2s) and H4 (H4R3me2s), was reduced in the gonads of *prmt5*-null zebrafish. This resulted in the downregulation of several Piwi pathway proteins, including Zili, and Vasa. In addition, various genes related to meiosis, gonad development and sexual differentiation were dysregulated in the gonads of *prmt5*-null zebrafish. Our results revealed a novel mechanism associated with *prmt5,* i.e. *prmt5* apparently controls germ cell development in vertebrates by catalyzing arginine methylation of the germline-specific proteins Zili and Vasa.

KEY WORDS: Germ cell, *Prmt5*, Zebrafish, *Zili*, *Ziwi*, *Vasa*

INTRODUCTION

Arginine methylation is a post-translational modification in histone and non-histone proteins that is known to affect numerous cellular activities, including RNA processing, DNA repair, protein-protein interactions and gene regulation (Karkhanis and Joshi, 2011; Wesche et al., 2017). Arginine methylation is catalyzed by a group of nine arginine methytransferases (Prmt1-Pmrt9), which are classified into three subgroups depending on the modification they catalyze (Blanc and Richard, 2017). Protein arginine methyltransferase 5 (Prmt5), a type II arginine methyltransferase that symmetrically dimethylates arginine in nuclear and cytoplasmic proteins (Agarwal et al., 2016), is involved in a variety of cellular processes, including ribosome biogenesis (Agarwal et al., 2016), Golgi apparatus assembly, cellular differentiation, germ cell development and tumorigenesis (Karkhanis and Joshi, 2011; Stopa et al., 2015).

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The roles of *Prmt5* in embryonic development has been extensively investigated in mouse models (Tee et al., 2010). *Prmt5*-null mice suffer from early embryonic lethality between embryonic days 3.5 and 6.5 due to the abrogation of pluripotent cells in blastocysts (Tee et al., 2010). Knocking down of *Prmt5* with a short interfering RNA (shRNA) in mouse embryonic stem cells (ESCs) results in the downregulation of pluripotency-associated RNAs and the upregulation of differentiation genes (Tee et al., 2010). *Prmt5* also functions together with the master germline determinant *Blimp1* during gastrulation to repress somatic cell differentiation and influence a germline fate (Ancelin et al., 2006). Thus, *Prmt5* is considered to be important for safeguarding pluripotency and preventing differentiation. The conditional loss of *Prmt5* in early primordial germ cells (PGCs) causes complete male and female sterility, possibly owing to the disruption of genome defense functionality during preimplantation development and the disruption of PGCs during global DNA demethylation (Kim et al., 2014). However, it appears that *Prmt5* is not required for PGC specification in mice (Tee et al., 2010). In *Drosophila melanogaster*, a homozygous mutation in the female *dart5* gene (homologous to *Prmt5*) results in grandchildless, while female flies are fertile, but their progeny lack PGCs (Li et al., 2015). Nevertheless, in mice, *Prmt5* acts at the conclusion of PGC reprogramming I to promote the proliferation and expression of the gonadal germline program; *Prmt5* is thus required for germ cell survival in mice (Li et al., 2015; Wang et al., 2015a).

Multiple lines of evidence indicate that *Prmt5* plays an essential role in germ cell development (Ancelin et al., 2006; Gonsalvez et al., 2006; Kim et al., 2014; Li et al., 2015; Tee et al., 2010; Wang et al., 2015a,b). Because histones are well-defined targets of *Prmt5*, arginine methylation of histones (e.g. H2AR3me2s and H4R3me2s) by *Prmt5* is considered an important mechanism that influences affecting germ cell development (Ancelin et al., 2006; Kim et al., 2014; Tee et al., 2010; Wang et al., 2015a).

Prmt5 catalyzes the arginine methylation of Piwi proteins in *Drosophila* and mice (Kirino et al., 2009; Vagin et al., 2009). Piwi proteins are an animal-specific class of the Argonaute family proteins that are specifically expressed in germ cells (Houwing et al., 2007). The function of Piwi proteins in germline development is evolutionarily conserved from invertebrates to vertebrates (Houwing et al., 2007). In *Drosophila*, Piwi proteins are required for the maintenance of germline stem cells (Cox et al., 1998, 2000), and Piwi protein loss results in both germline and embryonic defects (Aravin et al., 2001; Sarot et al., 2004; Vagin et al., 2006). The two Piwi-related *Caenorhabditis elegans* genes, *prg-1* and *prg-2*, are also required for the maintenance of mitotic germline stem cells (Cox et al., 1998). However, mutations in the mouse Piwi genes *Miwi* (*Pwil1*) and *Mili* (*Pwil2*) affect only the meiotic progression of developing sperm, but not the maintenance of mitotic germ cells (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004). Zebrafish

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also has two Piwi genes, *ziwi* and *zili* (Huang et al., 2011). In zebrafish, the *piwi* (*ziwi*) gene is expressed in both the male and the female gonads (Houwing et al., 2007). Loss of *ziwi* results in progressive loss of germ cells (Houwing et al., 2007). *zili* is required for germ cell differentiation and meiosis (Houwing et al., 2008).

Prmt5 also plays a role in germ cell fate (Kirino et al., 2010). In addition to arginine methylation of Piwi proteins, *Prmt5* methylates arginine residues in Vasa proteins; this function is conserved across phyla (Kirino et al., 2010). Vasa expression is exclusively restricted to the germ cell lineage and is a universal marker of animal germ cells (Hay et al., 1988; Lasko and Ashburner, 1988). *Vasa* is required for germ cell formation (Liang et al., 1994; Yajima and Wessel, 2011) and is widely conserved across invertebrate and vertebrate species, including *Drosophila*, *Xenopus*, zebrafish, mouse and human (Raz, 2000). Zebrafish *vasa* is required for germ-cell differentiation and maintenance (Hartung et al., 2014). In *Xenopus,* the *vasa* gene homolog (XVLG1) is expressed in oocytes and embryos, and is required for the formation of germ cells (Ikenishi and Tanaka, 1997; Komiya et al., 1994). In mice, the protein expression of the *Vasa* homolog (MVH, DDX4) is restricted to the germ cell lineage (Leroy et al., 1989), and loss of function of the MVH protein inhibits the proliferation and differentiation of spermatocytes, leading to male sterility.

Although it is clear that *Prmt5* plays an important role in germ cell development, and that many germ-cell-specific proteins, including Piwi and Vasa, are arginine methylated, whether *Prmt5* affects germ cell development by catalyzing the arginine methylation of these germ cell-specific proteins remains unclear. Additionally, owing to the early embryonic lethality of *Prmt5*-null mice (Tee et al., 2010), the functions of *Prmt5 in vivo*, besides the defects in germ cell development suggested by conditional loss of *Prmt5* in mice (Kim et al., 2014; Li et al., 2015; Wang et al., 2015a), remain unknown. Here, we use CRISPR/Cas9 to generate *prmt5*-null zebrafish, which cause incompletely penetrant lethality and sterility.

RESULTS

Targeted disruption of *prmt5* resulted in a low survival rate, a male phenotype and infertility

Prmt5 is evolutionarily conserved across humans, mice and zebrafish (*Danio rerio*) (Fig. S1). By sgRNA/Cas9-mediated *prmt5* disruption, we identified two mutants in the *prmt5* gene: $prmt^{ihb1994/ihb1994}$ (mutant 1; M1) and $prmt5^{\ ihb1995/ihb1995}$ (mutant 2; M2) (Fig. S2A). Each of the two alleles of *prmt5* encoded a different truncated peptide (Fig. S2B). Loss of the Prmt5 protein was confirmed using whole-body lysates (Fig. S2C,D).

From the embryonic stage to adulthood, *prmt5* heterozygotes ($prmt5^{+/-}$) were indistinguishable from wild-type siblings ($prmt5^{+/+}$). To obtain *prmt5* homozygotes ($prmt5^{-/-}$), we mated $prmt5^{+/-}$ (♀)×$prmt5^{+/-}$ (♂). We genotyped the offspring at 15 days post-fertilization (dpf). We noticed that the number of $prmt5^{-/-}$ zebrafish was lower than expected based on Mendel's ratio (1:2:1), which suggested that some $prmt5^{-/-}$ zebrafish might die at the larval stage. In our zebrafish-rearing system, zebrafish sex could easily be determined by body shape and gonad dissection at 2 months post-fertilization (mpf). We compared the observed number of fish of each of the three genotypes obtained by the $prmt5^{+/-}$×$prmt5^{+/-}$ mating between 2 mpf and 4 mpf with the expected number (Fig. 1A). The observed numbers and sexual ratios of wild-type and $prmt5^{+/-}$ zebrafish were very close to those expected (Fig. 1A). However, the observed number of $prmt5^{-/-}$ zebrafish was significantly lower than the expected (Fig. 1A). Although the mean initial growth rate of the $prmt5^{-/-}$ zebrafish was relatively slower than the wild-type or $prmt5^{+/-}$ zebrafish, the mean body weight of the $prmt5^{-/-}$ zebrafish at 4 mpf was similar to the wild type and $prmt5^{+/-}$. Therefore, $prmt5^{-/-}$ zebrafish were viable, even though their initial survival rate was low.

Interestingly, $prmt5^{-/-}$ zebrafish were always phenotypically male-like, based on pigmentation hue, abdomen shape, testes-like gonads and the presence of male-specific breeding tubercles (BTs) (or epidermal tubercles, ETs) on their pectoral fins (Fig. 1B). We therefore evaluated the fertilization rate of the three genotypes of adult males: wild type ($prmt5^{+/+}$), heterozygotes ($prmt5^{+/-}$) and homozygotes ($prmt5^{-/-}$). When mated to wild-type females, all $prmt5^{-/-}$ zebrafish exhibited male mating behaviors and induced female egg-laying, but the eggs collected from these matings were unfertilized. In the $prmt5^{+/+}$ (♂)×wild type (♀) or $prmt5^{+/-}$ (♂)×wild type (♀) matings, the fertilization rate was similar (Fig. 1C). These data suggest that $prmt5^{-/-}$ adults were completely infertile. In addition, the gonadosomatic index (GSI) of $prmt5^{-/-}$ was significantly lower than that of either $prmt5^{+/+}$ or $prmt5^{+/-}$ (Fig. 1D).

Characters similar to those of the $prmt5^{-/-}$ ($prmt5^{ihb1994/ihb1994}$) zebrafish, including low survival rate, male phenotype and infertility were also observed in $prmt5^{ihb1995/ihb1995}$ (mutant 2; M2) (Fig. S3A). To exclude off target effects, we performed a complementation test. We crossed $prmt5^{+/ihb1994}$ heterozygotes (♀) with $prmt5^{+/ihb1995}$ heterozygotes (♂) and obtained $prmt5^{ihb1994/ihb1995}$ mutant. As shown in Fig. S3B,C, the phenotypes exhibited in $prmt5^{ihb1994/ihb1995}$ were consistent with those in $prmt5^{ihb1994/ihb1994}$ (M1) and $prmt5^{ihb1995/ihb1995}$ (M2). In addition, after back-crossing $prmt5^{+/ihb1994}$ with wild type (strain AB, disallowing offspring-parent mating) for six generations, we validated the phenotypes in F8 $prmt5^{ihb1994/ihb1994}$ by crossing $prmt5^{+/ihb1994}$ (♀, F7)×$prmt5^{+/ihb1994}$ (♂, F7). These data might exclude off-target effects. In subsequent experiments, we primarily used $prmt5^{ihb1994/ihb1994}$ (mutant 1; M1) and focused on gonadogenesis in $prmt5^{-/-}$ zebrafish.

Our results suggested that *prmt5*-null zebrafish have low survival rates but are viable to adulthood, which differs from the *Prmt5*-null mice. Furthermore, the targeted disruption of *prmt5* caused a male infertile phenotype.

The gonads of $prmt5^{-/-}$ zebrafish failed to differentiate into normal testes or ovaries

We examined the expression of the Prmt5 protein in the ovaries and testes of adult wild-type zebrafish. The Prmt5 protein was clearly expressed in the ovaries and testes of adult wild-type zebrafish (4 dpf), implicating *prmt5* in ovarian and testicular functions (Fig. S4).

Zebrafish first develop oocytes between 10 and 25 dpf (Uchida et al., 2002). It has been suggested that exposure to differentiated oocytes around 2-3 weeks of age is required for female development (Houwing et al., 2008). At 20 dpf, abnormalities in $prmt5^{-/-}$ gonads were clearly indicated by a pyknotic mass and by the presence of numerous atretic oogonia compared with the gonads of $prmt5^{+/+}$ zebrafish; the $prmt5^{+/+}$ gonads also exhibited a loose structure, with a more prominent ovarian cavity and mitotic oogonia compared with $prmt5^{-/-}$ (Fig. 2A,B). Between 30 and 45 dpf, the gonads of the $prmt5^{+/+}$ zebrafish began to differentiate into testes (Fig. 2C,F) or ovaries (Fig. 2D,G), as indicated by the presence of spermatogonia, spermatocytes and spermatids (Fig. 2C,F) or by the presence of an increasing number of early and late stage IB perinucleolar oocytes (Fig. 2D,G). In contrast, $prmt5^{-/-}$ zebrafish gonads failed to differentiate into ovaries or normal testes; instead, a testis-like structure containing some spermatogonia cysts developed (Fig. 2E,H). Between 68 and 81 dpf, almost half of

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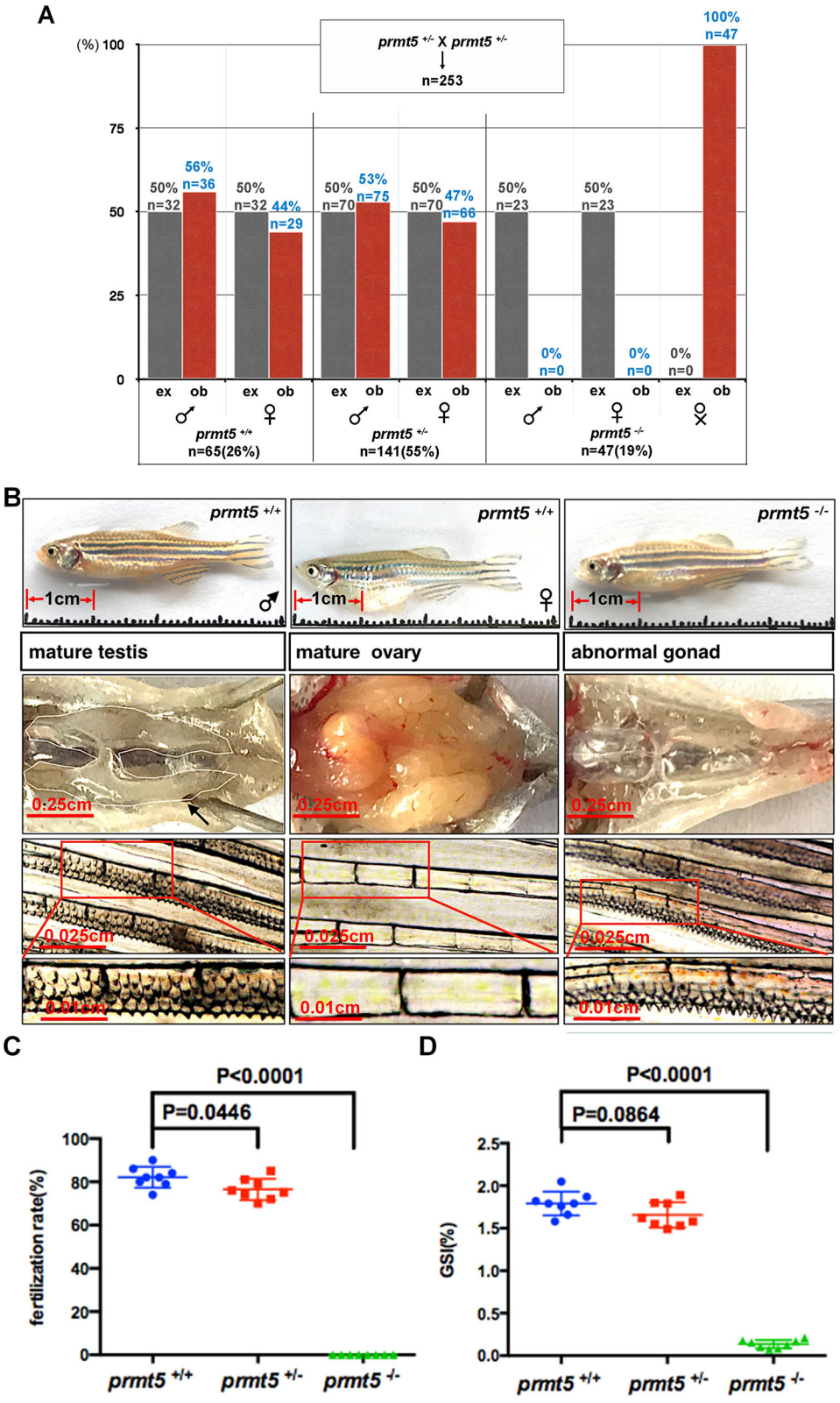

Fig. 1. Loss of *prmt5* in zebrafish reduces larval survival and generates an infertile male-like phenotype. (A) The 253 offspring of *prmt5*$^{+/-}$ (♀)×*prmt5*$^{+/-}$ (♂) matings; percentages of expected (ex, gray bars) and observed (ob, red bars). The observed percentage of *prmt5*$^{-/-}$ zebrafish (19%) was less than expected (25%). (B) The testes/ovaries and breeding tubercle in *prmt5*-null zebrafish (*prmt5*$^{-/-}$) and their wild-type siblings (*prmt5*$^{+/+}$) at 150 dpf. All *prmt5*$^{-/-}$ adults had atrophied testes, whereas *prmt5*$^{+/+}$ adults had normal testes/ovaries. Both adult male *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ zebrafish had breeding tubercles (arrow), which were not observed in adult female *prmt5*$^{+/+}$ zebrafish (*n*=10, respectively). Red boxes indicate the positions of the magnified sections underneath. (C) The fertilization rate of *prmt5*$^{+/+}$, *prmt5*$^{+/-}$ and *prmt5*$^{-/-}$ male zebrafish. Bars show means of eight independent experiments; error bars show s.d. (*n*=8, respectively). (D) The gonado-somatic indexes (GSI) of *prmt5*$^{+/+}$, *prmt5*$^{+/-}$ and *prmt5*$^{-/-}$ male zebrafish (*n*=8, respectively). Differences between two groups were determined by unpaired two-tailed Student's *t*-test. Bars show means of eight independent experiments; error bars show s.d.

the *prmt5*$^{+/+}$ zebrafish had mature testes that were filled with germ cells at different stages of spermatogenesis: spermatogonia, spermatocytes, spermatids and spermatozoa (Fig. 2I,L). The remaining *prmt5*$^{+/+}$ zebrafish had mature ovaries that were filled with oocytes at different stages of oogenesis: stages IB, II, III and IV (Fig. 2J,M). However, spermatogenesis in the *prmt5*$^{-/-}$ zebrafish was almost halted at 68-81 dpf, and only few spermatogonia cyst were detected (Fig. 2K). Eventually, the gonads of *prmt5*$^{-/-}$ were filled with Sertoli and Leydig cells (Fig. 2N). These data suggested that the gonads of *prmt5*$^{-/-}$ zebrafish failed to differentiate into normal testes or ovaries, thereby developing testis-like tissue without generating sperm.

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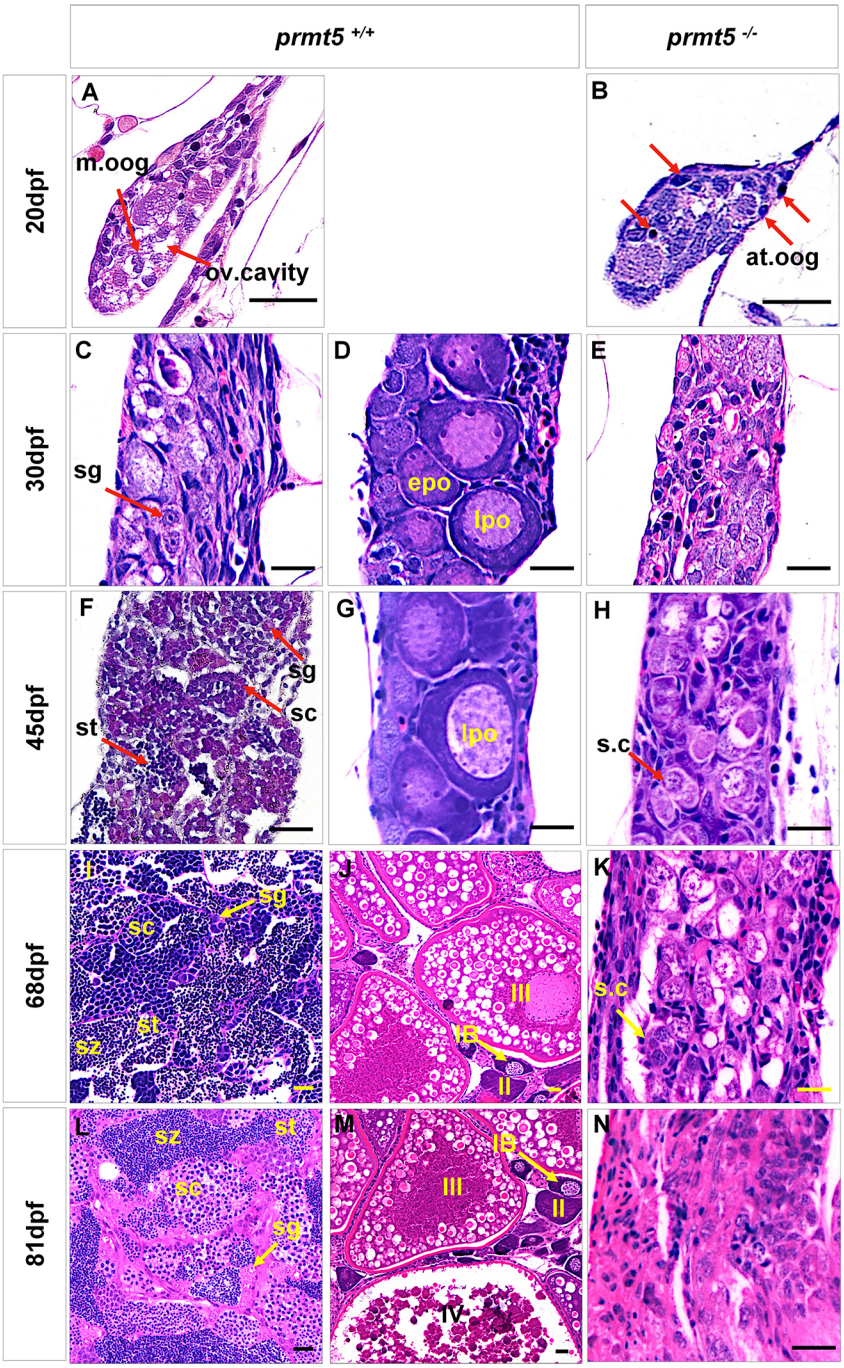

Fig. 2. The gonads of *prmt5*$^{-/-}$ zebrafish fail to differentiate into normal testes or ovaries, as shown by histological comparisons of gonadogenesis. (A,B) At 20 dpf, *prmt5*$^{-/-}$ gonads contained a pyknotic mass and many atretic oogonia (at.oog, indicated by red arrows), while the *prmt5*$^{+/+}$ gonads had a loosened structure with a prominent ovarian cavity (ov.cavity) and many mitotic oogonia (m.oog). (C-H) Between 30 and 45 dpf, the *prmt5*$^{+/+}$ gonads began to differentiate into testes (C,F) or ovaries (D,G), as indicated by the presence of spermatogonia (sg), spermatocytes (sc) and spermatids (st), or by the increasing numbers of early stage IB perinucleolar oocytes(epo) and late stage IB perinucleolar oocytes (lpo). In contrast, the *prmt5*$^{-/-}$ gonads displayed a testis-like morphology containing only a few spermatogonia cysts (s.c). (I-N) Between 68 and 81 dpf, *prmt5*$^{+/+}$ zebrafish possessed either mature testes (I,L), containing germ cells at different stages of spermatogenesis (spermatogonia, sg; spermatocytes, sc; spermatid, st; spermatozoa, sz), or mature ovaries (J,M), containing germ cells at different stages of oogenesis (IB, II, III or IV). The *prmt5*$^{-/-}$ gonads contained few spermatogonia cysts (s.c) (K) and instead contained several Sertoli cells (N). Scale bars: 20 µm; *n*=6 for each stage.

Loss of *prmt5* disrupts primordial germ cell (PGC) migration in early embryogenesis, leading to reduction of primordial germ cell number and causing a male-like gene expression profile during sexual differentiation

Vasa is germ cell-specific transcript (Yoon et al., 1997), that has been used for labeling germ cells (Braat et al., 1999; Houwing et al., 2008). Because early reduction of primordial germ cells promotes testis formation in zebrafish (Houwing et al., 2007; Tzung et al., 2015), to determine whether the deficiency in gonad development exhibited in *prmt5*$^{-/-}$ zebrafish resulted from a reduction in germ cells, we examined the number of germs cells by *vasa* staining during early embryogenesis. At the 18-somite stage, in *prmt5*$^{+/+}$ embryos, PGCs migrated and gathered at the genital ridge (Fig. 3Aa,c). However, in *prmt5*$^{-/-}$ embryos at 18 s, fewer PGCs migrated to the genital ridge, and their distribution was relatively incompact (Fig. 3Ab,d). In the *prmt5*$^{+/+}$ embryos at prim-5, PGCs were more compact at the genital ridge (Fig. 3Ae,g). In contrast, in the *prmt5*$^{-/-}$ embryos at prim-5, more PGCs migrated to ectopic positions, far from the genital ridge (Fig. 3Af,h). Quantitative analysis indicated that more PGCs in *prmt5*$^{-/-}$ embryos migrated to ectopic positions compared with the *prmt5*$^{+/+}$ embryos during early embryogenesis (Fig. 3Ba). It was proposed that the ectopic PGCs might be selectively eliminated by apoptosis, whereas PGCs that correctly migrate to the genital ridges might be resistant to death by apoptosis (Schlueter et al., 2007). Of note, at 18 s, no significant difference in total numbers of PGCs was detected between *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ embryos (Fig. 3Bb). However, from prim-5 stage to the 54 dpf stage, the total number of PGCs in *prmt5*$^{-/-}$ embryos was relatively lower than those of the

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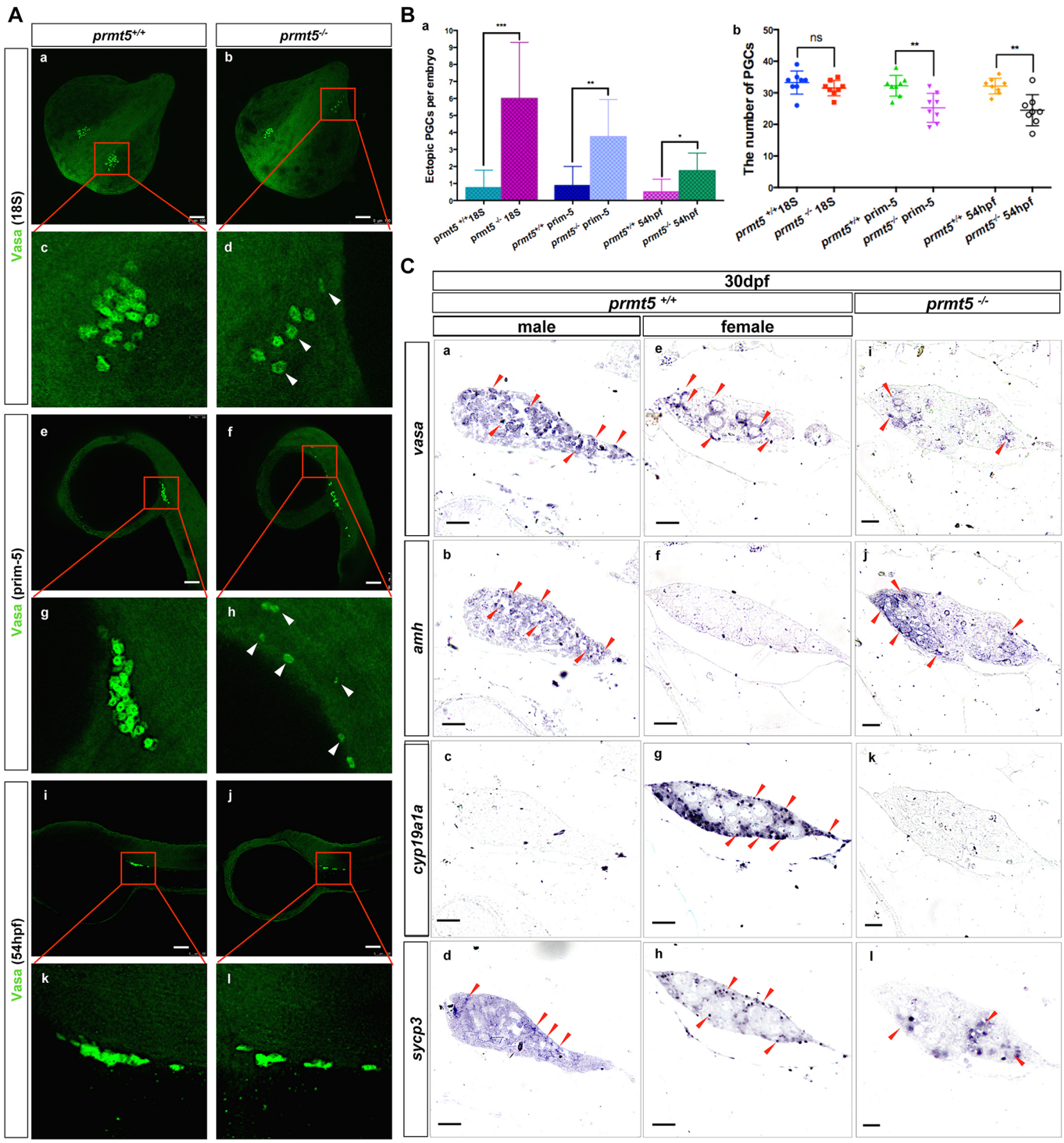

Fig. 3. Loss of *prmt5* disrupts primordial germ cell migration and causes a male-like gene expression profile during sexual differentiation. (A) Vasa in embryos at the 18-somite stage (18 s), prim-5 stage (prim5) and 54 hpf. n=80 for each stage. Arrowheads indicate the cells with relative higher gene expression. (Ba) Mean numbers of ectopic PGCs in *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ zebrafish embryos at the 18-somite stage, prim-5 stage and 54 hpf. (Bb) Scatterplot of PGC numbers in *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ zebrafish embryos at 18-somite stage, prim-5 stage and 54 hpf. Differences between two groups were determined by unpaired two-tailed Student's *t*-test. ns, not significant. *P<0.05; **P<0.01; ***P<0.001. (C) *vasa*, *amh*, *cyp19a1a* and *sycp3* in the gonads of *prmt5*$^{-/-}$ and *prmt5*$^{+/+}$ zebrafish during sexual differentiation (30 dpf). (a-d) Male gonads; (e-h) female gonads. Red arrowheads indicate gene expression. (b,c) Upregulation of *amh* and downregulation of *cyp19a1a* in gonads of *prmt5*$^{+/+}$ males. (f,g) Downregulation of *amh* and upregulation of *cyp19a1a* in gonads of *prmt5*$^{-/-}$ females. (j,k) Upregulation of *amh* and downregulation of *cyp19a1a* in gonads of *prmt5*$^{-/-}$ zebrafish. (d,h) Vigorous meiosis indicated by *sycp3* staining in gonads of *prmt5*$^{+/+}$ zebrafish. (i) There are few signs of meiosis in gonads of *prmt5*$^{-/-}$ zebrafish. *n*=6 for each staining. Scale bars: 100 µm (A); 50 µm (C).

prmt5$^{+/+}$ embryos (Fig. 3Bb). In fact, it has been reported that PGC mitosis in most fish ceases by midsomitogenesis and the PGCs remain mitotically quiescent during migration (Timmermans and Taverne, 1989). Therefore, loss of *prmt5* might disrupt PGC migration during early embryogenesis, resulting in apoptosis instead of proliferation defects, and leading to a reduction of primordial germ cell number.

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To further investigate how *prmt5* loss affects gonad development, we examined the gene expression levels of the early male somatic cell marker gene (*amh*), the early female somatic cell marker gene (*cyp19a1a*) and the meiosis marker gene (*sycp3*) during sexual differentiation by whole-mount *in situ* hybridization (Houwing et al., 2008; Siegfried and Nüsslein-Volhard, 2008). At 30 dpf, the number of germ cells labeled by *vasa* in *prmt5*$^{-/-}$ zebrafish (Fig. 3Ci) was reduced compared with *prmt5*$^{+/+}$ male gonads (Fig. 3Ca) or female gonads (Fig. 3Ce). In the *prmt5*$^{+/+}$ male gonads, *cyp19a1a* expression was low (Fig. 3Cc), but *amh* was high (Fig. 3Cb). Conversely, in *prmt5*$^{+/+}$ female gonads, the expression of *cyp19a1a* was high (Fig. 3Cg) and *amh* was undetectable (Fig. 3Cf). Notably, in the *prmt5*$^{-/-}$ gonads, *amh* (Fig. 3Cj) and *cyp19a1a* (Fig. 3Ck) levels were comparable with that in the *prmt5*$^{+/+}$ male gonads (Fig. 3Cb,c), exhibiting a male expression profile. This further supports the observation that the *prmt5*$^{-/-}$ zebrafish exhibits the male-like phenotype. Additionally, vigorous meiosis was clearly indicated by *sycp3* staining in the *prmt5*$^{+/+}$ gonads (Fig. 3Cd,h) compared with that in the *prmt5*$^{-/-}$ gonads (Fig. 3Cl). This suggested a disruption of meiosis in *prmt5*$^{-/-}$ gonads. Thus, these results suggested that loss of *prmt5* might cause defects in gonad differentiation and meiosis, resulting in the development of a male-like phenotype.

Deletion of *prmt5* led to germ cell apoptosis

To further determine the reasons why *prmt5* loss causes defects in germ cell development at later stages, we examined apoptotic cells in the developing gonads of *prmt5*$^{-/-}$ zebrafish. At 21 dpf, the gonadal germ cells were readily detected in gonadal cross-sections of both *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ zebrafish (Fig. 4A,B; circled by dashed white lines) (Houwing et al., 2007; Miao et al., 2017; Rodriguez-Mari et al., 2010). It was clear that more germ cells in the gonads of the *prmt5*$^{-/-}$ zebrafish had undergone apoptosis than in the *prmt5*$^{+/+}$ zebrafish (Fig. 4A,B). In adult zebrafish (125 dpf), the gonads of the *prmt5*$^{-/-}$ zebrafish had more apoptotic cells compared with the *prmt5*$^{+/+}$ zebrafish (Fig. 4C,D). These results suggested that *prmt5* is required for the survival and maintenance of germ cells.

Prmt5 is required for the arginine methylation of Zili and Vasa proteins as well as for H2R8me2s and H4R3me2s

The male phenotype and deficient germ cell development exhibited by the *prmt5*-null zebrafish resembled the phenotypes of *ziwi*, *zili* and *vasa* mutants (Houwing et al., 2008, 2007; Hartung et al., 2014). In addition, the arginine methylation of Vasa and Piwi proteins is essential for germline development (Huang et al., 2011; Chen et al., 2009; Kirino et al., 2009, 2010). We thus hypothesized that *prmt5* loss leads to the disruption of arginine methylation in the Vasa and Piwi proteins.

First, we quantified Prmt5 protein expression, the symmetrical dimethylation of arginine (with the anti-Sym11 antibody), and the asymmetrical dimethylation of arginine (with the anti-Asym24 antibody). We also investigated the symmetrical dimethylation of H3R8 and H4R3 (H3R8me2s and H4R3me2s), two well-defined

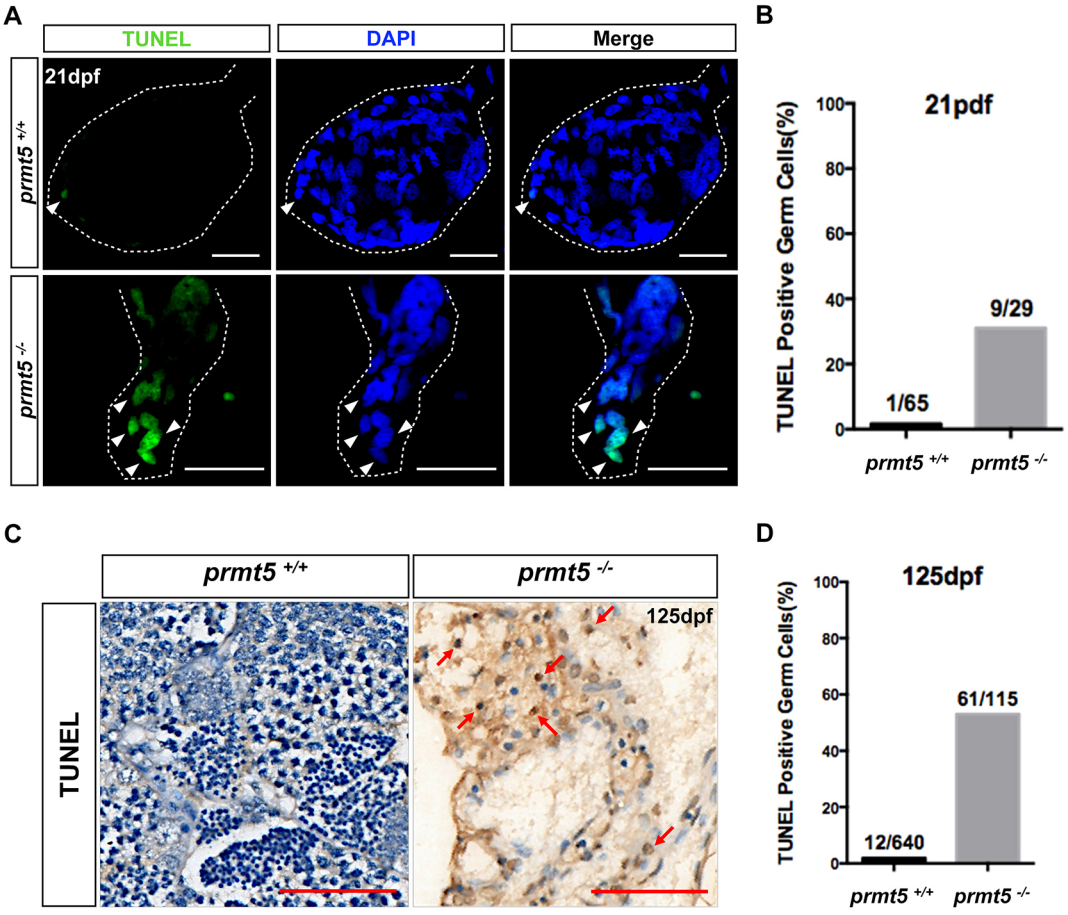

Fig. 4. Deletion of *prmt5* in zebrafish leads to germ cell apoptosis. (A) Apoptotic cells in the gonads (white dashed lines) of *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ zebrafish at 21 dpf. More apoptotic cells (green; white arrowheads) are found in the *prmt5*$^{-/-}$ zebrafish gonads than in *prmt5*$^{+/+}$ zebrafish gonads (*n*=4, respectively). (B) The ratios of apoptotic cells in *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ zebrafish at 21 dpf. (C) Apoptotic cells (arrows) in the gonads of *prmt5*$^{+/+}$ and *prmt5b*$^{-/-}$ zebrafish at 125 pdf; *n*=4, respectively. (D) The ratios of apoptotic cells in *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ zebrafish at 125 dpf. Scale bars: 20 µm.

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targets of Prmt5 (Ancelin et al., 2006; Kim et al., 2014; Pal et al., 2004; Stopa et al., 2015). Prmt5 was ubiquitously expressed but was most highly expressed in the testes (Fig. 5A). Notably, the symmetrical dimethylation of arginine was higher in both the testes and ovaries, whereas H3R8me2s was highest in the testes (Fig. 5A). We also observed the symmetrical dimethylation of arginine in the testes and ovaries of adult wild-type zebrafish (4 mpf) using immunofluorescent assays (Fig. S5). These data indicated that *prmt5* plays an important role in the testes and, to a lesser extent, in the ovaries. As expected, no Prmt5 protein expression was detected in *prmt5*-null gonads (Fig. 5B). We did not identify the symmetrical dimethylation of arginine, H3R8me2 s or H4R3me2s in any $prmt5^{-/-}$ gonads that were evaluated (Fig. 5B). Interestingly, the protein expression levels of Ziwi, Zili and Vasa were dramatically reduced in the $prmt5^{-/-}$ gonads compared with the $prmt5^{+/+}$ testes (Fig. 5B). These data suggested that the deletion of *prmt5* might impact Ziwi, Zili and Vasa protein expression. Alternatively, owing to fewer germ cells in the $prmt5^{-/-}$ gonads compared with the $prmt5^{+/+}$ testes, it might also cause lower signaling of Ziwi, Zili and Vasa proteins in the $prmt5^{-/-}$ gonads.

We then used anti-Ziwi, anti-Zili and anti-Vasa antibodies to pull down endogenous Ziwi, Zili and Vasa proteins in the zebrafish testes, and examined their levels of arginine methylation using the anti-Sym11 antibody (against the symmetrical dimethylation of arginine). We identified symmetrical arginine dimethylation of both Zili and Vasa (Fig. 5C). To our surprise, we did not detect

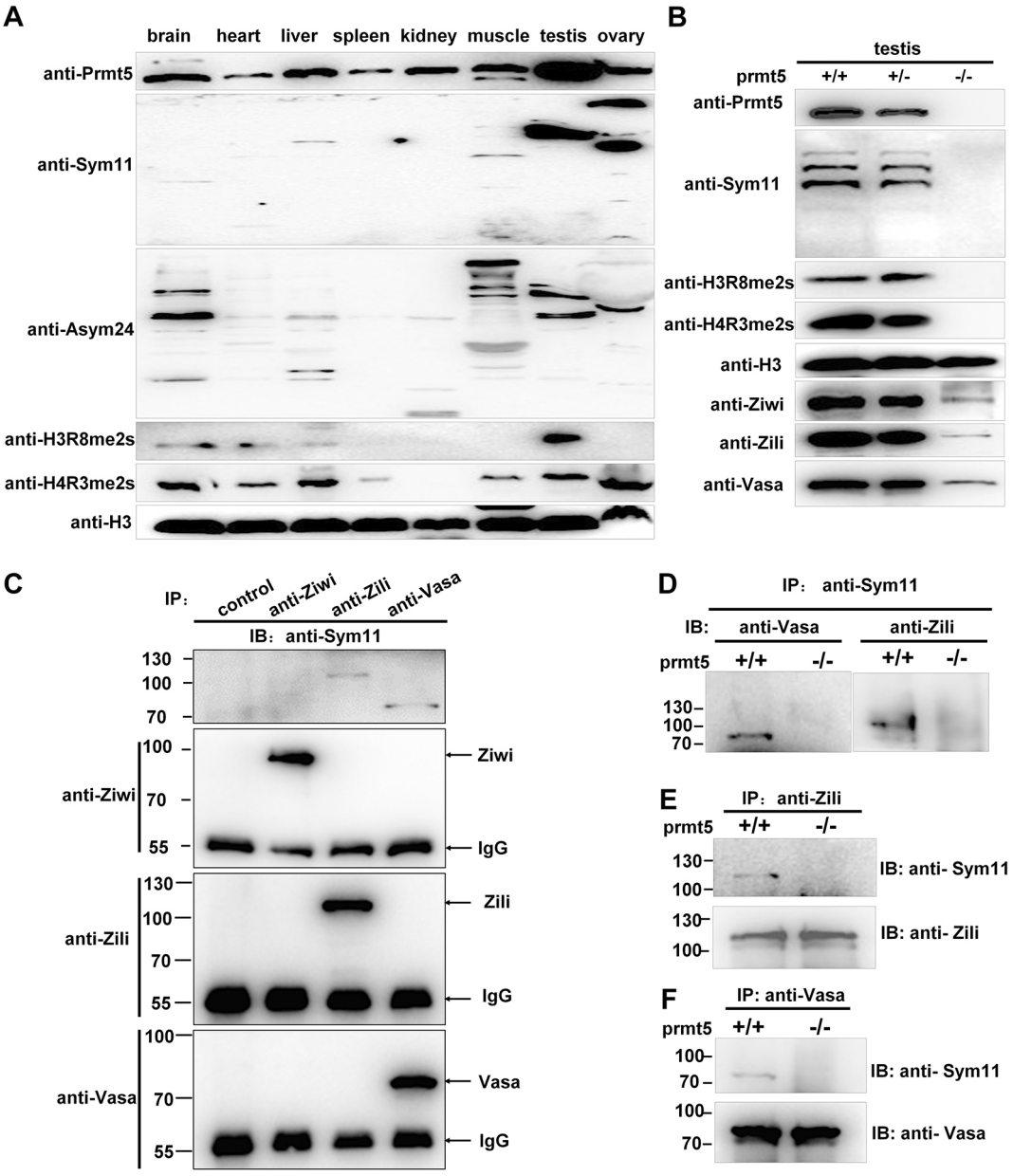

Fig. 5. *prmt5* is required for the arginine methylation of Vasa and Zili proteins. (A) Prmt5, symmetric dimethylarginine (sDMA), asymmetric dimethylarginine (aDMA), H3R8me2s, H4R3me2s and H3 in the tissues of adult zebrafish at 3 mpf. sDMA (anti-Sym11) and aDMA (anti-Asym24) panels have been vertically compressed to optimize display layout. (B) Prmt5, sDMA, aDMA, H3R8me2s, H4R3me2s, Ziwi, Zili and Vasa in the testes of adult (4 mpf) $prmt5^{+/+}$ and $prmt5^{+/-}$, and the gonads of $prmt5^{-/-}$ zebrafish. (C) sDMA of Zili and Vasa, but not Ziwi in the testes of adult zebrafish (4 mpf). (D) In gonads of $prmt5^{-/-}$ zebrafish, sDMA of Zili and Vasa was lacking. (E,F) The lack of sDMA of Zili and Vasa in $prmt5^{-/-}$ gonads.

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symmetrical arginine dimethylation of the Ziwi protein (Fig. 5C) (Chen et al., 2009). It has been shown that zebrafish Tudor-domain-containing protein 1(Tdrd1) interacts with both Ziwi and Zili (Huang et al., 2011). The binding of the Tudor domain to the Piwi protein requires symmetrically dimethylated arginine (sDMA) residues in the N-terminal tail of the Piwi protein (Kirino et al., 2009; Nishida et al., 2009; Reuter et al., 2009; Vagin et al., 2009). However, such sDMA residues were only identified in the Zili protein, not in the Ziwi protein (Huang et al., 2011). This might explain the reason why symmetrically dimethylated arginine residues were not detected in the zebrafish Ziwi protein. These data suggested that, *in vivo*, arginine symmetrical dimethylation mainly occurs on the Zili protein.

To further test the levels of arginine methylation of Vasa and Zili, we used the anti-Sym11 antibody to pull down proteins in the testes of *prmt5*$^{+/+}$ zebrafish and the gonads of *prmt5*$^{-/-}$ zebrafish. We then examined the proteins using anti-Vasa and anti-Zili antibodies. In the *prmt5*$^{+/+}$ testes, sDMA was detected in the Vasa and Zili proteins (Fig. 5D). However, in the *prmt5*$^{-/-}$ gonads, sDMA was not detected in either the Vasa and the Zili proteins (Fig. 5D). We then used anti-Zili and anti-Vasa antibodies to pull down Zili and Vasa from the testes of *prmt5*$^{+/+}$ zebrafish and the gonads of *prmt5*$^{-/-}$ zebrafish. The protein expression levels of Zili and Vasa were much lower in the gonads of *prmt5*$^{-/-}$ zebrafish compared with the testes of the *prmt5*$^{+/+}$ zebrafish. Based on the protein levels of Zili and Vasa detected in the pilot experiments, we adjusted the loading amounts to ensure that the protein levels of Zili and Vasa were the same in the *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ zebrafish. We then examined Zili and Vasa arginine methylation using anti-Sym11 antibody. Consistent with the results of the anti-Sym11 antibody pull down, sDMA was detected in the Zili and Vasa proteins from the *prmt5*$^{+/+}$ zebrafish, but not from *prmt5*$^{-/-}$ zebrafish (Fig. 5E and F).

To further determine whether the deletion of *prmt5* impacts Vasa and Zili protein expression. We performed Flow cytometry analysis (Gazitt et al., 2004). Interestingly, in addition to the reduction of Vasa- and Zili-positive cells, the expression levels of Vasa and Zili indicated by the mean fluorescence intensity were also decreased in the gonads of *prmt5*$^{-/-}$ compared with the testes of *prmt5*$^{+/+}$ (Fig. S6). These finding indicated that loss of *prmt5* not only causes defects in germ cell development, but also induces downregulation of Vasa and Zili.

These data suggest that *prmt5* is required for arginine methylation of the Zili and Vasa proteins, and stabilizing Zili and Vasa. Moreover, *prmt5* is also required for H3R8me2s and H4R3me2s.

Vasa and Zili were methylated at specific arginine residues, and Prmt5 directly catalyzes the symmetric dimethylarginine of Vasa and Zili

To identify the specific dimethylated arginine residues of Vasa and Zili, and to further confirm these arginine residues by mutation assays would provide more direct evidence to support that Vasa and Zili are dimethylated by Prmt5 in zebrafish. Therefore, we performed mass spectrometry analysis of Vasa and Zili after co-immunoprecipitation using anti-Vasa antibody or anti-Zili antibody, respectively. Fig. 6A shows that the dimethylated arginine residues of Vasa and Zili have high scores (>39.0), and Fig. 6B indicates the dimethylated arginine residues in the Vasa or Zili peptides. Seven arginine residues in Vasa, including R101, R105, R177, R179, R183, R197 and R201, and two arginine residues in Zili, including R68 and R221, were indeed dimethylated *in vivo* (Fig. 6A,B). Subsequently, we used an *in vitro* methylation assay to examine whether Vasa and Zili were directly dimethylated by Prmt5 using an anti-Sym 11 antibody. As expected, the Vasa mutant with seven arginines to lysines (Vasa-7M: R101K, R105K, R177K, R179K, R183K, R197K and R201K) and the Zili mutant with two arginines to lysines [Zili (1-133aa)-R68/221K] could not be dimethylated by Prmt5; however, their corresponding wild-type peptides [Vasa and Zili (amino acids 1-300)] could be readily dimethylated by Prmt5 (Fig. 6C,D). These data suggest that *prmt5* catalyzes arginine methylation in the Zili and Vasa proteins at specific arginine residues *in vivo*.

Deletion of *prmt5* reduces symmetric dimethylarginine of Vasa and Zili during early embryogenesis

We earlier showed that the deletion of *prmt5* causes a reduction in symmetric dimethylarginine of Vasa and Zili in adult zebrafish (4 mpf) by western blotting (Fig. 5B,D,E,F). However, the reduction of germ cells in early embryogenesis resulting from deletion of *prmt5* might cause stage differences in GC development between *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ adult zebrafish. If the arginine dimethylation occurred at only a specific stage of GC development and that stage was missing in *prmt5*$^{-/-}$ zebrafish, then dimethylation should not be detected. Therefore, to clarify this concern, we further selected zebrafish at the earlier stage (1 mpf) to examine symmetric dimethylarginine of Vasa and Zili. Because of the difficulty in obtaining enough gonad tissues from 1-month-old larvae to conduct western blotting when compared with adult zebrafish (4 mpf), we used immunofluorescence staining. Overall, fewer germ cells were detected in the gonads of the *prmt5*$^{-/-}$ zebrafish compared with the gonads of the *prmt5*$^{+/+}$ zebrafish at 1 mpf stage (Fig. S7). In order to reliably compare symmetric dimethylarginine levels in Vasa and Zili between *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ gonads, we selected the tissue sections with comparable numbers of germ cells from *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ gonad sections (1 mpf stage) for subsequent assays. We examined the expression patterns of Vasa and Prmt5, or Zili and Prmt5. In both ovaries and testes of 1 mpf larvae, Prmt5 was co-expressed with Vasa or Zili (Fig. 7A,B). In *prmt5*$^{-/-}$ gonads, the Vasa-positive cells were comparable with those of in *prmt5*$^{+/+}$ gonads, but symmetric dimethylarginine in Vasa was almost undetectable (Fig. 7C). Similar results were obtained for the Zili protein (Fig. 7D). These data suggest that loss of *prmt5* caused a reduction in symmetric dimethylarginine in Vasa and Zili at the early stage. Based on the dramatic reduction in symmetric dimethylarginine in Vasa and Zili but substantial existence of Vasa and Zili proteins in the gonads of 1-month-old *prmt5*$^{-/-}$ larvae, it appeared that the observed difference in symmetric dimethylarginine in Vasa and Zili between *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ zebrafish was not simply a consequence of the relevant stage not being present in *prmt5*$^{-/-}$ zebrafish.

Deletion of *prmt5* reduces H3R8me2s and H4R3me2s levels in the gonads

Histones are primary targets of Prmt5, and arginine methylation of histones by Prmt5 has been shown to play an important role in gonadogenesis (Ancelin et al., 2006; Kim et al., 2014; Wang et al., 2015a). Thus, to investigate whether *prmt5* loss impacts H3R8me2s and H4R3me2s, two classic targets of Prmt5 catalysis were employed (Kim et al., 2014; Pal et al., 2004). We quantified H3R8me2s and H4R3me2s levels in the testes of adult zebrafish using specific antibodies.

Similar to evaluation of symmetric dimethylarginine levels in Vasa and Zili, 1-month-old larvae (30 dpf) were examined to avoid potential effects due to differences in PGC staging. As shown in Fig. 8A,B, we could not detect both H3R8me2s and H4R3me2s in the *prmt5*$^{-/-}$ gonads by immunofluorescence staining, but they were obvious in the *prmt5*$^{+/+}$ gonads (Fig. 8A,B). This suggested

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A

Protein	Modified sequences	Position	Score for location
Vasa	_DGGGDESGKR(dimethyl)GFGRGGFR_	197	131.24
Vasa	_GFGR(dimethyl)GGFR_	201	107.32
Vasa	_GR(dimethyl)GGFR(di)GGFR_	101	105.1
Vasa	_GR(di)GGFR(dimethyl)GGFR_	105	105.1
Vasa	_GR(dimethyl)GR(di)GGFR(di)GGFR_	177	39.702
Vasa	_GR(di)GR(dimethyl)GGFR(di)GGFR_	179	39.702
Vasa	_GR(di)GR(di)GGFR(dimethyl)GGFR_	183	39.702
Zili	_FGR(dimethyl)GITQSIAAEPLVGMA_	68	44.587
Zili	_SEESISFLGR(dimethyl)_	221	68.893

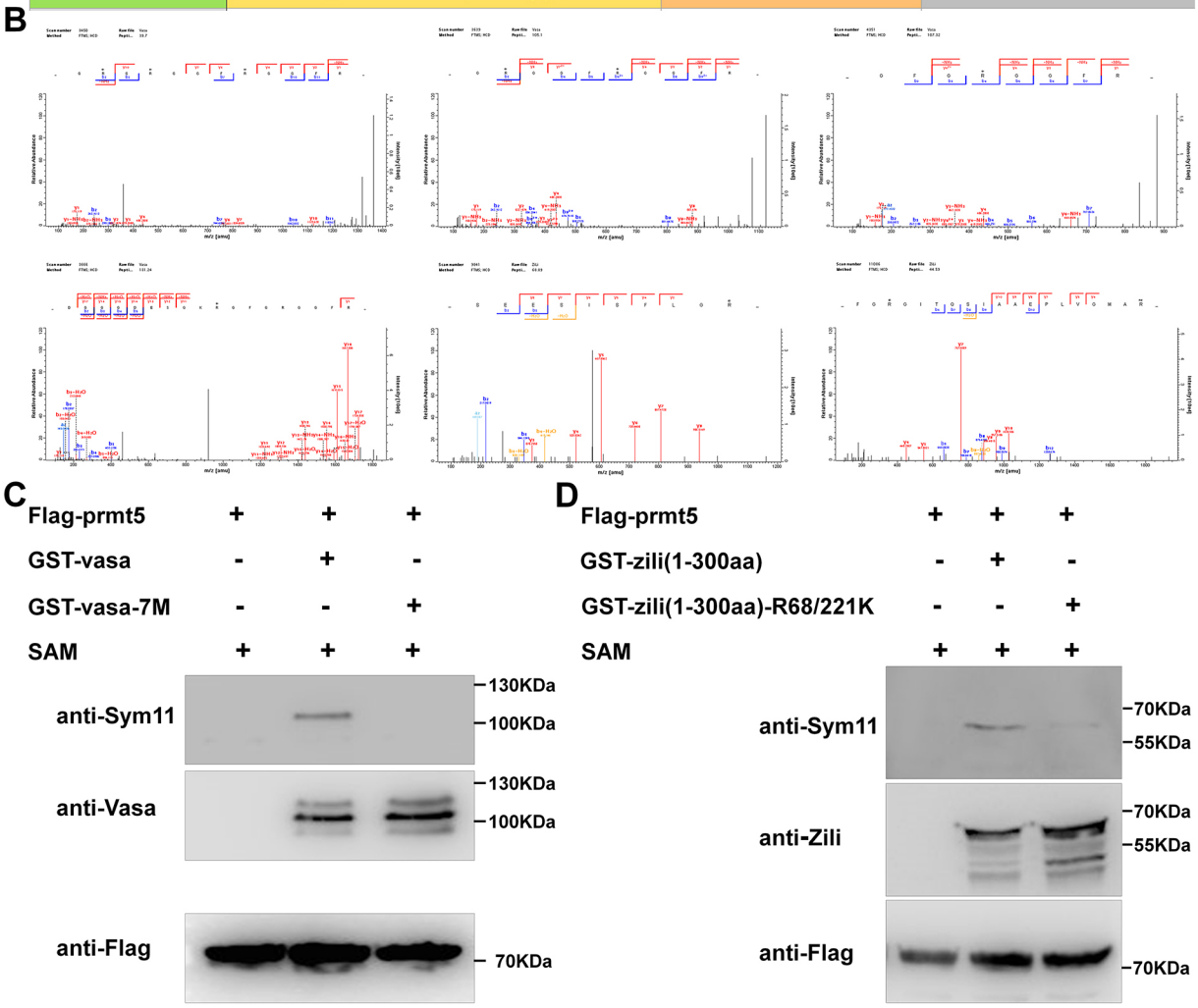

Fig. 6. The dimethylation of arginine in Vasa and Zili is identified and Prmt5 catalyzes symmetric dimethylation of arginine in Vasa and Zili *in vitro*. (A) Dimethylarginine regions in Vasa and Zili. Vasa and Zili were purified from zebrafish testes (4 mpf) and further used for mass spectrometric analysis. (B) The representative pictures of the corresponding MS/MS spectra of methylated peptides from Vasa and Zili annotated with a comprehensive series of b and y fragment ions. (C) Prmt5 catalyzed symmetric dimethylation of arginine in Vasa *in vitro*, but not in the mutants. (D) Prmt5 catalyzed symmetric dimethylation of arginine in Zili (1-300 aa) *in vitro*, but not in the mutant Zili(1-300 aa)-R68/221K.

that loss of *prmt5* caused a reduction in H3R8me2s and H4R3me2s during early embryogenesis.

At 90 dpf, *prmt5*$^{-/-}$ gonads lacked spermatocytes and contained a few spermatogonia, but some pyknotic cells were observed (Fig. 8C). In contrast, the testes of the wild-type siblings (*prmt5*$^{+/+}$) contained abundant spermatogonia, spermatocytes, spermatid and spermatozoa (Fig. 8C). From 104 to 125 dpf, degenerate lumens gradually formed and enlarged in the *prmt5*$^{-/-}$ gonads, although Leydig cells remained (Fig. 8D,E).

From 90 to 125 dpf, levels of both H3R8me2s and H4R3me2s were much lower in the gonads of the *prmt5*$^{-/-}$ zebrafish compared with those of the *prmt5*$^{+/+}$ zebrafish (Fig. 8C-E). However, as development progressed, levels of H3R8me2s and H4R3me2s in different cell types extensively varied in both the *prmt5*$^{-/-}$ and the

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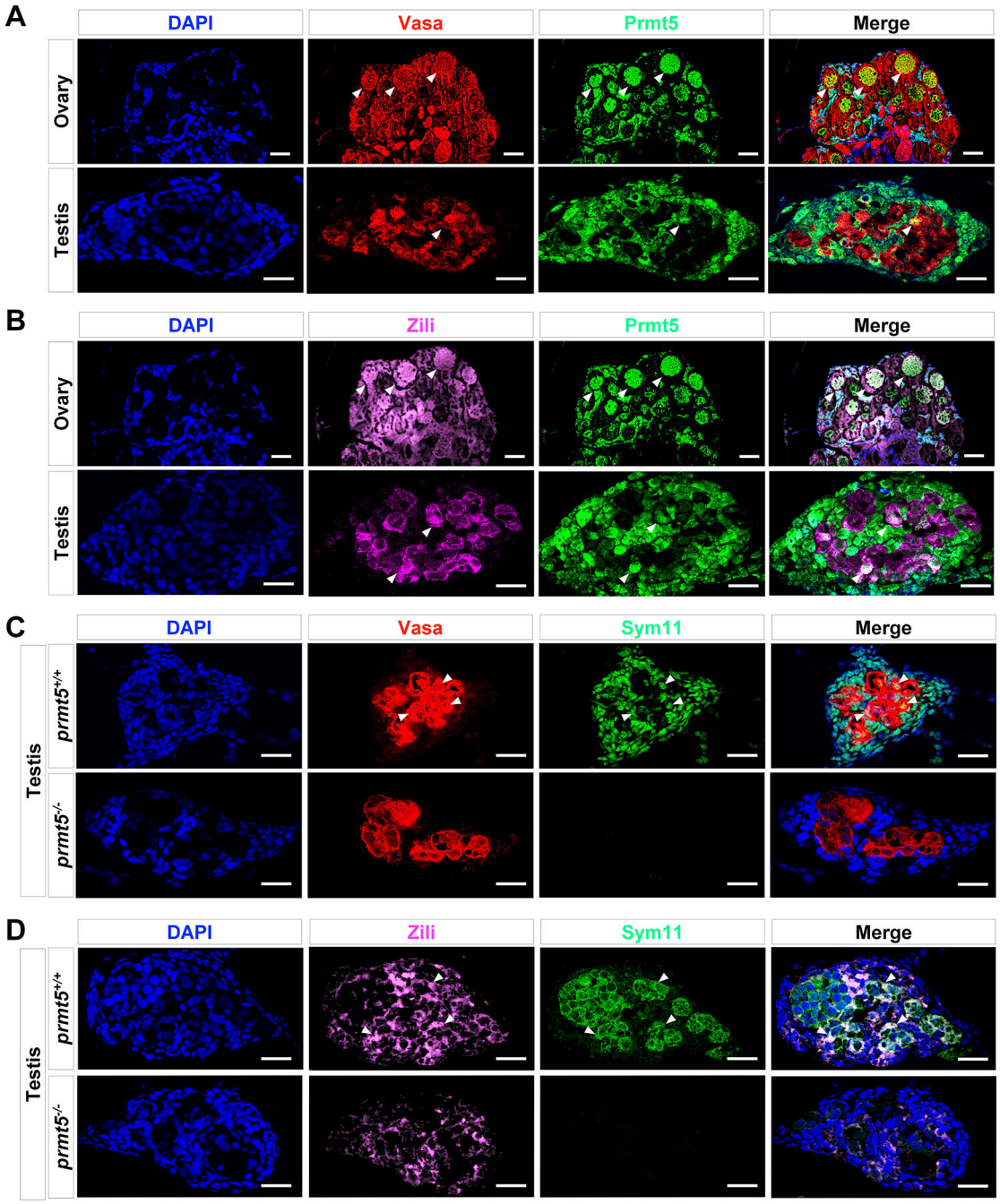

Fig. 7. Co-expression of Prmt5 with Vasa or Zili in both ovaries and testis, and disruption of *prmt5* leads to reduction of symmetric dimethylarginine levels in Vasa and Zili. (A) Vasa (red), Prmt5 (green) and DNA (DAPI staining; blue) in wild-type zebrafish ovaries (*n*=6) and testes (*n*=6) at 1 mpf. Vasa-positive and Prmt5-positive cells are indicated (white arrowheads). (B) Zili (red), Prmt5 (green) and DNA (DAPI staining; blue) in wild-type zebrafish ovaries (*n*=6) and testis (*n*=6) at 1 mpf. Zili-positive and Prmt5-possitive cells are indicated (white arrowheads). (C) Vasa (red), Sym11 (symmetric dimethylarginine antibody, green) and DNA (DAPI staining, blue) in *prmt5*$^{+/+}$ testes (*n*=6) and *prmt5*$^{-/-}$ gonads (*n*=6) at 1 mpf. Vasa-positive and Sym11-positive cells are indicated (white arrowheads). (D) Zili (red), Sym11 (green) and DNA (DAPI staining, blue) in *prmt5*$^{+/+}$ testes (*n*=6) and *prmt5*$^{-/-}$ gonad (*n*=6) at 1 mpf. Zili-positive and SYM11-positive cells are indicated (white arrowheads). Scale bars: 20 µm.

prmt5$^{+/+}$ zebrafish. In the *prmt5*$^{+/+}$ testes, both H3R8me2s and H4R3me2s were strongly detected in the spermatogonia, spermatocytes and spermatid (st) (Fig. 8C-E). At 90 dpf, either H3R8me2s or H4R3me2s were lower in *prmt5*$^{-/-}$ gonad cells, compared with the *prmt5*$^{+/+}$ gonad cells. Notably, H3R8me2s and H4R3me2s persisted in the Sertoli cells of the *prmt5*$^{-/-}$ gonads, but these modified histones were not detected in Leydig cells (Fig. 8D-F). This phenomenon may be worth further study. Our results suggest that *prmt5* loss also impacts H3R8me2s and H4R3me2s during gonadal development.

Deletion of *prmt5* leads to the dysregulation of genes associated with gonadal development

To identify the mechanisms by which *prmt5* loss affects germ cell development, we examined the expression levels of various gonadal development-related genes, including *vasa*, *dnd*, *ziwi*, *sycp3*,

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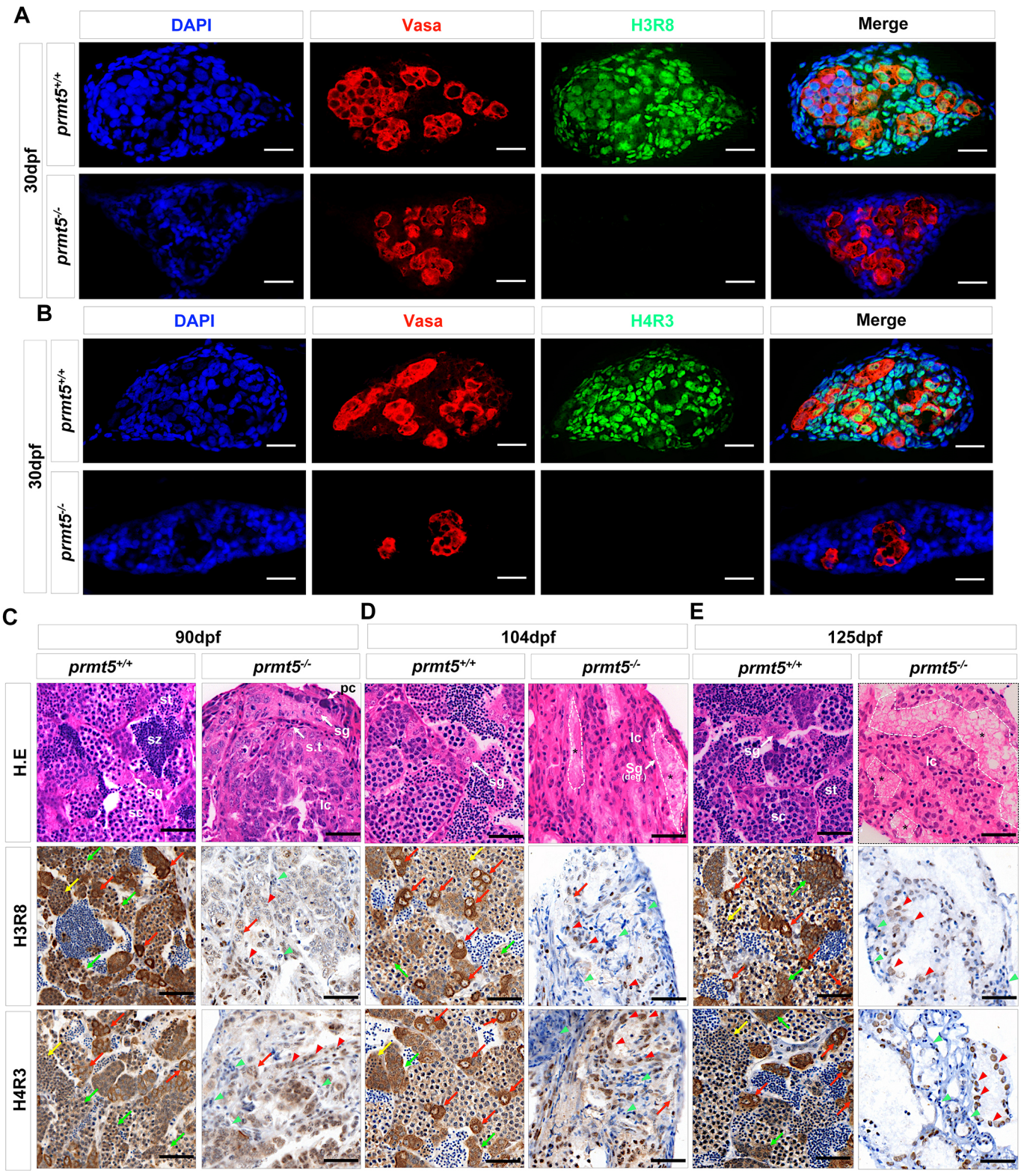

Fig. 8. See next page for legend.

cyp11c1, *sox9a*, *amh* and *cyp19a1a* (Hartung et al., 2014; Houwing et al., 2007; Liu and Collodi, 2010; Siegfried and Nüsslein-Volhard, 2008; Sun et al., 2013). At 1 mpf (during sexual differentiation), the gene expression levels of *vasa*, *dnd1*, *ziwi*, *sycp3* and *cyp19a1a* were much lower in the *prmt5*$^{-/-}$ gonads than in the *prmt5*$^{+/+}$ gonads (Fig. S8A). In contrast, the gene expression levels of *cyp11c1* and *amh* were higher in the *prmt5*$^{-/-}$ gonads than in the *prmt5*$^{+/+}$ gonads. The gene expression of *sox9a* was similar in the *prmt5*$^{-/-}$ and *prmt5*$^{+/+}$ gonads. Thus, *prmt5*$^{-/-}$ gonads potentially began to differentiate into testes at the beginning of the sexual differentiation period. At 2 mpf (after sexual differentiation), the gene expression levels of *vasa*, *dnd*, *ziwi* and *sycp3* remained lower

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Fig. 8. The deletion of *prmt5* in zebrafish results in reduction of H3R8me2s and H4R3me2s. (A,B) Vasa (red), H3R8me2s (green) (A), H4R3me2s (green) (B) and DNA (DAPI staining; blue) in *prmt5*$^{+/+}$ testes (*n*=6) and *prmt5*$^{-/-}$ gonads (*n*=6) at 1 mpf. No H3R8me2s-positive cells (A) or no H4R3me2s-positive cells (B) were detected in *prmt5*$^{-/-}$ gonads. (C-E) Hematoxylin and Eosin staining, and H3R8me2s and H4R3me2s staining in wild-type sibling controls (*prmt5*$^{+/+}$) and *prmt5* homozygous mutants (*prmt5*$^{-/-}$). At 90 dpf (C), the testes of *prmt5*$^{+/+}$ zebrafish showed typical testicular morphology, with abundant spermatogonia (sg), spermatocytes (sc), spermatid (st) and spermatozoa (sz). s.t, seminiferous tube. The gonads of *prmt5*$^{-/-}$ had pyknotic cells (pc), but few spermatogonia (sg) and no spermatocytes (sc). At 104 dpf (D), in the gonads of *prmt5*$^{-/-}$ zebrafish, lumens (indicated with white dashed lines) began to enlarge, while remaining germ cells were replaced by Leydig cells (lc) and Sertoli cells (SC). The spermatogonia in the lumens gradually degenerated (Sg deg., asterisk). At 125 dpf (E), in the gonads of *prmt5*$^{-/-}$ zebrafish, lumens (indicated with white dashed lines) underwent further enlargement, germ cells were replaced by Leydig cells (lc) and the spermatogonia in the lumens degenerated. Spermatogonia, red arrows; spermatocytes, green arrows; Leydig cells, green arrowheads; Sertoli cells, red arrowheads. Scale bars: 20 µm.

in the *prmt5*$^{-/-}$ gonads when compared with the *prmt5*$^{+/+}$ gonads (Fig. S8B). Interestingly, in the *prmt5*$^{-/-}$ gonads at 2 dpf, *cyp11c1* and *amh* were downregulated when compared with the *prmt5*$^{+/+}$ gonads, but *cyp19a1a* was upregulated. This implied that the *prmt5*$^{-/-}$ gonads were not exactly testes, even though they appeared phenotypically male. At 4 mpf, the expression patterns of the gonadal development-related genes were similar to those at 2 mpf, except for *cyp11c1* in the *prmt5*$^{-/-}$ gonads: at 4 mpf, *cyp11c1* expression was similar in the *prmt5*$^{+/+}$ gonads and the *prmt5*$^{-/-}$ gonads (Fig. S8C). These results suggested that the deletion of *prmt5* leads to the dysregulation of gonadal development-associated genes, resulting in defects in gonad development.

Based on the data obtained in this study, we propose a working model for *prmt5* in zebrafish germ cell development (Fig. S9). During gonad development, prmt5 catalyzes the symmetrical arginine dimethylation of germ cell-specific proteins (e.g. Vasa and Zili) in gonad cells, leading to the stabilization of Piwi pathway proteins. The Piwi pathway regulates piRNA biogenesis and modulates germline-specific gene expression. In contrast, Prmt5 also catalyzes the symmetrical arginine dimethylation of histones (including H3R8me2s and H4R3me2s) to regulate germline-specific gene expression. Therefore, *prmt5* maintains germ cell survival, differentiation and meiosis, as well as inhibition of apoptosis.

DISCUSSION

The mechanisms by which *prmt5* affects germ cell development

The role of *prmt5* in germ cell development was first described in *Drosophila*: the genetic disruption of *dart5*, which is the fly ortholog of human *PRMT5*, results in male sterility and a slight reduction in female fecundity (Gonsalvez et al., 2006). Therefore, *dart5* is considered to be essential to germ-cell specification and maintenance (Gonsalvez et al., 2006). In mice embryos between embryonic day 7.5 (E7.5) and E11.5, *Prmt5* cooperates with *Blimp* (a key regulator of PGC specification) to establish and propagate unipotent PGCs and to promote PGC specification (Ancelin et al., 2006; Tee et al., 2010). The conventional knocking out of *Prmt5* in mice causes embryonic lethality before implantation, indicating that *Prmt5* plays an essential role in early mouse development (Tee et al., 2010). The conditional loss of *Prmt5* in PGCs causes complete male and female sterility, suggesting that *Prmt5* also plays a crucial role in germ cell development (Kim et al., 2014; Li et al., 2015; Wang et al., 2015a,b). However, *Prmt5* may not be required for PGC specification in mice, but may instead keep PGCs in the cell cycle after specification (Li et al., 2015). Here, gonads of *prmt5*$^{-/-}$ zebrafish failed to differentiate into normal testes or ovaries, leading to the expression of an infertile male phenotype. Further investigation suggested that *prmt5* was required for germ cell survival, maintenance and differentiation, re-enforcing the essential role of *prmt5* in germ cell development. We also noticed that *prmt5* loss in zebrafish reduced, but did not eliminate, the PGCs marked by Vasa, suggesting that *prmt5* might not be required for PGC formation. However, because we were unable to mate *prmt5*$^{+/-}$ (♂) with *prmt5*$^{-/-}$ (♀) due to the infertility/non-viability of *prmt5*-null females, we could not rule out the possibility that the maternal *prmt5* is required for PGC formation.

Even though the importance of *Prmt5* in germ cell development is well recognized, the mechanisms by which *prmt5* affects germ cell development remain largely unknown. Multiple *Prmt5* targets have been identified to date (Stopa et al., 2015). Although *Prmt5* is a well-defined Sm-protein methyltransferase, *Prmt5*-induced Sm protein methylation does not affect germ-cell development in *Drosophila* (Gonsalvez et al., 2006). Based on the interactions between Blimp and Prmt5 in a mouse model, it has been proposed that H2A/H4 R3 methylation by Prmt5 regulates mouse germ-cell lineage (Ancelin et al., 2006). Similarly, it is thought that Prmt5 methylates H2AR3, H3R2 and H4R3 to affect mouse germ cell development (Tee et al., 2010; Wang et al., 2015a,b). *Prmt5* may also influence PGCs by protecting genomic integrity during global DNA demethylation, resulting in the downregulation of the LINE1 and IAP transposons, and inhibition of the DNA damage response (Kim et al., 2014). As histones are classic *Prmt5* targets (Blanc and Richard, 2017; Karkhanis and Joshi, 2011; Stopa et al., 2015; Wesche et al., 2017), it is understandable that *Prmt5* function has primarily been explained in the context of the arginine methylation of histones (Ancelin et al., 2006; Kim et al., 2014; Tee et al., 2010; Wang et al., 2015a,b). However, histone proteins are ubiquitously expressed, so the disruption of histone function may have whole body effects, and may not be restricted to specific tissues. In addition, the loss of *Prmt5* function causes some specific phenotypes in addition to embryonic lethality (Batut et al., 2011; Liu et al., 2015). Indeed, the tissue-specific phenotypes generated as a result of *Prmt5* loss might be a consequence of its role modifying tissue-specific genes.

In *Drosophila*, Piwi proteins are arginine methylated by PRMT5, which is probably required for PGC specification (Gonsalvez et al., 2006). In mice, arginine-methylated Miwi and Mili have been identified, which are required for binding to Tudor domains of Tdrd proteins. This implies that the arginine methylation of Miwi and Mili may play a role in germinal granule/nauge formation, germ cell specification and germ cell differentiation (Chen et al., 2009). Interestingly, zebrafish Tdrd1 Tudor domains specifically interact with arginine-methylated Zili protein, suggesting that the arginine methylation of Zili is essential for efficient Piwi pathway activity and proper nuage formation (Huang et al., 2011). Indeed, *Drosophila* PRMT5 is required for the arginine methylation of Vasa *in vivo*, and arginine methylated Vasa proteins have been identified in mice and *Xenopus*, implying a role in germline development (Kirino et al., 2010). Therefore, the arginine methylation of Piwi and Vasa protein by Prmt5 may be essential for germ cell development. However, to our knowledge, no studies have yet shown this connection *in vivo*, particularly in a vertebrate model. Here, we find that zebrafish *prmt5* is required for germ cell survival, maintenance and differentiation. In addition, the loss of *prmt5* reduced arginine methylation of Zili and Vasa, thereby

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destabilizing the proteins in the Piwi pathway. This result suggested a possible mechanism underlying the role of *prmt5* in germ cell development.

We also noticed that *prmt5* loss reduced H3R8me2s and H4R3me2s levels in the gonads. Interestingly, Vasa mRNA expression was also dramatically reduced in the *prmt5*-null gonads, suggesting that Vasa was also regulated by *prmt5* at the transcriptional level. Indeed, it is possible that the dysregulation of histone arginine methylation affects *vasa* at the transcriptional level. Therefore, arginine methylation of germline-specific proteins, including Zili and Vasa might operate synergistically with arginine methylation of histones by *prmt5* to control germ cell development during gonadogenesis.

During the early embryonic stage (prim5), we observed that there were fewer germ cells in *prmt5*-null embryos. Vasa tracing indicated that the migration of PGCs was disturbed after disruption of *prmt5*, leading to ectopic location of PGCs out of the genital ridge. This might subsequently cause apoptosis of PGCs (Dumstrei et al., 2004; Schlueter et al., 2007; Tzung et al., 2015). In 21 dpf and 125 dpf embryos, we also noticed that more germ cells underwent apoptosis, suggesting that disruption of *prmt5* might mainly induce apoptosis instead of causing proliferation defects in germ cells. It appears that the mechanisms of *prmt5* deletion in inducing germ cell apoptosis differ between the early and the later embryonic stages. At the early embryonic stages (from prim5 to 54 hpf), the deletion of *prmt5* initially affects PGC migration, subsequently causing apoptosis. However, at the later stages (21 dpf and 125 dpf), it seems that the deletion of *prmt5* does not affect germ cell migration, but still induces apoptosis. Elucidation of the different mechanisms involved in the phenomenon is warranted.

prmt5 plays an important role in snRNP homeostasis, which facilitates an essential step in the formation of translatable mRNAs (Fischer et al., 2011; Matera and Wang, 2014; Meister and Fischer, 2002; Prusty et al., 2017). Therefore, disruption of *prmt5* might also influence mRNA splicing process of certain genes, leading to defects of gonadogenesis that were exhibited by the *prmt5*-null zebrafish (Horiuchi et al., 2018; Kasowitz et al., 2018). Further investigate this will help us to fully understand the mechanisms by which *prmt5* influences germ cell development.

In zebrafish, in addition to *prmt5*, other Prmt genes also exist, similar to mammals (Dong et al., 2007; Hung and Li, 2004; Tsai et al., 2011). However, it appears that other Prmt genes are not redundant with *prmt5* in influencing germ cell development because disruption of *prmt5* alone causes dramatic defects of gonad differentiation in zebrafish.

prmt5 affects the proteins of the Piwi pathway

Multiple lines of evidence indicate that the Piwi pathway is required for efficient transposon silencing, germline differentiation and meiosis, which are essential for germ cell development (Ghildiyal and Zamore, 2009; Houwing et al., 2008, 2007; Huang et al., 2011). The zebrafish Piwi proteins consist of Ziwi and Zili (Houwing et al., 2008). The Piwi pathway also include Vasa and the Tudor domain-containing proteins (Huang et al., 2011; Kirino et al., 2010). Tdrd1 has been identified as molecular scaffold that is crucial for efficient Piwi-pathway activity and proper nuage formation (Huang et al., 2011). It appears that the arginine methylation of Zili is required before Tdrd association and all subsequent functions (Chen et al., 2009; Huang et al., 2011). In addition, it is evident that Piwi pathway proteins collaborate to control piRNA biogenesis, which silences transposons to influence germ cell development in the gonads (Huang et al., 2011). Therefore, these major components of the Piwi pathway (Zili, Ziwi, Tdrd1 and Vasa) should act reciprocally.

Here, we not only detected an abrogation in arginine methylation of Zili and Vasa after *prmt5* loss, but also observed that other Piwi-pathway components, such as Ziwi and Tdrd1, are also affected. This result further supported the close relationship among Zili, Vasa, Ziwi and Tdrd1 (Houwing et al., 2008, 2007). Arginine methylation directly enhances the stability of Zili and Vasa, which in turn indirectly affects the stability of Ziwi and Tdrd1. However, the observed increase in the stability of the Zili and Vasa after arginine methylation requires further investigation using biochemical approaches.

MATERIALS AND METHODS

Generation of *prmt5*-null zebrafish

We used CRISPR/Cas9 to disrupt *prmt5* in zebrafish. *prmt5* sgRNA was designed using CRISPR design tool (crispr.mit.edu). The zebrafish codon-optimized Cas9 plasmid was digested with XbaI, then purified and transcribed using the T7 mMessagem Machine Kit (Ambion) (Liu et al., 2016a). pUC19-gRNA vector was used for amplifying *prmt5* sgRNA (Liu et al., 2016b). The primers for amplifying *prmt5* sgRNA template were as follows: 5′-TGTAATACGACTCACTATAGGTGGAACAGCGGCATAC-ACGTTTTAGAGCTAGAAATAG-3′ and 5′-AAAAGCACCGACTCGG-TGCC-3′. sgRNA was synthesized using the Transcript Aid T7 High Yield Transcription Kit (Fermentas).

Zebrafish (*D. rerio*) strain AB was raised, maintained, reproduced and staged according to standard protocols. Cas9 RNA (0.75-1.25 ng/per embryo) and sgRNA (0.075 ng/per embryo) were mixed and injected into embryos at the one-cell stage. Mutations were initially detected using a HMA assays as previously described (Liu et al., 2016a). If the HMA results were positive, then the remaining embryos were raised up to adulthood as the F0 generation, and were then back-crossed with wild-type zebrafish (strain AB) to generate the F1 generation, which were genotyped by HMA initially and confirmed by sequencing of targeting sites. Heterozygous F1s were back-crossed with wild-type zebrafish (strain AB; disallowing offspring-parent mating) to generate the F2 generation. F2 adults carrying the target mutation were inter-crossed to generate the F3 offspring. The F3 generation contained wild-type (+/+), heterozygote (+/−) and homozygote (−/−) individuals. In addition, to exclude off target effects, during phenotype analysis of *prmt5*-null zebrafish, we also kept back-crossing $prmt5^{+/-}$ zebrafish with wild-type zebrafish (strain AB; disallowing offspring-parent mating) for six generations. The F7 $prmt5^{+/-}$ zebrafish were used for cross-breeding, and $prmt5^{-/-}$ (F8) and $prmt5^{+/+}$ (F8) zebrafish were used for phenotype validation. The primers used to identify mutants were: 5′-CAAGACCTGTCCTGTTTGATGA-3′ and 5′-AGCAGGTCAG-ATCGTGTTTG-3′.

The two novel mutants were named following zebrafish nomenclature guidelines (wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Conventions#ZFINZebrafishNomenclatureGuidelines-1.3): $prmt5^{ihb1994/ihb1994}$ (M1) (zfin.org/ZDB-ALT-180319-10), and $prmt5^{ihb1995/ihb1995}$ (M2) (zfin.org/ZDB-ALT-180319-11). To further exclude off target effects, we also performed a complementation test by mating $prmt5^{ihb1994/+}$ (♀)×$prmt5^{ihb1995/+}$ (♂) and validated the phenotypes in the mutant $prmt5^{ihb1994/ihb1995}$.

Zebrafish were maintained in a recirculating water system according to standard protocols. All experiments with zebrafish were approved by the Institutional Animal Care and Use Committee of Institute of Hydrobiology, Chinese Academy of Sciences under protocol number 2016-001.

Antibodies

The antibodies used were as follows: anti-Ziwi antibody was raised in rabbits against the synthetic peptide, MTGRARARSRGRGRGQE-C; anti-Zili antibody was raised in rabbits against the synthetic peptide, MDPKRPTFPSPPGVIRA-C; and anti-Tdrd1 antibody was raised in rabbits against the synthetic peptide, C-RRPATGPSSLSPRGP. Anti-sera were subsequently purified against the synthetic peptide (ABclonal). Other

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antibodies used were as follows: anti-Vasa (GTX128306; GeneTex, 1:1000), anti-sDMA (SYM11; Millipore, 1:1000), anti-aDMA (ASYM24; Millipore, 1:1000), anti-H3R8me2s (A2374, ABclonal, 1:1000), anti-H4R3me2s (A3159, ABclonal, 1:1000), anti-PRMT5 (A11335, ABclonal, 1:1000), anti-H3(A2348, ABclonal, 1:1000), anti-β-actin (AC004, ABclonal, 1:1000), anti-Flag antibody (F1804, 1:1000 for immunoblot analysis, Sigma-Aldrich) and Alexa Fluor 488 goat anti-rabbit IgG (A11008, 1:1000).

Western blot and co-immunoprecipitation

Western blotting and co-immunoprecipitation were performed using antibodies mentioned above. The tissues were homogenized for 1 min at 4°C and centrifuged for 15 min at 13,523 ***g*** at 4°C. The supernatants were used for western blotting or co-immunoprecipitation. For co-immunoprecipitation, protein G Sepharose beads (GE Healthcare), antibody (1:250) and tissue lysate were incubated overnight at 4°C while rotating. The beads were washed three times with RIPA buffer (P0013B, Beyotime) and then boiled with 1× SDS sample loading buffer, separated using 12% SDS-PAGE and transferred to a polyvinylidenedifluoride membrane (Millipore). The Fuji Film LAS4000 mini-luminescent image analyzer was used to image the blots.

Immunohistochemistry and immunofluorescence

Immunohistochemical staining and immunofluorescence staining were performed using the standard protocols. The antibodies used for immunohistochemistry and immunofluorescence are mentioned above.

In situ hybridization

Whole-mount *in situ* hybridization was performed as previously described (Liu et al., 2009). Briefly, paraffin sections were washed with PBST (1× phosphate-buffered saline with 0.1% Tween) and treated with proteinase K for 5 min at room temperature. Subsequently, the sections were fixed in buffered 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 20 min, incubated in preheated HYB (hybridization) buffer at 68°C for 1 h [200 ml HYB buffer: 100 ml formamide, 50 ml 20× SSC (pH 7.0), 1 g Torula yeast ribonucleic acid, 200 µl heparin (50 mg/ml) and 47.8 ml distilled H_2O]. Sections were then hybridized with individual probes overnight (12-16 h) at 68°C. After hybridization, the slides were washed with preheated wash buffer (68°C) (200 ml wash buffer: 100 ml formamide, 10 ml 20× SSC, 200 µl Tween, 89.8 ml distilled H_2O) and MABT [1 l MABT: 200 ml 5× MAB (500 mM maleic acid, 150 mM NaCl, adjusted to pH 7.5 with NaOH), 1 ml 100% Tween20, 799 ml DEPC-treated water]. The sections were blocked with blocking buffer, i.e. MABT:blocking reagent [dissolved (10%w/v) in 1× MAB by shaking and heating]:FBS at a ration of 8:1:1, for at least 1 h at room temperature and incubated with anti-DIG antibody (1:2000) overnight at 4°C. The sections were washed three times (15 min/each) with MABT and staining buffer NTMT [50 ml NTMT contains 5 ml 1 M Tris (pH 9.5), 2.5 ml 1 M $MgCl_2$, 1 ml 5 M NaCl, 50 µl 100% Tween, 50 µl 2 M tetramisol and 41.4 ml DEPC-treated water] twice (5 min/wash). Finally, the sections were incubated with the staining solution (staining solution contains 10 ml staining buffer NTMT, 1 g polyvinylpyrrolidone and 200 µl NBT/BCIP mix) in the dark at room temperature.

RNA isolation and semi-quantitative real-time PCR

Total RNA was extracted from zebrafish testes or ovaries using RNA Isolation Plus Reagent (Takara). Double-stranded cDNA was synthesized using the Revert Aid TM first-strand cDNA synthesis kit (Fermentas). The primers for amplifying *ef1a*, *vasa*, *dnd*, *ziwi*, *sycp*, *cyp11c1*, *sox9a*, *amh* and *cyp19a1a* are listed in Table S1.

Histological analysis and fertility assessment

We dissected the gonadal tissues from *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ adult zebrafish (4 mpf) after anesthesia and calculated the GSI (gonad weight/body weight ×100%). The gonads were fixed in 4% PFA overnight at 4°C and embedded in paraffin wax. The sections (7 µm) were stained with Hematoxylin and Eosin, and examined under a microscope (Olympus BX53). The developmental staging of oogenesis and spermatogenesis were based on previous descriptions (Braat et al., 1999; Leal et al., 2009; Maack and Segner, 2003; Siegfried and Nüsslein-Volhard, 2008; Sun et al., 2013).

The fertilization rate of male adult zebrafish with three genotypes (*prmt5*$^{+/+}$, *prmt5*$^{+/-}$ and *prmt5*$^{-/-}$) obtained by mating *prmt5*$^{+/-}$ (♀)×*prmt5*$^{+/-}$ (♂) was evaluated. All males were mated with wild-type females once a week and the valid mating trials were repeated for more than 3 weeks. After 24 h, the numbers of normal developed embryos were counted as fertilized. The fertilization rate was calculated as the ratio of fertilized eggs divided by the total number of eggs spawned.

Apoptosis assays

TUNEL assays were performed to detect apoptotic cells in the gonads of *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ at 21 dpf (FITC labeling) or 125 dpf (biotin labeling).

Mass spectrometry analysis

To determine arginine methylation sites of Zili and Vasa, we first conducted co-immunoprecipitation to enrich Zili and Vasa proteins from the testis of zebrafish (4 mpf) using anti-Zili and anti-Vasa antibodies respectively. We then separated the immunoprecipitates using 10% SDS-PAGE. After Coomassie Brilliant Blue staining, we excised the band corresponding to the Zili or Vasa protein. Subsequently, the purified Zili or Vasa protein was digested by trypsin and analyzed by online nanoflow LC-MS/MS using the Ultimate 3000 nano-LC system (Dionex) connected to a LTQ-Orbitrap Elite (Thermo Fisher Scientific) mass spectrometer. Samples were injected onto the analytical C18-nanocapillary LC column (C18 resin with 3 µm particle size, 15 cm length 150 mm length×75 µm inner diameter, Acclaim PepMap RSLC, Thermo Fisher Scientific) and eluted at a flow rate of 300 nl/min with a 130 min gradient from 5% solvent B (90% ACN/0.1% formic acid, v/v) to 50% solvent B. The peptides were then directly ionized and sprayed into an Orbitrap Elite mass spectrometer using a nanospray ion source. The mass spectrometer was operated in a data-dependent mode with an automatic switch between MS and MS/MS acquisition. Full MS spectra from m/z 350 to 1800 were acquired with a resolution of 60,000 at m/z 400 in profile mode. Following every survey scan, up to 15 of the most intense precursor ions were selected for MS/MS fragmentation by high-energy collisional dissociation (HCD) with normalized collision energy of 30%. The dynamic exclusion duration was set to be 120 s with a repeat count of one and a ±10 ppm exclusion window. All acquired raw data were processed with pFind software (version 3.1.2) and searched against the NCBI Human Protein Database (www.ncbi.nlm.nih.gov/protein). Then the arginine methylation sites of Zili and Vasa were determined. The estimated false discovery rate (FDR) of peptide identification was <1%.

Flow cytometry

Single cell suspension of *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ gonads (2 mpf) was washed with phosphate-buffered saline (PBS) (pH 7.2) twice, and then fixed and permeabilized using the paraformaldehyde-methanol method. Subsequently, the permeabilized cells were washed and incubated with anti-Vasa or anti-Zili antibodies for 60 min at 4°C. The cells were then washed and incubated with fluorescein isothiocyanate (FITC)-labeled affinity-purified goat anti-rabbit IgG for 60 min at 4°C. After incubation, the cells were washed twice with PBS (pH 7.2), suspended in PBS and then analyzed using a flow cytometer (CytoFlex S). The mock-treated single cell suspension of *prmt5*$^{+/+}$ and *prmt5*$^{-/-}$ gonads was run in parallel and served as negative controls. Ten-thousand positive cells were counted in each treated group. Data analysis was performed using CytExpert 2.0 software. The percentage of positive cells and the mean fluorescence intensity were determined in the defined regions compared with the mock controls based on FITC fluorescence histograms.

Plasmid construction

The wild-type zebrafish Zili and Vasa constructs were kindly provided by René F. Ketting. Zebrafish Zili-1-300 aa, zebrafish Vasa and their mutants Zili-1-300(R68/221K), Vasa-7M (R101K, R105K, R177K, R179K, R183K, R197K, R201K) were constructed using PCR-based mutagenesis and cloned into the PGEX-4T vector. Zebrafish *prmt5* was amplified from zebrafish complementary DNA (cDNA) using PCR and subcloned into pCMV-Flag vector.

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In vitro methylation assay

HEK-293T cells were transfected with Flag-prmt5. After transfection for 24 h, the cells were harvested and lysed with the RIPA buffer. Total cell extracts were incubated with ANTI-FLAG M2 Affinity Gel (Sigma-Adrich) and co-immunoprecipitated. The co-immunoprecipitated Flag-Prmt5 was incubated with the recombinant GST-Vasa, GST-Vasa-7M, GST-Zili (1-300) or GST-Zili(1-300)-R68/221K proteins (1.5 µg). Methylation reactions were conducted using methylation buffer [50 mM Tris-HCl, (pH 8.5), 5 mM $MgCl_2$ and 4 mM DTT] containing 100 µM S-adenosylmethionine (SAM) (B9003S, BioLabs) for 2 h at 30°C. The reactions were stopped by adding 2× SDS loading buffer and boiling the samples for 5 min. The methylation reaction products were separated by SDS-PAGE, transferred to PVDF membranes and analyzed by western blotting using anti-SYM11, anti-Vasa and anti-Zili antibodies.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: W.X., J.Z.; Methodology: J.Z.; Validation: J.Z., G.Y., X.C., F.R.; Formal analysis: W.X., J.Z.; Investigation: W.X., J.Z., F.R.; Resources: D.Z., X.L., G.Y., X.C., C.X., G.O., J.W.; Data curation: D.Z., C.X.; Writing - original draft: W.X., J.Z.; Writing - review & editing: W.X., J.Z.; Visualization: J.Z., X.L.; Supervision: W.X.; Funding acquisition: W.X.

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Supplementary information

Supplementary information available online at
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