Dnmt3aa But Not Dnmt3ab Is Required for Maintenance of Gametogenesis in Nile Tilapia

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Research

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Dnmt3aa but not dnmt3ab is required for maintenance of gametogenesis in Nile tilapia

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Abstract

Background: Dnmt3a, a de novo methyltransferase, is essential for both male and female germ line DNA methylation. Only one Dnmt3a is identified in mammals, and homozygous mutation of Dnmt3a is lethal, while two Dnmt3a, dnmt3aa and dnmt3ab, are identified in teleosts due to the third round of genome duplication, and homozygous mutation of dnmt3aa and dnmt3ab is viable in zebrafish. Dnmt3aa and dnmt3ab were demonstrated to have essential and non-overlapped functions on modulating behavioral control, however, their function in gonadal development is unclear in fish.

Results: In this study, the expression patterns of dnmt3aa and dnmt3ab in developing gonads of Nile tilapia was analyzed by quantitative real time PCR and fluorescence in situ hybridization. Both dnmt3aa and dnmt3ab displayed sexually dimorphic expression in developing gonads. Dnmt3aa was widely expressed in gonadal germ cells and somatic cells, highly expressed in oogonia, phase I and II oocytes and granulosa cells in ovaries and spermatogonia and spermatocytes in testes, while dnmt3ab was mainly expressed in ovarian granulosa cells and
testicular spermatocytes. Mutation of *dnmt3aa* and *dnmt3ab* was achieved by CRISPR/Cas9 in tilapia. Lower GSI (Gonadosomatic index), increased apoptosis of oocytes and spermatocytes and significantly reduced sperm quality were observed in *dnmt3aa*−/+ mutants, while no obvious phenotype was observed in *dnmt3ab*−/+ mutants. Consistently, the expression of apoptotic genes was significantly increased in *dnmt3aa*−/+ mutants. In addition, *dnmt3aa* and *dnmt3ab* were found to have certain compensatory effects in the gonads. The global DNA methylation level in ovaries and testes of *dnmt3aa*−/+ mutants was decreased significantly, compared with that of *dnmt3ab*−/+ mutants and WT.

**Conclusions:** Taken together, our results suggest that *dnmt3aa*, not *dnmt3ab*, plays important roles in maintaining gametogenesis in teleost. Our results enrich the understanding of the function of DNA methyltransferases in gonads of non-mammalian vertebrates.

**Keywords:** Nile tilapia; DNA methylation; Dnmt3a; Gonad development; gametogenesis.
Background

DNA methylation, a mechanism of epigenetics plays crucial role in the control of development related gene expression during gametogenesis and early embryogenesis [1, 2]. During germ cells development, epigenetic reprogramming occurs dynamically, remodeling of DNA methylation marks in particular [3, 4]. At day 7.5 of early embryo (E7.5) in mouse, global DNA methylation of primordial germ cells (PGCs) is erased. Later, De novo DNA methylation proceeds differentially between male and female germ cells, earlier in spermatogenesis than in oogenesis. In the female germ cells, de novo DNA methylation occurs in arrested oocytes in meiotic prophase I. However, in the male germ cells, it takes place in mitotically arrested prespermatogonia before birth [5-7].

DNA methylation is catalyzed by a group of enzymes called DNA methyltransferases (dnmts), including Dnmt1 and Dnmt3. Dnmt1 is involved in the methylation of hemimethylated DNA and thus called maintenance DNA methyltransferase, while Dnmt3 is able to place methylation marks on previously unmethylated CpGs of DNA and thus mainly responsible for the de novo DNA methylation during development [8, 9]. In mammals, Dnmt3 subfamily is composed of three members, Dnmt3a, Dnmt3b, and Dnmt3l [10]. Recently, Dnmt3c, a novel rodent specific member of the de novo dnmts, has been identified to regulate DNA methylation in the male germline [11]. Of these, Dnmt3a, Dnmt3b, and Dnmt3c have been proven to have catalytic activities in vivo, whereas Dnmt3l is a catalytically inactive DNA methyltransferase cofactor [9, 12].

Studies on mammals have shown that Dnmt3a, Dnmt3c and Dnmt3l, not Dnmt3b, are required for gametogenesis. During the development of male germ cells in mouse, Dnmt3a
exhibited dynamic expression patterns and it is highly expressed in spermatogonia and spermatocytes [13, 14]. Male germ cells without Dnmt3a or Dnmt3l undergo meiotic catastrophe, impaired spermatogenesis, which results in no spermatocytes, spermatids or spermatozoa and significantly reduced testis size in Dnmt3a conditional mutant mouse [15-18].

In female mouse, Dnmt3a is expressed in follicles and stromal cells at different developmental stages, and exist in cytoplasm and nucleus of oocytes and granulosa cells [19, 20]. Dnmt3a or Dnmt3l null oocytes fail to acquire methylation during oocytes growth, which leads to abnormal embryonic development after fertilization [15, 17].

The teleost specific whole genome duplication has increased the dnmts copy number [21, 22]. Two Dnmt3a paralogous genes (dnmt3aa and dnmt3ab) have been isolated and identified in tilapia, zebrafish, flatfish and ricefield eel [21, 23-25]. Studies on dnmt3aa and dnmt3ab in fish have been mainly focused on their expression in gonads. In Nile tilapia and bluehead wrasse, the expressions of dnmt3aa and dnmt3ab are significantly higher in the testes than in the ovaries, and significantly increased during the sex reversal from female to male [21, 26]. In ricefield eel, dnmt3aa and dnmt3ab are highly expressed in spermatocytes of testes, with the expression of dnmt3aa significantly increased during the female to male sex reversal [25, 27].

These studies suggest that dnmt3aa and dnmt3ab may play important roles in gonadal development of fish, however, their detailed expression profiles during the sex determination and differentiation and gonadal development of teleost are unclear. Recently, some research groups reported the mutation of dnmt3aa and dnmt3ab in zebrafish, demonstrating their critical function on behavior regulation [28], temperature adaptation [29] and brain neural development [30]. Nevertheless, the roles of dnmt3aa and dnmt3ab in gonadal development of fish remain
Nile tilapia (*Oreochromis niloticus*) is an important farmed fish with a stable XX-XY sex determination system. The availability of genetic all-XX and all-XY fish [31], short spawning cycle (14 days), together with the availability of high-quality genome sequences [32], have made it an excellent model for the study of gene expression and function in relation to reproduction and fertility. Particularly, genome editing technique has been established in tilapia by our group [33]. In the present study, we clarified the precise expression profiles of *dnmt3aa* and *dnmt3ab* during the key stages of gonadal development in female and male, uncovered their roles in reproduction and fertility in teleosts and the possible mechanism involved, by homozygous mutant establishment and phenotype analyzes in Nile tilapia.

**Results**

**Expression patterns and cellular localization of *dnmt3aa* and *dnmt3ab* in developing gonads.**

Generally, several key biological events occur at different time points during gonadal development in tilapia, such as sex determination and differentiation at 5-10 dah (day after hatching), the initiation of germ cells meiosis and oogenesis in the XX gonads (ovaries) at 30 dah, the initiation of spermatogenesis in the XY gonads (testes) at 90 dah, and sperm maturation in the XY gonads and vitellogenesis in the XX gonads at 180 dah. In this study, we analyzed the expression patterns and cellular localization of *dnmt3aa* and *dnmt3ab* in female and male gonads of tilapia from 5 to 180 dah.

Ontogeny analyses showed that *dnmt3aa* and *dnmt3ab* displayed sexually dimorphic expression profiles in developing gonads (Additional file 1: Fig. S1). *Dnmt3aa* and *dnmt3ab*
were expressed in gonads from as early as 5 dah, with relatively higher expression in the testes than the ovaries. In the testes, *dnmt3aa* was up-regulated from 5 to 180 dah. In the ovaries, *dnmt3aa* was up-regulated from 5 dah and reached the highest level at 120 dah, and maintained this level till 180 dah (Additional file 1: Fig. S1a). *Dnmt3ab* was up-regulated from 5 to 120 dah and maintained at relatively high level in ovaries and testes at 120 and 180 dah (Additional file 1: Fig. S1b). Generally, higher expression was observed for *dnmt3aa* than *dnmt3ab* at all the time point examined.

We identified the cell populations expressing *dnmt3aa* and *dnmt3ab* in gonads by fluorescence *in situ* hybridization (FISH). *dnmt3aa* was widely expressed in the gonads of tilapia, highly expressed in oogonia, phase I and II oocytes and granulosa cells of ovaries (Fig. 1a-d), spermatogonia and spermatocytes of testes (Fig. 1e-h). *Dnmt3ab* was mainly expressed in granulosa cells of ovaries (Fig. 1i-l) and spermatocytes of testes (Fig. 1m-p). In contrast, no signal for *dnmt3aa* and *dnmt3ab* mRNA was detected in the gonads using the sense probe (Additional file 1: Fig. S2).

**Establishment of tilapia *dnmt3aa* and *dnmt3ab* mutant lines by CRISPR/Cas9.**

The gRNA sites containing *Hpy 188III* and *Mly I* adjacent to protospacer adjacent motif (PAM) were selected in the third and second exon of *dnmt3aa* and *dnmt3ab*, respectively (Fig. 2a, b). Genomic DNA extracted from 20 pooled injected embryos was used as a template for PCR amplification and mutation assays. Complete digestion of the PCR products from *dnmt3aa* and *dnmt3ab* with *Hpy 188III* and *Mly I*, respectively, produced two fragments in the control groups, while an intact DNA fragment was observed in embryos injected with both Cas9 mRNA and target gRNA (Fig. 2a, b). Representative Sanger sequencing results from the uncleaved bands
were listed. In-frame and frame-shift deletions induced at the target sites were confirmed by Sanger sequencing (Fig. 2a, b).

F1 generation fish were obtained by crossing chimeric XY F0 males and WT XX females. Heterozygous F1 offspring with a deletion of 4 bp for *dnmt3aa* and 5 bp for *dnmt3ab*, were selected to breed the F2 generation (Fig. 2c). Further, homozygous mutant fish of *dnmt3aa* and *dnmt3ab* were validated by Sanger sequencing (Fig. 2d, e). Frame-shift mutations led to premature termination of translation of *dnmt3aa* at amino acid 126 and *dnmt3ab* at amino acid 36 (Fig. 2f, g). Restriction enzyme digestion assay identified the *dnmt3aa*+/+, *dnmt3aa*+/-, *dnmt3aa*−/−, *dnmt3ab*+/+, *dnmt3ab*+/- and *dnmt3ab*−/− individuals (Fig. 2h, i). The loss of *dnmt3aa* and *dnmt3ab* mRNA was confirmed by reverse transcription PCR (RT-PCR) using a specific primer on the target site (Fig. 2j).

**Gonadal morphology and histology of *dnmt3aa*+/− and *dnmt3ab*+/− female mutants.**

To investigate the role of *dnmt3aa* and *dnmt3ab* in follicular development, we analyzed the gross morphology and histology of WT, *dnmt3aa*+/− and *dnmt3ab*+/− ovaries at different developmental stages. Morphological observation showed that the *dnmt3aa*+/− ovaries atrophied at 60 dah, while there was no difference in gonad morphology between *dnmt3ab*+/− and WT (Fig. 3a-c). We selected three different sampling points (part1, part2 and part3) from *dnmt3aa*+/− ovaries, including the gonad smaller part, the thicker part and the atrophy part, for histological observation. The results showed that at 60 dah, WT and *dnmt3ab*+/− ovaries were full of oogonia, phase I and phase II oocytes, while *dnmt3aa*+/− ovaries had only a few oogonia and oocytes (Fig. 3a’-c’). Consistently, statistical analysis showed that the GSI of *dnmt3aa*+/− fish was significantly lower than that of WT fish, and the number of follicles at different developmental
stages was significantly reduced, while there was no significant difference between $dnmt3ab^{-/-}$ and WT fish (Fig. 3d, e).

To assess whether the atrophied ovaries could be recovered, we further analyzed two more developmental stages at 120 dah and 240 dah. Morphological observations showed that $dnmt3aa^{-/-}$ ovaries were atrophied and degenerated, while $dnmt3ab^{-/-}$ ovaries developed normally at 120 and 240 dah (Fig. 3f-h, k-m). Histological observation showed that the WT and $dnmt3ab^{-/-}$ ovaries were filled with phase II and phase III oocytes at 120 dah, while only a few phase II and phase III oocytes existed in $dnmt3aa^{-/-}$ ovaries (Fig. 3f’-h’). At 240 dah, the oocytes of WT, $dnmt3aa^{-/-}$ and $dnmt3ab^{-/-}$ females were matured, less oogonia, phase I, phase II, phase III and phase IV follicle cells was observed in $dnmt3aa^{-/-}$ ovaries than the $dnmt3ab^{-/-}$ and WT ovaries (Fig. 3k’-m’). Consistently, statistical analysis showed that the GSI and the number of follicles of $dnmt3aa^{-/-}$ was significantly decreased (Fig. 3i, j, n, o). In addition, the number of follicles in $dnmt3aa^{-/-}$ ovarian smaller part (part1) and atrophy part (part3) was also significantly reduced (Additional file 1: Fig. S3). Taken together, these results suggest that homozygous mutation of $dnmt3aa$ resulted in reduced follicles and ovarian atrophy and degeneration in tilapia.

**Gonadal morphology and histology of $dnmt3aa^{-/-}$ and $dnmt3ab^{-/-}$ male mutants.**

The gross morphology and histology of the testes in the WT, $dnmt3aa^{-/-}$ and $dnmt3ab^{-/-}$ fish were analyzed at 60, 120 and 240 dah. At 60 dah, no obvious difference was observed in the testicular morphology among them (Fig. 4a-c). Histological examination showed that the testes of WT, $dnmt3aa^{-/-}$ and $dnmt3ab^{-/-}$ fish were full of spermatogonia (Fig. 4a’-c’) and with no difference in number (Fig. 4e). At 120 dah, the testes of $dnmt3aa^{-/-}$ fish were smaller and more
transparent than those of the WT fish (Fig. 4f-h). Histological examination showed that the testes of WT and dnmt3ab<sup>−/−</sup> fish were full of spermatogenic cells at different developmental stages, while less spermatocytes were observed in dnmt3aa<sup>−/−</sup> testes (Fig. 4f’-h’). Statistical analysis showed that the GSI and the number of spermatocytes in the testes of dnmt3aa<sup>−/−</sup> fish were significantly lower than those of WT fish (Fig. 4i, j). At 240 dah, morphological observation showed that the testes of the dnmt3aa<sup>−/−</sup> fish, but not the WT and dnmt3ab<sup>−/−</sup> fish, was transparent (Fig. 4k-m). Histological examination showed that dnmt3aa<sup>−/−</sup> fish had fewer spermatocytes (Fig. 4l’), compared with the dnmt3ab<sup>−/−</sup> fish and the WT fish (Fig. 4m’).

Consistently, compared with the WT fish, the GSI and spermatocytes number of dnmt3aa<sup>−/−</sup> fish were significantly reduced at 240 dah (Fig. 4n, o).

**Germ cells apoptosis in dnmt3aa<sup>−/−</sup> and dnmt3ab<sup>−/−</sup> gonads.**

Germ cells in the ovaries of dnmt3aa<sup>−/−</sup> fish were significantly decreased. In order to further evaluate the effect of dnmt3aa and dnmt3ab mutation on ovary development of tilapia, we examined the total and apoptotic germ cells in WT, dnmt3aa<sup>−/−</sup> and dnmt3ab<sup>−/−</sup> fish by Vasa (germ cells marker) and TUNEL immunofluorescence co-staining (Fig. 5a-o). The number of Vasa positive cells in dnmt3aa<sup>−/−</sup> ovaries was significantly reduced compared with that of WT fish, but there was no difference between dnmt3ab<sup>−/−</sup> and WT fish (Fig. 5p). TUNEL assay showed that there was a large number of germ cells in apoptosis in dnmt3aa<sup>−/−</sup> ovaries (Fig. 5j), but there was no obvious germ cells apoptosis in ovaries of the dnmt3ab<sup>−/−</sup> and WT fish (Fig. 5e, o). Statistical analysis showed that the number of apoptotic germ cells in the dnmt3aa<sup>−/−</sup> ovaries was significantly increased compared with that in the dnmt3ab<sup>−/−</sup> and WT ovaries (Fig. 5q). In zebrafish, medaka and tilapia, significant decrease of ovarian germ cells led to female
to male sex reversal [33-36]. Therefore, we further examined the expression of cyp19a1a, the female pathway key gene, and found that it was still expressed in the dnmt3aa−/− ovaries (Additional file 1: Fig. S4). It is worth noting that in the F0 generation mutants with high mutation rate (75%) at another dnmt3aa target, we also observed a significant reduction of germ cells in the ovaries (Additional file 1: Fig. S5).

Similar to the situation observed in ovaries, the number of spermatocytes in the testes of dnmt3aa−/− fish decreased significantly at 120 dah. In order to further evaluate the effect of dnmt3aa and dnmt3ab mutation on testes development of tilapia, we examined the total and apoptotic spermatocytes in WT, dnmt3aa−/− and dnmt3ab−/− fish by Sycp3 (spermatocyte marker) and TUNEL immunofluorescence co-staining (Fig. 6a-o). Statistical analysis showed that the number of Sycp3 positive cells in dnmt3aa−/− fish was significantly lower than that of WT fish, while no significant difference in the number of Sycp3 positive cells was observed in dnmt3ab−/− and WT fish (Fig. 6p). A large number of apoptotic spermatocytes, significantly higher than that of WT and dnmt3ab−/− fish, was observed in dnmt3aa−/− fish (Fig. 6j, q).

Sperms quality of WT, dnmt3aa−/− and dnmt3ab−/− XY fish.

The semen were obtained from the mature WT, dnmt3aa−/− and dnmt3ab−/− mutants by in vitro extrusion at 240 dah, and analyzed with a computer-assisted sperm analyzer after 1:10 dilution. The sperms from dnmt3aa−/− mutants displayed lower activity compared with those from the WT and dnmt3ab−/− fish (Fig. 7a-c). In addition, the sperm concentration of dnmt3aa−/− mutants was significantly lower than that of WT and dnmt3ab−/− fish (Fig. 7d). Further analysis showed that the percentage of progressive sperms in dnmt3aa−/− fish was significantly lower than that in WT and dnmt3ab−/− fish (Fig. 7e), and the proportion of immotile sperms was
significantly higher than that in WT and \( dnmt3ab^{-/+} \) fish (Fig. 7f). Furthermore, the VSL (Straight linear velocity) (Fig. 7g), VCL (Curvilinear velocity) (Fig. 7h) and BCF (Beat frequency of sperms flagella) (Fig. 7i) of sperms from the \( dnmt3aa^{-/+} \) fish were significantly lower than those of the WT and \( dnmt3ab^{-/+} \) fish. Morphologically, similar to the WT sperms, the sperms from the \( dnmt3ab^{-/+} \) mutants were characterized with straight and long tail, while the sperms from \( dnmt3aa^{-/+} \) mutants consisted of some abnormal sperms with curly and short tail from Papanicolaou staining and scanning electron microscope analysis (Fig. 7 j-l, Additional file 1: Fig. S6).

**Apoptosis genes expression and compensatory expression of \( dnmt \) family genes in \( dnmt3aa^{-/+} \) and \( dnmt3ab^{-/+} \) gonads.**

As mentioned above, there was a large number of germ cells in apoptosis in the gonads of \( dnmt3aa^{-/+} \) fish. Thus, we further analyzed the expression of apoptosis genes in 60 dah XX and 120 dah XY gonads by quantitative real time PCR (qRT-PCR). The results showed that the expression of apoptosis genes \( baxa, baxb, caspase3a, caspase3b \) and \( caspase8 \) was significantly increased in \( dnmt3aa^{-/+} \) ovaries at 60 dah, but there was no significant difference between \( dnmt3ab^{-/+} \) and WT ovaries (Fig. 8a). Gonads of \( dnmt3aa \) and \( dnmt3ab \) homozygous mutants were analyzed for gene compensatory expression. The homozygous mutation of \( dnmt3ab \) caused a compensatory increase of \( dnmt3aa \) expression at 60 dah. However, there was no significant difference in the expression of other \( dnmts \) between the mutants and WT fish (Fig. 8b). At 120 dah, the expression of apoptosis genes \( baxa, baxb, caspase3b \) and \( caspase8 \) in \( dnmt3aa^{-/+} \) males was significantly increased (Fig. 8c). Interestingly, we found that \( dnmt3aa \) and \( dnmt3ab \) compensated for each other in the homozygous mutant males at 120 dah (Fig. 8d).
The global DNA methylation level of WT, \(dnmt3aa^{-/-}\) and \(dnmt3ab^{-/-}\) gonads.

The global DNA methylation status in WT, \(dnmt3aa^{-/-}\) and \(dnmt3ab^{-/-}\) gonads was examined using 5-methylcytosine (5-mC) antibody, which could detect 5-methylcytosine but not unmethylated cytosine. Immunoreactive signals were predominantly present in the nuclei of oocytes and granulosa cells in ovaries (Fig. 9a-c) and spermatogonia, spermatocytes and spermatozoa in testes (Fig. 9d-f). The immunoreactive signals were strong in spermatocytes and spermatozoa but weak in spermatogonia (Fig. 9d-f). Statistical analysis showed that the 5-mC levels in ovaries and testes of \(dnmt3aa^{-/-}\) fish were significantly lower than those of WT and \(dnmt3ab^{-/-}\) fish. There were no significant differences of the 5-mC levels in ovaries and testes between \(dnmt3ab^{-/-}\) and WT fish (Fig. 9g, h). Further analysis showed that the 5-mC levels of granulosa cells in ovaries and spermatogonia, spermatocytes and spermatozoa in testes of \(dnmt3aa^{-/-}\) fish were significantly lower than those of \(dnmt3ab^{-/-}\) and WT fish (Additional file 1: Fig. S7). These results showed that mutation of \(dnmt3aa\) significantly reduced the 5-mC levels in tilapia ovaries and testes.

Discussion

DNA methylation, mediated by \(dnmts\), is required for proper embryonic development and for the formation of mature functional germ cells [38]. \(Dnmt3a\), one of the \(dnmts\), is responsible for \textit{de novo} methylation of mammalian germ cells and plays crucial roles in mammalian gonads development [17, 39-40]. In teleost, there are two \(Dnmt3a\) paralogs, \(dnmt3aa\) and \(dnmt3ab\), due to the third round of genome duplication [21]. The expression patterns and roles of \(dnmt3aa\) and \(dnmt3ab\) in gonadal development remain poorly understood in teleost. In the present study, we examined the expression patterns of both \(dnmt3aa\) and \(dnmt3ab\), mutated them, and
analyzed the gonadal phenotypes and discussed possible mechanisms in the farmed fish Nile tilapia.

Different expression patterns of *dnmt3aa* and *dnmt3ab* in gonad indicate the subfunctionalization in teleost.

Gene expression patterns are important aspects of gene regulation and function analysis. The analysis of *Dnmt3a* expression patterns in gonads is mainly focused on mammals [19, 41], but rarely reported in fish. In humans, *Dnmt3a* mRNA expression is detected in the ovarian follicles from primordial to secondary follicles, granulosa cells, and germinal vesicle (GV) and metaphase II (MII) oocytes [42]. In addition, *Dnmt3a* is expressed in human spermatogenic cells, including spermatogonia cells, primary spermatocytes, secondary spermatocytes, and round spermatids [43]. In rhesus monkey, *Dnmt3a* mRNA is expressed in follicles at different developmental stages [44]. In female mouse, Dnmt3a protein is localized around the nucleus of GV oocytes and in the cytoplasm of the MII oocytes [45]. In male mouse, *Dnmt3a* is expressed at mRNA and protein levels in male germ cells, at a high level in type A spermatogonia, slightly decreased in type B spermatogonia, and further decreased in preleptotene and pachytene spermatocytes [13-14]. In general, Dnmt3a is expressed in mammalian follicles and granulosa cells of ovaries and spermatogenic cells of testes. There are few studies on the cellular localization of *dnmt3aa* and *dnmt3ab* in teleost except for a report describing immunoreactive signals for Dnmt3a (Dnmt3aa and Dnmt3ab) in male germ cells, particularly in spermatocytes in ricefield eels [25]. In this study, we comprehensively studied the expression patterns and their cellular localization of *dnmt3aa* and *dnmt3ab* in the ovaries and testes of tilapia. *Dnmt3aa* and *dnmt3ab* displayed sexually dimorphic expression profiles in developing gonads with
higher expression in testes than in ovaries. Furthermore, higher expression was observed for

\textit{dnmt3aa} than \textit{dnmt3ab} at all the time point examined, suggesting that \textit{dnmt3aa} may play more

important roles in gonadal development than \textit{dnmt3ab}. In tilapia, \textit{dnmt3aa} was highly expressed

in oogonia, phase I and II oocytes and granulosa cells, while \textit{dnmt3ab} was mainly expressed in

granulosa cells of the ovaries. Both were highly expressed in spermatogonia and spermatocytes

of the testes. These results demonstrated that the expression of \textit{dnmt3aa} and \textit{dnmt3ab} in Nile

tilapia was basically consistent with that in mammals. Interestingly, in ovaries, \textit{dnmt3aa} was

expressed in both germ cells (oogonia and oocytes) and somatic cells (granulosa cells), while

\textit{dnmt3ab} was only expressed in somatic cells (granulosa cells). The different expression

patterns of \textit{dnmts} in gonads are probably essential for the acquisition of a sex specific

methylation pattern \cite{46}. The different expression patterns of \textit{dnmt3aa} and \textit{dnmt3ab} in the

gonads of tilapia suggest that they may play different roles in gonadal development of tilapia.

**The function of \textit{Dnmt3a (dnmt3aa and dnmt3ab)} in gonadal development of vertebrates**

The development of germ cells in gonads is a highly ordered process, which initiate in the

growth of fetus and complete in adults \cite{6}. DNA methylation, as an important epigenetic

modification, has important implications for gamete integrity and formation of functionally

mature germ cells in mammals \cite{7}. During germ cells development, DNA methylation

reprogramming occurred dynamically \cite{3, 4}. Both male and female PGCs undergo genome-

wide DNA demethylation to reach a very low DNA methylation level at E13.5 \cite{47, 48}. After

E13.5, the male germ line is globally remethylated before birth, whereas the female germ line

regains DNA methylation during oocytes growth after birth. During this process, \textit{Dnmt3a} is

essential for both male and female germ lines \textit{de novo} methylation \cite{5, 17, 49}. 



During follicular development after birth, DNA methylation is thought to play important roles in the regulation of gene expression in oocytes. In the process of oocytes maturation from GV to MII stages, global DNA methylation gradually increases and reaches the highest level in MII oocytes [48]. Genomic imprints are established during this process [50]. Conditional knockout of Dnmt3a in mouse oocytes results in failure of DNA methylation establishment in oocytes, significant reduction of DNA methylation, loss of maternal imprints, and death of offspring in uterus [5, 17, 39, 49-51].

DNA methylation is also crucial for somatic granulosa cells that surround oocytes during follicular development. Granulosa cells are essential for producing competent oocytes. Correctly established DNA methylation in the granulosa cells is important for the regulation of expression of genes related to follicular development [52]. It is noteworthy that granulosa cells undergo dynamic DNA methylation changes to repress or activate the genes required for their proliferation and differentiation during follicular development in human [53, 54]. Studies in human and bovines have shown that the methylation levels of granulosa cells in ovaries decrease with age [53, 55, 56]. Abnormal DNA methylation in the granulosa cells was associated with the age-related decline of ovarian function, oocyte quality, and altered gene expression, and changing Dnmt3a gene expression in granulosa cells causes impaired oocyte maturation in the GV and MII oocytes and subsequent abnormal embryonic development [20, 57]. In this study, homozygous mutation of dnmt3aa resulted in partial gonadal degeneration, oocytes apoptosis and significant increase of apoptosis genes, suggesting that dnmt3aa plays important roles in the development of tilapia oocytes. In mice, oocytes conditional knockout Dnmt3a are capable to fertilize, different from the phenotype observed in tilapia in which global...
mutation of *dnmt3aa* caused oocytes apoptosis and ovarian degeneration. The discrepancy might be attributed to the following reasons: (1) The oocyte does not develop in isolation but in instead highly dependent on surrounding granulosa cells of the intact ovarian follicle. The process of oocytes development is supported by granulosa cells, and the bidirectional communication between oocytes and granulosa cells is important for the development and maturation of the oocytes [58-60]. The abnormal methylation, significant decrease of 5-mC level, of granulosa cells affected the development of oocytes and resulted in oocytes apoptosis in *dnmt3aa*−/− mutants in tilapia. (2) Unlike viviparous mammals, fish eggs are megalolecithal eggs. The oocytes development requires a large amount of yolk accumulation. Insufficient vitellogenin synthesis in liver led to oocytes development arrest and increased oocytes apoptosis in *dnmt3aa*−/− mutants. (3) Studies have shown that some apoptosis genes (*caspase3*, *caspase8*) are directly regulated by DNA methylation [61, 62]. Mutation of *dnmt3aa* decreased DNA methylation promoted the expression of apoptosis genes and caused the apoptosis of oocytes in tilapia.

DNA methylation also plays crucial roles in normal spermatogenesis. Previous studies have demonstrated that male germ cells in mice have a highly distinct epigenetic pattern, characterized by a unique genome-wide pattern of DNA methylation. During spermatogenesis, global remethylation is established from spermatogonia to spermatocytes, and continues in the round spermatids and spermatozoa [63, 64]. It has been reported that abnormal DNA methylation in spermatogenic cells due to genetic failure, environmental factors and disturbed expression of the *dnmts* may lead to spermatogenic impairments [65-67]. Indeed, azoospermia studies in human have shown that significant changes in *dnmts* expression and DNA
methylation levels in spermatogenic cells may lead to male infertility [68]. In male mouse, conditional mutation of Dnmt3a in germ cells led to meiotic catastrophe, impaired spermatogenesis and small testes with no spermatocytes, spermatids and spermatozoa [17]. In this study, significantly higher apoptotic signals (TUNEL staining) and apoptotic genes expression were observed in spermatocytes of the dnm3aa mutant testes, suggesting that dnm3aa plays important roles in spermatogenesis in tilapia. However, viable sperms were produced in dnm3aa−/− fish even though the sperm concentration, motility, and flagella beat frequency were significantly lower than those of WT fish, which is different from the phenotype of Dnmt3a conditional mutation in mice. Interestingly, a significant up-regulation of dnm3ab expression in dnm3aa−/− testes was observed. The compensatory increase of dnm3ab in the testes of dnm3aa−/− mutants may be one of the reasons for these differences. In human, low sperms motility is associated with decreased sperms methylation [69]. Consistently, lower 5-mC level of spermatozoa and lower sperm mobility were observed in dnm3aa−/− mutants of tilapia.

It is worth noting that homozygous mutation of Dnmt3a in mice was fatal, whereas mutation of either dnm3aa or dnm3ab in zebrafish and tilapia was viable [28, 29, 70]. In this study, different spatial-temporal expression profiles of dnm3aa and dnm3ab, up-regulation of dnm3ab in dnm3aa−/− gonad and up-regulation of dnm3aa in dnm3ab−/− gonad were observed. These results indicate the subfunctionalization and compensation of dnm3aa and dnm3ab in tilapia, and might be possible reasons for the survival of these mutants. As suggested previous study [71], the existence of duplicate genes probably increased the survival opportunities of the species. Our study provided another model for the notion.
Conclusions

In this study, we demonstrated that both $dnmt3aa$ and $dnmt3ab$ displayed sexually dimorphic expression in developing gonads. $Dnmt3aa$ was highly expressed in oogonia, phase I and II oocytes and granulosa cells in ovaries and spermatogonia and spermatocytes in testes, while $dnmt3ab$ was mainly expressed in ovarian granulosa cells and testicular spermatocytes. Mutation of $dnmt3aa$ resulted in lower GSI, increased apoptosis of oocytes and spermatocytes and significantly reduced sperm quality, while no obvious phenotype was observed in $dnmt3ab$ homozygous mutants. The level of 5-mC in $dnmt3aa^{-/-}$ mutants testes and ovaries decreased significantly, while there was no difference between $dnmt3ab^{-/-}$ mutants and WT. Our results suggest that $dnmt3aa$, not $dnmt3ab$, plays important roles in maintaining normal gametogenesis in teleost. Our results enrich the understanding of the function of DNA methyltransferase in gonads of non-mammalian vertebrates.
Methods

Animals rearing conditions

Nile tilapia (*Oreochromis niloticus*) was reared in recirculating aerated freshwater tanks at 26 °C under a natural photoperiod. All-XX progenies were obtained by crossing a pseudomale (XX male, producing sperms after hormonal sex reversal) with a normal XX female. All-XY progenies were obtained by crossing an YY super male with an XX female. Animal experiments were conducted in accordance with the regulations of Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University, China. (No. IACUC-20181015-12, 15 October 2018).

Establishment of *dnmt3aa* and *dnmt3ab* homozygous mutant line by CRISPR/Cas9

CRISPR/Cas9 was performed to knockout *dnmt3aa* and *dnmt3ab* in tilapia as described previously [33]. Briefly, the guide RNA of *dnmt3aa* and *dnmt3ab* and Cas9 mRNA were co-injected into one-cell-stage embryos at a concentration of 500 and 1000 ng/µL, respectively. Twenty injected embryos were collected 72 h after injection. Genomic DNA was extracted from pooled control and injected embryos and used to access the mutations. DNA fragments spanning the target site was amplified. The mutated sequences were analyzed by restriction enzyme digestion with *Hpy* 188III and *Mly* I and Sanger sequencing.

Heterozygous F1 offspring were obtained by F0 XY male founders mated with WT XX females. The F1 fish were genotyped by fin clip assay and the individuals with frame-shift mutations were selected. XY male and XX female siblings of F1 generation, carrying the same mutation, were mated to generate homozygous F2 mutants. The F1 mutant fish which carried
4 and 5 base-pairs deletion were used for construction of F2 \( dnmt3aa \) and \( dnmt3ab \) mutants, respectively. The \( dnmt3aa \) and \( dnmt3ab \) F2 mutants were screened using restriction enzyme digestion and Sanger sequencing. The genetic sex of each fish was determined by genotyping using sex-linked marker (marker 5) as described previously [31].

**Gonad morphological and histological analysis**

The fish were sampled at 60, 120 and 240 dah. Briefly, the fish were anesthetized with MS-222 (Sigma-Aldrich, St. Louis, USA) and the gonad morphology of the mutants and WT fish was imaged by stereomicroscope (Leica, Bensheim, Germany) after abdominal anatomy. Then gonads were removed and the body and gonad weight were measured on an analytical balance. The gonadosomatic index was calculated. Then the gonads were fixed in Bouin’s solution for 24 h at room temperature, dehydrated and embedded in paraffin. The fixed samples were then processed as follows: serial dehydration in 70, 80 and 90% ethanol for 1.5 h each, 95% ethanol for 2 h, and 100% ethanol three times for 1 h each; sequential clearance in xylene and ethanol mixture (1:1) for 30 min and xylene twice for 30 min each; and infiltration in paraffin 2 h. The samples were sectioned at 5 μm thickness using the Leica microtome (Leica Microsystems, Wetzlar, Germany). The sections were deparaffinized, hydrated, stained with hematoxylin and eosin (H&E) as described previously [72]. Photographs were taken under Olympus BX51 light microscope (Olympus, Tokyo, Japan). Sibling WT fish were used as control for phenotype analysis.

**Gene expression analyses by qRT-PCR**

Gonads of the WT, \( dnmt3aa^{-/-} \) and \( dnmt3ab^{-/-} \) fish were dissected at different developmental
stages (5, 30, 60, 90, 120 and 180 dah) for gene expression assay. Total RNA (1.0 μg) was extracted and reverse transcribed using PrimeScript RT Master Mix Perfect Real Time Kit according to the manufacturer's instructions (Takara, Dalian, China). qRT-PCR was performed on an ABI7500 qRT-PCR machine, according to the protocol of SYBR Premix Ex TaqTM II (Takara, Dalian, China). The relative abundance of key genes in the gonad was evaluated using the formula \( R = 2^{-\Delta\Delta C_t} \) [73]. The reference gene gapdh was used to normalize the expression values. Primer sequences used in this study were listed in Additional file 2: Table S1.

**Germ cells counting**

The gonads of 5 fish of each genotype (WT, \( dnmt3aa^{-/-} \) and \( dnmt3ab^{-/-} \) fish) were dissected at 60, 120 and 240 dah. Fixed ovaries and testes by Bouin’s solution were embedded in paraffin and sectioned at 5 μm thickness. Germ cells from the median sections of testes (n=5) and different parts (part1, part2 and part3) of ovaries were counted for statistical analyses. The histological classification of the follicles and spermatogenic cells were performed as described previously [74, 75].

**Immunofluorescence (IF), TUNEL assay and Immunohistochemistry (IHC)**

The rabbit polyclonal antibodies (Vasa, Cyp19a1a and Sycp3) were prepared by our laboratory. The dilution and specificity of these antibodies have been analyzed previously [76-78]. For IF, Alexa Fluor 488- and 594- conjugated secondary antibodies (Invitrogen, Shanghai, China) were diluted 1:500 in blocking solution and incubated with tissue to detect the primary antibodies. The nuclei were stained by 4’, 6-diamidino-2-phenylinidole (DAPI) (Invitrogen, Carlsbad, USA). Apoptosis of germ cells was evaluated by staining paraffin sections of WT, \( dnmt3aa^{-/-} \) and
mutant fish at 60 (ovaries) and 120 dah (testes) with in situ cell death detection kit, TMR red terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) system (Roche, Mannheim, Germany) according to the manufacturer's protocol. Apoptotic germ cells in the testes (entire median section) and ovaries (part 2) (n=5) were counted for statistical analysis. Fluorescence signals were captured by confocal microscopy (Olympus FV3000) (Olympus, Tokyo, Japan). The assessment of global DNA methylation in WT, dnmt3aa−/− and dnmt3ab−/− gonads was performed with immunohistochemistry using the anti-5-methylcytosine antibody (MABE146, Merck Millipore) according to previous reports [20, 25]. Photographs were taken under an Olympus BX51 light microscope (Olympus, Tokyo, Japan). Finally, the positive signals were quantified using image J software (National Institutes of Health, Bethesda, MD, USA) according to the instructions.

Fluorescence in situ hybridization (FISH)

The tilapia dnmt3aa and dnmt3ab open reading frames were amplified with specific primers (Additional file 2: Table S1), and the amplicons were cloned into pGEM-T Easy Vector. The sense and anti-sense RNA probes were labeled with digoxigenin (DIG) by in vitro transcription using an RNA labeling kit (Roche, Mannheim, Germany). The gonads of fish were sampled at indicated time. The fish gonads were fixed in 4% paraformaldehyde in PBS and processed for serial paraffin sectioning at 5 μm thickness. The sections were deparaffinized, rehydrated and digested with proteinase K (4 μg/mL; Roche, Mannheim, Germany) at 37 °C for 15 min, followed by hybridization with DIG-labeled RNA probes at 60 °C overnight. The sections were washed with 50% formamide/2×SSC for 30 min, 2×SSC for 20 min, and 0.2×SSC for 20 min. The slides were incubated for 30 min at room temperature in a humidified chamber with Anti-
DIG-POD (Roche, Mannheim, Germany) diluted by 200 times in DIG2 buffer. Then the sections were washed with DIG1 buffer. After washing, the TSA Plus Fluorescein System (PerkinElmer, Boston, USA) was used for the amplification of hybridization signals. The nuclei were stained by DAPI (Invitrogen, Carlsbad, USA) staining. Fluorescence signals were captured by confocal microscopy (Olympus FV3000) (Olympus, Tokyo, Japan).

Sperm mobility analysis

Sperm concentration, sperm motility, curvilinear velocity (VCL), straight line velocity (VSL) and beat frequency of sperm flagella (BCF) were examined by computer assisted sperm analysis using the Sperm Quality Analyzer according to the manufacturer’s instructions (Zoneking Software, China). Briefly, sperms collected from WT, *dnmt3aa*−/− and *dnmt3ab*−/− XY fish (n=5) at 240 dah were diluted at 1:10 with phosphate buffer saline, and one drop semen was dripped into the counting pool of the sperm counting board, and placed on the operating platform of a Leica DM500 light microscope (Leica, Bensheim, Germany).

Sperms Papanicolaou staining and scanning electron microscope analysis

Semens from WT, *dnmt3aa*−/− and *dnmt3ab*−/− fish were collected by *in vivo* extrusion and then 1 μl of drained semens mixed with 9 μl of double distilled water were applied to clean slides which were dried naturally and then stained by Papanicolaou staining. Photographs were taken under Olympus BX51 light microscope (Olympus, Tokyo, Japan). To further examine sperms morphology, semens were collected from WT, *dnmt3aa*−/− and *dnmt3ab*−/− XY fish for scanning electron microscope analysis. In brief, sperm specimens were pre-fixed using 2.5 % glutaraldehyde, rinsed three times with phosphate buffer (pH 7.2), and dehydrated in ascending graded ethanol. Then the dehydrated samples were put into a drying basket and dried with
critical point dryer. The surface of the dried samples was treated with electric conduction, and
the specimens were observed under a Zeiss Evo MA/LS10 (Zeiss, Oberkochen, Germany)
scanning electron microscope.

Data analyses

All data were presented as mean ± SD from at least three independent experiments. Different
letters above the error bar indicate statistical differences at P < 0.05 as determined by one-way
ANOVA followed by Tukey's post-hoc test. Statistics analyses were performed using GraphPad
Prism 8 software package (GraphPad Software, La Jolla, USA). In all analyses, P < 0.05 was
considered to be statistically significant.

Abbreviations

5-mC: 5-methylcytosine; dah: day after hatching; dnmnt: DNA methyltransferase; FISH:
Fluorescence in situ hybridization; GSI: gonadosomatic index; IF: immunofluorescence; IHC:
Immunohistochemistry; PAM: protospacer adjacent motif; qRT-PCR: quantitative reverse
transcription polymerase chain reaction; RT-PCR: reverse transcription PCR.

Ethical Approval and Consent to participate

Animal experiments were conducted in accordance with the regulations of Guide for Care and
Use of Laboratory Animals and were approved by the Committee of Laboratory Animal
Experimentation at Southwest University, China. (No. IACUC-20181015-12, 15 October 2018).

Consent for publication

All authors have consented to publication.
Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Wang, D.S. and Sun, L.N. conceived and designed the experiments; Wang, F.L., Qin, Z.L. and Li, Z.Q. analyzed the data; Wang, F.L., Li, Z.Q. Yang, S.Y. performed the experiments; Wang, D.S., Wang, F.L. and Sun, L.N. wrote the manuscript. All authors read and approved the manuscript.

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**Figure Legends**

**Fig. 1** Cellular localization of *dnmt3aa* (a-h) and *dnmt3ab* (i-p) in developing gonads by fluorescence *in situ* hybridization. *Dnmt3aa* was widely expressed in the gonads of tilapia, especially in oogonia, phase I and II oocytes and granulosa cells of ovaries (a-d), spermatogonia and spermatocytes of testes (e-h). *Dnmt3ab* was mainly expressed in granulosa cells of ovaries (i-l), and spermatocytes of testes (m-p). OC, oocytes; OG, oogonia; GC, granulosa cells; SC, spermatocytes; SG, spermatogonia; dah, day after hatching. Red fluorescence represents the *dnmt3aa* and *dnmt3ab* signals. Blue fluorescence represents the DAPI signals. Arrow indicates the positive signals. White boxes indicate the regions magnified in d and i.

**Fig. 2** Establishment of *dnmt3aa* and *dnmt3ab* mutant lines. (a, b) Gene structure of *dnmt3aa* and *dnmt3ab* showing the target site and the *Hpy* 188III and *Mly* I restriction site. The Cas9 mRNA and gRNA were added as indicated. Sanger sequencing results from the uncleaved bands were listed. The PAM was marked in light green. Deletions were marked by dashes (-) and numbers to the right of the sequences in parentheses indicated the loss of bases for each allele. The mutant fish which carried 4 and 5 base-pairs deletion were used for homozygous mutant construction of *dnmt3aa* and *dnmt3ab*, respectively. WT, wild type. (c) Schematic diagram showing the breeding plans of *dnmt3aa* and *dnmt3ab* F0 to F2 fish. (d, e) Sequencing results of *dnmt3aa* and *dnmt3ab* genes from WT and homozygous mutant fish. (f, g) Schematic diagram of Dnmt3aa and Dnmt3ab wild type (WT) and the predicted truncated protein. (h, i) Identification of F2 genotypes by restriction enzyme digestion assay. (j) RT-PCR analysis of *dnmt3aa* and *dnmt3ab* mRNA expression in gonads of mutants and WT fish. The 3' sequences of forward primer were designed on the target site, which is indicated by white box. No band
was amplified in the homozygous mutants, while one band corresponding to \textit{dnmt3aa} and
\textit{dnmt3ab} mRNA was amplified in the WT XY testes. \textit{gapdh} was used as internal control.

\textbf{Fig. 3} Morphological and histological analyses of WT, \textit{dnmt3aa}^{-/−} and \textit{dnmt3ab}^{-/−} ovaries at 60, 120 and 240 dah. (a-c, a’-c’, f-h, f’-h’, k-m, k’-m’) Morphological and histological observation. (d, i, n) Gonadosomatic index (GSI) (n=10). (e, j, o) Statistical analysis of germ cells counting (n=5). Follicles from the median sections (part2) of ovaries were counted for statistical analyses. (a-c, f-h) Gonads were fixed with Bouin's solution. Different letters above the error bar indicate statistical differences at P < 0.05 as determined by one-way ANOVA followed by Tukey's post-hoc test. Results were presented as mean ± SD in d, e, i, j, n and o. Scale bar in a-c, f-h, k-m, 1 cm. dah, day after hatching; OC, oocytes; OG, oogonia; GC, granulosa cells.

\textbf{Fig. 4} Morphological and histological analyses of WT, \textit{dnmt3aa}^{-/−} and \textit{dnmt3ab}^{-/−} testes at 60, 120 and 240 dah. (a-c, a’-c’, f-h, f’-h’, k-m, k’-m’) Morphological and histological observation. (d, i, n) Gonadosomatic index (GSI) (n=10). (e, j, o) Statistical analysis of germ cells counting (n = 5). Germ cells from the median sections of testes were counted for statistical analyses. (a-c) Gonads were fixed with Bouin's solution. Different letters above the error bar indicates statistical differences at P < 0.05 as determined by one-way ANOVA followed by Tukey's post-hoc test. Results were presented as mean ± SD in d, e, i, j, n and o. Scale bar in a-c, f-h, k-m, 1 cm. Scale bar in a’-c’, f’-h’, k’-m’, 10 μm. dah, day after hatching; PSC, primary spermatocytes; SG, spermatogonia; SPT, spermatids; SPZ, spermatozoa; SSC, secondary spermatocytes.

\textbf{Fig. 5} Apoptosis detection in WT, \textit{dnmt3aa}^{-/−} and \textit{dnmt3ab}^{-/−} ovaries at 60 dah. Increased
apoptosis of germ cells in the ovaries of $dnmt3aa^{-/-}$ fish at 60 dah. (a, f, k) Histological analyses of germ cell by H&E staining. (b, g, l) Nuclei were counterstained with DAPI. (c, h, m) Green fluorescence represents the Vasa signals. (d, i, n) Red fluorescence represents the TUNEL positive signals. (e, j, o) Co-localization of some Vasa and TUNEL signals as indicated by orange color in tilapia ovaries. (p) Germ cells count of WT, $dnmt3aa^{-/-}$ and $dnmt3ab^{-/-}$ ovaries at 60 dah. (q) TUNEL positive germ cells in the median section of the ovaries (part2) (n=5). Results were presented as mean ± SD. Different letters above the error bar indicate statistical differences at P < 0.05 as determined by one-way ANOVA followed by Tukey's post-hoc test. 
dah, day after hatching. Scale bar, 10 μm.

**Fig. 6** Apoptosis detection in WT, $dnmt3aa^{-/-}$ and $dnmt3ab^{-/-}$ testes at 120 dah. Increased apoptosis of spermatocytes in the testes of $dnmt3aa^{-/-}$ fish at 120 dah. (a, f, k) Histological analyses of germ cell by H&E staining. (b, g, l) Nuclei were counterstained with DAPI. (c, h, m) Green fluorescence represents the Sycp3 signals. (d, i, n) Red fluorescence represents the TUNEL positive signals. (e, j, o) Co-localization of some Sycp3 and TUNEL signals as indicated by orange color in tilapia testes. (p) Sycp3 positive cells count of WT, $dnmt3aa^{-/-}$ and $dnmt3ab^{-/-}$ testes at 120 dah. (q) TUNEL positive cells in the entire median section of the testes (n=5). Results were presented as mean ± SD. Different letters above the error bar indicate statistical differences at P < 0.05 as determined by one-way ANOVA followed by Tukey's post-hoc test. dah, day after hatching. Scale bar, 10 μm.

**Fig. 7** Sperms quality analyses of WT, $dnmt3aa^{-/-}$ and $dnmt3ab^{-/-}$ XY fish at 240 dah. (a-c) The tracks of motile sperms from WT, $dnmt3aa^{-/-}$ and $dnmt3ab^{-/-}$ XY fish. Pink, blue and green present grade A, B, C sperms, respectively. (d-i) The physiological characteristics of WT,
dnmt3aa−/− and dnmt3ab−/− sperms (n=5). PR, progressive sperms; IM, immotile sperms. VSL, straight linear velocity; VCL, curvilinear velocity; BCF, Beat frequency of sperms flagella. Results were presented as mean ± SD. Different letters above the error bar indicate statistical differences at P < 0.05 as determined by one-way ANOVA followed by Tukey's post-hoc test. (j-l) Sperms morphology examination by scanning electron microscope.

Fig. 8 Detection of apoptosis genes and analysis of compensatory expression of dnmt family genes in dnmt3aa−/− and dnmt3ab−/− fish at 60 dah (a, b) and 120 dah (c, d). WT, wild type. The reference gene gapdh was used to normalize the expression values. Results were presented as mean ± SD. Different letters above the error bar indicate statistical differences at P < 0.05 as determined by one-way ANOVA followed by Tukey's post-hoc test. dah, day after hatching.

Fig. 9 5-methylcytosine (5-mC) staining of WT, dnmt3aa−/− and dnmt3ab−/− gonads at 120 dah. (a-c) 5-mC staining of WT, dnmt3aa−/− and dnmt3ab−/− ovaries. Positive signals were observed in the nuclei of oocytes and granulosa cells. (d-f) 5-mC staining of WT, dnmt3aa−/− and dnmt3ab−/− testes. Positive signals were observed in spermatogonia, spermatocytes and spermatozoa. The positive signals correspond to the brownish color. (g) Statistical analysis of relative 5-mC staining level in WT, dnmt3aa−/− and dnmt3ab−/− ovaries. (h) Statistical analysis of relative 5-mC staining level in WT, dnmt3aa−/− and dnmt3ab−/− testes (n = 5, and five sections were counted per sample). The IHC positive signals were quantified using image J software according to the instructions. Results were presented as mean ± SD. Different letters above the error bar indicate statistical differences at P < 0.05 as determined by one-way ANOVA followed by Tukey's post-hoc test. OC, oocytes; GC, granulosa cells; SC, spermatocytes; SG, spermatogonia; SZ, spermatozoa. Scale bar, 10 μm.
Fig. 1
Fig. 2
Fig. 3

|       | 60 dah | 120 dah | 240 dah |
|-------|--------|---------|---------|
| WT    | ![Image](a.png) | ![Image](f.png) | ![Image](k.png) |
| *Shh*-α | ![Image](b.png) | ![Image](g.png) | ![Image](l.png) |
| *Shh*-β | ![Image](c.png) | ![Image](h.png) | ![Image](m.png) |

| d | 60 dah | e | 60 dah | i | 120 dah | j | 120 dah | n | 240 dah | o | 240 dah |
|---|--------|---|--------|---|---------|---|---------|---|---------|---|---------|
|   | ![Image](d.png) | ![Image](e.png) | ![Image](f.png) | ![Image](g.png) | ![Image](i.png) | ![Image](j.png) | ![Image](n.png) | ![Image](o.png) |
Fig. 6

|   | 120 dah H.E. | DAPI | Syep3 | TUNEL | Merge |
|---|--------------|------|-------|-------|-------|
| WM |               |      |       |       |       |
| f  |               |      |       |       |       |
| k  |               |      |       |       |       |

- Relative Syep3 protein: [Bar Graph]
- Relative TUNEL protein: [Bar Graph]
Fig. 7

WT  |  dnmt3aa^{+/−}  |  dnmt3ab^{+/−}
---|---|---

**Figures:**
- **a:** Microscopy images of different genotypes.
- **d, e, f, g, h, i:** Graphs showing sperm concentration, percentage, and other parameters.
- **j, k, l:** Additional microscopy images.
Additional file 1: Figure legends

**Fig. S1 (a-b)** Ontogenetic expression of *dnmt3aa* and *dnmt3ab* by qRT-PCR. Results were presented as mean ± SD. *gapdh* was selected as the internal control. Different letters above the error bar indicate statistical differences at P < 0.05, as determined by one-way ANOVA followed by Tukey's post-hoc test. dah, day after hatching.

**Fig. S2 (a-p)** Cellular localization of *dnmt3aa* and *dnmt3ab* in tilapia testes and ovaries at different developmental stages by FISH. No signal for *dnmt3aa* and *dnmt3ab* were detectable in the ovaries and testes using the sense probe. dah, day after hatching.

**Fig. S3 (a-d, f-i, k-n)** Histological observation WT and *dnmt3aa*−/− ovaries of part1 and part3 at 60, 120 and 240 dah. (e, j, o) Statistical analysis the number of WT and *dnmt3aa*−/− part1 and part3 ovarian follicles at 60, 120 and 240 dah. Results were presented as mean ± SD. Different letters above the error bar indicate statistical differences at P < 0.05 as determined by one-way ANOVA followed by Tukey's post-hoc test. dah, day after hatching.

**Fig. S4** Expression analysis of *cyp19a1a* (female pathway key gene) in WT (a-c) and *dnmt3aa*−/− ovaries (d-f). Green fluorescence represents the Cyp19a1a signals (white arrows). Blue fluorescence represents the DAPI signals.

**Fig. S5** Knockdown *dnmt3aa* by CRISPR/Cas9 in another target site. (a) Gene structures of *dnmt3aa* showing the second target site and the *Sau* 96I restriction site. The Cas9 mRNA and gRNA were added as indicated. Sanger sequencing results from the uncleaved bands were listed. The PAM was marked in light green. (b-d) Histological analysis ovaries of WT and *dnmt3aa* highly efficient knockdown females. (e-g) Analysis of Vasa (germ cells marker) expression in WT and *dnmt3aa* knockdown ovaries by IF. Green fluorescence represents the Vasa signals.
Fig. S6 Papanicolaou staining analysis of WT, *dnmt3aa*−/− and *dnmt3ab*−/− sperms. d, e, f are the amplification of a, b and c, respectively.

Fig. S7 (a) Statistical analysis relative 5-mC staining level of granulosa cells in WT, *dnmt3aa*−/− and *dnmt3ab*−/− ovaries at 120 dah. (b) Statistical analysis relative 5-mC staining level of WT, *dnmt3aa*−/− and *dnmt3ab*−/− spermatogonia, spermatocytes and spermatozoa in testes at 120 dah. Results were presented as mean ± SD. Different letters above the error bar indicate statistical differences at P < 0.05 as determined by one-way ANOVA followed by Tukey's post-hoc test. SG, spermatogonia; SC, spermatocytes; SZ, spermatozoa. dah, day after hatching.
Fig. S1

(a) dnmt3aa

(b) dnmt3ab

Relative mRNA Level vs. Time (dah) for XX and XY genotypes.

 binnen bars indicate significant differences (p < 0.05).
| Sense probe | 5 dah | 30 dah | 90 dah | 180 dah |
|------------|-------|--------|--------|---------|
| *dnmt3aa*  | ![Fig. S2](image) | ![Fig. S2](image) | ![Fig. S2](image) | ![Fig. S2](image) |
|            | ![Fig. S2](image) | ![Fig. S2](image) | ![Fig. S2](image) | ![Fig. S2](image) |
| XY         | ![Fig. S2](image) | ![Fig. S2](image) | ![Fig. S2](image) | ![Fig. S2](image) |
| *dnmt3ab*  | ![Fig. S2](image) | ![Fig. S2](image) | ![Fig. S2](image) | ![Fig. S2](image) |
|            | ![Fig. S2](image) | ![Fig. S2](image) | ![Fig. S2](image) | ![Fig. S2](image) |
Fig. S6

|            | WT | dnmt3aa"" | dnmt3ab"" |
|------------|----|-----------|-----------|
| a          | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| d          | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |

Fig. S7

![Graph](graph1.png)

![Graph](graph2.png)
### Additional file 2: Table S1. All primer sequences used in this study.

| Primer name                       | Sequence (5'-3')                                         | Purpose               |
|----------------------------------|---------------------------------------------------------|-----------------------|
| gRNA-dnmt3aa-target 1-F          | TAATACGACTCATATAGGCAGACCA                               |                       |
|                                  | GGACAGTCCAGGTTTTTAGAGCTAGAA                              |                       |
| gRNA-dnmt3aa-target 2-F          | TAATACGACTCATATAGGAGCGA                                 |                       |
| gRNA-dnmt3ab-target 1-F          | CCGGCCTACCGAGGTTTTAGAGCTAGAA                            | CRISPR/Cas9           |
| gRNA-R                           | AGCACCGACTCGGTGCCAC                                     |                       |
| dnmt3aa-KO-T1-detect-F           | CCTGAAGAGCCTTCTTAGCCACA                                 |                       |
| dnmt3aa-KO-T1-detect-R           | ATGCATGTTAACAACCTCTGC                                   |                       |
| dnmt3ab-KO-detect-F              | TGTTGCATTTGCTCCACTCAACCA                                |                       |
| dnmt3ab-KO-detect-R              | TGTGAATCAGACCTAATCAGCTGTGC                              | Mutant screening       |
| dnmt3aa-KO-T2-detect-F           | AACCTGCCAGCATGTACTCG                                    |                       |
| dnmt3aa-KO-T2-detect-R           | TGTTGTGTGAAGGGGGAGG                                    |                       |
| dnmt3aa-RT-PCR-F                 | CAGTCCAGAGGAGGG                                        |                       |
| dnmt3aa-RT-PCR-R                 | TCTCCCTGCGGACTGGCTCG                                    |                       |
| dnmt3ab-RT-PCR-F                 | TGGACTCGGACCTGAT                                        | RT-PCR                |
| dnmt3ab-RT-PCR-R                 | CCTCGACACGGCAAGAGGAGG                                   |                       |
| gapdh-F                          | AAGCTCATTTCCTGTTAT                                      |                       |
| gapdh-R                          | CCTTTGTGTATTTTCTTG                                     |                       |
| dnmt3aa-ISH-F                    | CCCAGACACCAGAGAAGC                                      |                       |
| dnmt3aa-ISH-R                    | TGTGGTTGTAAGGGGGAGG                                    | ISH                   |
| dnmt3ab-ISH-F                    | TCCACCAAAGCTTTACCC                                       |                       |
| dnmt3ab-ISH-R                    | CCTGTCATGCGGAGGAT                                       |                       |
| dnmt1-qPCR-F                     | TGGCTCCACGTGATGAC                                       | qRT-PCR               |
| dnmt1-qPCR-R                     | AATTTGCTTGTCTCTCCGT                                     |                       |
| dnmt3aa-qPCR-F                   | TTGAGCCGGGAGGTA                                         |                       |
dnmt3aa-qPCR-R  CATGCCGACAGTGATGGAGT
dnmt3ab-qPCR-F  TCCACAAAGCTTTACCCCC
dnmt3ab-qPCR-R  CGGACGATACCCACAGTGAT
dnmt3bb.1-qPCR-F  AATGAGAACAGCCCCCTGAC
dnmt3bb.1-qPCR-R  CGCTCCTGAAGACCTTGGTCCG
dnmt3ba-qPCR-F  CGAAAGAGGACGACAACGT
dnmt3ba-qPCR-R  GTTCATGCCAGGCAGGTTTC
dnmt3bb.2-qPCR-F  CTTTACCTGAACCGGGACACA
dnmt3bb.2-qPCR-R  TATTCCTGGAAACGCACAGG
baxa-qPCR-F  TGCATCAGATTCACGATGAGTT
baxa-qPCR-R  ACGAGTCGGCATGCAAAGTA
baxb-qPCR-F  TGGCAATAAAGCAGTGACGA
baxb-qPCR-R  CCTCTCTTGCCACAAAGT

caspase3a-qPCR-F  GGAACAAATCAGGCGCTC

caspase3a-qPCR-R  CGTCAGTACCGTCCGCTGA

caspase3b-qPCR-F  ACTGTGGCGTCAGATGAAAGC

caspase3b-qPCR-R  GACCCTTGCAAGTGTTTCCCT

caspase8-qPCR-F  CCGCAACAGCAGTTCACTT

caspase8-qPCR-R  TCAGGAAGAGGGGTGGGATT

caspase9-qPCR-F  TTCCCTAGTAAGCTATCGCTGA

caspase9-qPCR-R  CTATGGAGGCCCTTGCGA

AMH-F5  ATGGGCTCCGAGACCTTGACTG  Genetic sex

AMH-R3  CAGAAATGTAGACGCCAGGTAT  identification