Heterozygous mutation of eEF1A1b resulted in spermatogenesis arrest and infertility in male tilapia, *Oreochromis niloticus*

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Eukaryotic elongation factor 1 alpha (eEF1A) is an essential component of the translational apparatus. In the present study, eEF1A1b was isolated from the Nile tilapia. Real-time PCR and Western blot revealed that eEF1A1b was expressed highly in the testis from 90 dah (days after hatching) onwards. In situ hybridization and immunohistochemistry analyses showed that eEF1A1b was highly expressed in the spermatogonia of the testis. CRISPR/Cas9 mediated mutation of eEF1A1b resulted in spermatogenesis arrest and infertility in the F₀ XY fish. Consistently, heterozygous mutation of eEF1A1b (eEF1A1b<sup>+/−</sup>) resulted in an absence of spermatocytes at 90 dah, very few spermatocytes, spermatids and spermatozoa at 180 dah, and decreased Cyp11b2 and serum 11-ketotestosterone level at both stages. Further examination of the fertilization capacity of the sperm indicated that the eEF1A1b<sup>+/−</sup> XY fish were infertile due to abnormal spermiogenesis. Transcriptomic analyses of the eEF1A1b<sup>+/−</sup> testis from 180 dah XY fish revealed that key elements involved in spermatogenesis, steroidogenesis and sperm motility were significantly down-regulated compared with the control XY. Transgenic overexpression of eEF1A1b rescued the spermatogenesis arrest phenotype of the eEF1A1b<sup>+/−</sup> testis. Taken together, our data suggested that eEF1A1b is crucial for spermatogenesis and male fertility in the Nile tilapia.

Eukaryotic translation elongation factor 1A (eEF1A) is one of the most abundant protein synthesis factors in eukaryotic cells. It is responsible for delivering aminoacylated tRNAs to the A site of the ribosome in a GTP-dependent reaction<sup>1</sup>. Eukaryotes possess a variable number of eEF1A genes with various expression patterns. Yeast (*Saccharomyces cerevisiae*) contains two eEF1A genes that are expressed almost equally in exponentially growing cells<sup>2</sup>. Fruit flies (*Drosophila melanogaster*) have two eEF1A genes, one of which is expressed only in certain life history stages<sup>3,4</sup>. In teleosts, only one eEF1A gene has been reported in zebrafish (*Danio rerio*), sea bream (*Sparus aurata*), Nile tilapia (*Oreochromis niloticus*) and medaka (*Oryzias latipes*)<sup>5–8</sup>. Recently, five eEF1A genes, referred to as eEF1A1-4 and 42Sp50, have been isolated and their relative expressions are different in ten tissues in flatfish (*Solea senegalensis*)<sup>9</sup>. In the African clawed frog (*Xenopus laevis*), three eEF1A genes are developmentally specific: the somatic form (*eEF-1S*) is present in embryos and most adult tissues, but is not detected in the germ cells; the oocyte form (*eEF-1O*), is present in germ cells and some adult tissues, but is not detected in the embryos; the 42Sp50 form, is detected only in oocytes<sup>10–12</sup>. In mammals, two eEF1A genes, named as eEF1A1 and eEF1A2, have been identified, eEF1A1, which is almost ubiquitously expressed, and eEF1A2, whose expression is restricted to some cell types in a few tissues<sup>13–16</sup>. Despite this, homozygous mutation of mammalian eEF1A2 is lethal during postnatal stages, possibly due to its critical function in translation elongation<sup>17–19</sup>. Although eEF1A is reported to be ubiquitously expressed in all tissues examined, its role in gonads remains to be elucidated. Gametogenesis is a complex process, during which numerous proteins are synthesized in the testis and ovary. Mutation of the elongation factors for proteins synthesis, may result in infertility in vertebrates, as was demonstrated for eEF4<sup>20</sup>. Besides its canonical function in translation elongation, eEF1A is also involved in other cellular processes, such as severing microtubule<sup>21</sup>, bundling F actin<sup>22</sup>, controlling cell apoptosis<sup>23</sup> and regulating protein degradation<sup>24,25</sup>. To date, only one report showed that eEF1A genes might be involved in the...
gmnotogenesis via non-canonical function. Oral administration of the eEF1A1 inhibitor gamendazole resulted in infertility in male mice, probably via disruption of actin-filament bundles related to the Sertoli cell-spermatisds ectoplasmic specialization junctions⁶. However, there have been no other reports on the role and mechanism of other eEFs, including eEF1A, in gametogenesis.

In addition to somatic cells, eEF1A is also expressed in germ cells. Proteins expressed in germ cells are directly related to gametogenesis. Disruption of genes expressed in germ cells often blocks gametogenesis²⁷-³⁰. Therefore, eEF1A might be directly involved in gametogenesis via protein synthesis in germ cells. Different eEF1A genes are reported to be expressed in the testis and ovary of Xenopus⁴¹,⁴². Our gonads transcriptomic data from gonads demonstrated that eEF1A1b and 42Sp50 are XY-enhanced and XX-specific genes in tilapia, respectively³¹. Taken together, we speculate that different elongation factors might be used for protein synthesis during spermatogenesis and oogenesis in lower vertebrates. Species with different eEF1A genes expressed in the testis and ovary would be a good model to study the diverse function of the eEF1A genes in spermatogenesis and oogenesis.

Nile Tilapia (Oreochromis niloticus), is a good model for the study of gene expression and function in fish due to the availability of monosex fish³², a short spawning cycle, many gonadal transcriptomes³¹ and published genome sequences⁴³. In the present study, expression of eEF1A1b was found to be enriched in XY males during tilapia gonadal development. Ontogenetic studies were conducted to determine its expression profile by real-time PCR and Western blot. The cellular localization of eEF1A1b was documented by in situ hybridization (ISH) and immunohistochemistry (IHC). We also mutated eEF1A1b and analyzed the gonadal phenotype, gene expression, serum 11-ketotestosterone (11-KT) level and fertility of the eEF1A1b deficiency and eEF1A1b⁻/⁻ fish.

Results

Phylogenetic and synteny analyses. As shown in the phylogenetic tree (supplemental Fig. S1), the phylogeny of eEF1A1 was split into two main clades. One clade included the eEF1A1 gene of tetrapods; and the other included the two distinct copies of eEF1A1 from teleosts that we named eEF1A1a and eEF1A1b.

Synteny analyses revealed the organization of the genomic region surrounding eEF1A1 genes in tetrapods and teleosts. eEF1A1a and its upstream gene sirc17a5, its downstream gene dsx3 and kcnq5a showed conserved synteny both in teleosts and tetrapods. eEF1A1b and its upstream gene ddi1 showed conserved synteny only in teleosts, and its downstream gene kcnq5b and rims1 showed conserved synteny only in tetrapods. Except kcnq5, no other conserved synteny of gene loci surrounding eEF1A1b were found (supplemental Fig. S2).

Tissue distribution. Analysis by IHC showed that eEF1A1b was highly expressed in the brain, heart, liver, intestine, kidney and testis, weakly expressed in the ovary, but was undetectable in gill, spleen, head kidney and muscle (Supplemental Fig. S3).

Expression profile of eEF1A1b in developing gonads. Real-time PCR demonstrated that expression of eEF1A1b was low from 10 to 90 dah, increased sharply from 90 dah in testis and slightly decreased from 150 to 180 dah; while no significant changes were found during all stages in ovary (Fig. 1a).

Western blot analyses revealed that eEF1A1b was expressed in both testis and ovary, with significantly higher expression in the testis than in the ovary from 90 dah, peaking at approximately 120 dah, and decreased to a much lower level at 300 dah (Fig. 1b).

Cellular localization of eEF1A1b in gonads. IHC analysis demonstrated that eEF1A1b was expressed in the spermatogonia of the testis, and in the oogonia and phase I oocytes of the ovary with eEF1A1b antisense probe (Fig. 2a, A and B). eEF1A1a, the paralogous gene of eEF1A1b, was expressed in the somatic cells of the testis, and in the oocytes and somatic cell of the ovary (Fig. 2a, C and D). In contrast, signal was detected in neither testis nor ovary with eEF1A1a and eEF1A1b sense probe (Fig. 2a, E-H).

Consistently, IHC analysis revealed that eEF1A1b was expressed exclusively in the spermatogonia of the testis from 5, 30, 90 and 180 dah (Fig. 2b, A-D), and was expressed in the oogonia of the ovary at 5 dah, later in the oogonia and phase I oocytes of the ovary at 30 to 180 dah (Fig. 2b, E-H).

Disruption of eEF1A1b by CRISPR/Cas9 and production of F₁ generation. A guide RNA containing BsaI adjacent to proto-spacer adjacent motif in the second exon of eEF1A1b was selected for mutation analysis (Fig. 3a). Genomic DNA from 20 pooled injected embryos and from fin of mutants was used as template for PCR amplification and mutation analyses. Complete digestion produced fragments of 425 and 102 bp in the control groups whereas an intact DNA fragment was observed in embryos injected with both Cas9 mRNA and target guide RNA (Fig. 3b). The mutation rate in the pooled embryos was approximately 97%. In-frame and frame-shift deletions induced at the target site were confirmed by Sanger sequencing. We screened 40 individual microinjected fish by restriction enzyme digestion at 90 dah, and found 38 to be mutant for eEF1A1b. All mutant fish had mutation rate over 90% (Supplemental Tab. S3). Twenty mutant fish were further confirmed by Sanger sequencing (Fig. 3c, d and e).

Effects of eEF1A1b deficiency on spermatogenesis and fertility in F₁ fish. Morphologically, all kinds of germ cells including spermatogonia, spermatocytes, spermatids and spermatozoa were present in the control testis at 90, 120, 150 and 180 dah (Fig. 4a, A-D). In contrast, in the eEF1A1b deficient testis only spermatogonia were observed at 90 and 120 dah, spermatocytes appeared at 150 dah and spermatozids and spermatozoa...
appeared at 180 dah (Fig. 4a, E-H). Additionally, fewer spermatozoa were observed in the efferent duct of the eEF1A1b deficient testis at 180 dah compared with the control.

By IHC, eEF1A1b was detected in the spermatogonia, and Cyp11b2 was observed in the Leydig cells in the control testis at 180 dah (Fig. 4b, A and B). In contrast, reduced expression of eEF1A1b and Cyp11b2 was observed in the eEF1A1b deficient testis (Fig. 4b, D and E). However, Vasa was detected in the spermatogonia and spermatocytes in the eEF1A1b deficient and control testis (Fig. 4b, C and F).

Western blot analyses revealed that the expression of eEF1A1b, Cyp11b2 and Vasa was significantly reduced in the eEF1A1b deficient testis compared with those of the control (Fig. 4c). Additionally, the size of the eEF1A1b deficient testis was significantly smaller than that of the control testis as demonstrated by gonadal somatic index (GSI, gonad weight/body weight × 100%) (Fig. 4d).

Fertility test showed that the eEF1A1b deficient XY fish were infertile (supplemental Tab. S4). When examined under microscope, the sperm of the eEF1A1b deficient fish were abnormal. The sperm from the control fish were normal with a flagella length of 16.2 ± 2.4 μm, and displayed strong motility. In contrast, the sperm from eEF1A1b deficient fish were characterized by short or absent flagella. The short-flagella sperms with a flagella length of 7.55 ± 3.16 μm, displayed weak motility, and the flagella-less sperms were stuck together, showing no motility (Fig. 4e; supplemental Fig. S5).

Effects of eEF1A1b deficiency on ovarian development in F0 fish. In contrast to the significant phenotypes of eEF1A1b deficiency in the testis, the ovary of most mutant fish showed normal development at 120 and 180 dah. Histological analysis demonstrated that folliculogenesis was normal in the ovary of the eEF1A1b deficient XX fish (Supplemental Fig. S6).

Effects of eEF1A1b heterozygous mutation on spermatogenesis and fertility in F1 fish. The heterozygous mutation of eEF1A1b (eEF1A1b+/−) XY fish with a 4 bp deletion were employed for phenotype analyses. Consistent with the phenotype of F0 generation, eEF1A1b+/− XY fish also displayed spermatogenesis arrest. Histologically, spermatogonia, spermatocytes and spermatids, were present at eEF1A1b+/+ testis, whereas only spermatogonia were observed in eEF1A1b+/− testis at 90 dah (Fig. 5a). By IHC, eEF1A1b and Oct 4 (spermatogonia maker), Phospho-histone h3 (Ph3, spermatocytes maker), Cyp11b2 were observed in the spermatogonia, spermatocytes and Leydig cells, respectively, of the eEF1A1b+/+ testis. In contrast, Oct 4 and reduced expression of eEF1A1b were observed in the spermatogonia, while no Ph3 and Cyp11b2 expression was detected in the eEF1A1b+/− testis. These results indicated that there were only spermatogonia, but no spermatocytes in the eEF1A1b+/− testis (Fig. 5a).

In addition, the eEF1A1b, cyp11b2 and vasa mRNA levels were found to be significantly down-regulated in the eEF1A1b+/− testis by real-time PCR (Fig. 5b). Further, significantly lower serum 11-KT level was detected in the eEF1A1b+/− XY fish compared with that of the eEF1A1b+/+ XY fish at 90 dah (Fig. 5c). Furthermore, the size of the eEF1A1b+/− testis was significantly smaller than that of the eEF1A1b+/+ testis as reflected by the GSI.
By histology, abundant spermatocytes, spermatids and spermatozoa were observed in the eEF1A1b+/+ testis at 120, 150 and 180 dah (Fig. 6, A-F). In contrast, in the testis of eEF1A1b−/− XY fish, just a few spermatocytes were observed at 120 dah, and a markedly reduced number of spermatocytes, spermatids and spermatozoa were observed at 150 and 180 dah (Fig. 6, G-L).

Fertility test showed that similar to the chimeric XY fish, eEF1A1b−/− XY fish were infertile (Fig. 7A). Morphological and biochemical analyses of sperm were performed to analyze the reason for male infertility in eEF1A1b−/− XY fish. The sperm of eEF1A1b+/+ XY fish were characterized by normal flagella, while the sperm of eEF1A1b−/− XY fish were flagella-less. Therefore, the ratio of sperm with morphological abnormalities to the total sperm was significantly higher in eEF1A1b−/− XY fish than that in the eEF1A1b+/+ XY fish (Fig. 7, B-D). Consequently, the sperm forward motility was lost in the eEF1A1b−/− XY fish (Fig. 7, F-H).

Gonadal transcriptome analysis of eEF1A1b+/− XY fish. Transcriptome sequencing of the testes from the eEF1A1b+/− XY fish at 180 dah yielded a total of 40,039,839 reads. The eEF1A1b+/− XY transcriptome data were analyzed using the transcriptome data from testis of 180 dah XY fish obtained earlier by our group as a control. The total gene counts of the eEF1A1b+/− XY fish (22,284) were slightly more than those of the

Figure 2. Cellular localization of eEF1A1b in tilapia testis and ovary at different developmental stages by ISH and IHC. (a) Cell type expressing eEF1A1a and eEF1A1b in tilapia gonads by ISH. eEF1A1b was detected in spermatogonia of the testis, while in the oogonia and phase I oocytes of the ovary at (A and B). eEF1A1a was detected in somatic cell of the testis, while in the oocytes and somatic cells of the ovary (C and D). (b) Cell type expressing eEF1A1b in tilapia gonads by IHC. Consistent with in situ hybridization results, eEF1A1b was detected in the spermatogonia of the testis from 5 to 180 dah (A–D), while in the oogonia of ovary at 5 dah, later in the oogonia and phase I oocytes of the ovary from 30 to 180 dah (E–H). SG, spermatogonia; SC, spermatocytes; ST, spermatids; OG, oogonia; OC, oocytes; I-IV, phase I to phase IV oocytes; Arrowheads indicate the positive signal.
**Transgene mediated rescue of spermatogenesis in eEF1A1b**

To rescue the spermatogenesis arrest phenotype of eEF1A1b<sup>+/−</sup> XY fish using a CMV promoter-derived expression vector. A specific band of 720 bp was amplified by CRISPR/Cas9. Gene structure of eEF1A1b was conducted in the eEF1A1b<sup>+/-</sup> testis when examined at 90 dah (Fig. 9b, A and B).

By IHC, Vasa and Ph3 were observed in the spermatogonia/spermatocytes and spermatocytes, and Cyp11b2 were observed in the Leydig cells of the XY fish (Fig. 8e). Consequently, the serum 11-KT level and GSI of the testis (Fig. 9c). Consistently, the serum 11-KT level in the eEF1A1b<sup>−/−</sup> testis compared with the control (Fig. 8c). In contrast, reduced Vasa, and no Ph3 signals were detected in the eEF1A1b<sup>+/-</sup> testis (Fig. 8d, A-D). In contrast, reduced eEF1A1b and Sf-1 expression and very sparse Starl and Cyp11b2 were detected in the eEF1A1b<sup>−/−</sup> testis (Fig. 8d, E-H). Consequently, the serum 11-KT level of the eEF1A1b<sup>−/−</sup> XY fish was significantly lower than that of eEF1A1b<sup>+/−</sup> XY fish (Fig. 8e).

**Discussion**

**Evolutionary implication of eEF1A1b.** In the present study, eEF1A1a and eEF1A1b genes were successfully identified from the Nile tilapia, zebrafish, medaka and fugu. Phylogenetic analyses of eEF1A1s from vertebrates demonstrated that eEF1A1a and eEF1A1b arose from the teleost genome duplication<sup>34</sup>. eEF1A1a has retained the ancestral functions, while eEF1A1b has evolved novel function/expression patterns. Moreover,
Figure 4. Effects of eEF1A1b deficiency on spermatogenesis and fertility in F₀. (a) Histological observations of testis from F₀ and control XY fish at 90, 120, 150 and 180 dah. eEF1A1b deficiency resulted in spermatogenesis arrest. All kinds of germ cells including spermatogonia, spermatocytes, spermatids and spermatozoa were present at the control testis at 90, 120, 150 and 180 dah (A-D). In contrast, in the F₀ testis only spermatogonia were observed at 90 and 120 dah, spermatocytes appeared at 150 dah, and spermatids and spermatozoa appeared at 180 dah (E-H). (b) Expression of eEF1A1b, Cyp11b2 and Vasa in the F₀ and control XY fish by IHC. By IHC, eEF1A1b was observed in the control testis (A) while almost undetectable in F₀ testis (D). Reduced Cyp11b2 expression was observed in Leydig cells in the F₀ testis (B) compared with the control testis (E). Vasa positive signals were detected in both the control and F₀ testis (C and F). Arrowheads indicate the positive signal. SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa. (c) Expression of eEF1A1b, Cyp11b2 and Vasa in the F₀ and control XY fish by Western blot. (d) Gonadal somatic index (GSI) of the F₀ and control XY fish. (e) Morphology and motility of sperm from 180 dah F₀ and control XY fish. The sperm from the control fish were with normal flagella, and displayed vigorous flagella activity and progressive movement (A); while the sperm from F₀ fish were characterized by short or absent flagella. The motility of short-flagella sperms was weak, and the flagella-less sperms were stuck together, showing no motility (B). F₀, eEF1A1b deficiency.
conserved synteny of eEF1A1a with kcnq5a and eEF1A1b with kcnq5b was observed in teleosts. Taken together, these results suggested that eEF1A1a and eEF1A1b were derived from the teleost-specific genome duplication.

The expression pattern and functional relevance of eEF1A1b in tilapia. Spatial-temporal gene expression patterns are important aspects of gene function analysis. Our previous data showed that eEF1A1a displayed no gender difference in expression, while eEF1A1b was a male-biased gene in tilapia gonad31. In the present study, both real-time PCR and Western blot analyses demonstrated that eEF1A1b showed sexually dimorphic expression from 70 dah, and was highly expressed in the spermatogonia, spermatocytes and Leydig cells, respectively, of the eEF1A1b+/+ testis. In contrast, Oct 4 and reduced expression of eEF1A1b were observed in the spermatogonia, while no Ph3 and Cyp11b2 expression was detected in the eEF1A1b+/− testis. Arrowheads indicate the positive signal. SG, spermatogonia; SC, spermatocytes; ST, spermatids. (b) Expression of eEF1A1b, cyp11b2 and vasa in the eEF1A1b+/+ and eEF1A1b+/− XY fish at 90 dah by real-time PCR. (c) Serum 11-KT levels of the eEF1A1b+/− and eEF1A1b+/+ XY fish. (d) GSI of eEF1A1b+/− and eEF1A1b+/+ XY fish at 90 dah. Results were expressed as mean ± SD. Different letters indicate statistical differences at P < 0.05 as determined by one-way ANOVA followed by post hoc test.

Figure 5. Effects of the heterozygous mutation of eEF1A1b on spermatogenesis and fertility. (a) IHC analyses of eEF1A1b, Oct 4 (spermatogonia maker), Ph3 (spermatocyte maker) and Cyp11b2 expression in the testes of eEF1A1b+/− and eEF1A1b+/+ XY fish at 90 dah. eEF1A1b and Oct 4, Ph3, Cyp11b2 were observed in the spermatogonia, spermatocytes and Leydig cells, respectively, of the eEF1A1b+/+ testis. In contrast, Oct 4 and reduced expression of eEF1A1b were observed in the spermatogonia, while no Ph3 and Cyp11b2 expression was detected in the eEF1A1b+/− testis. Arrowheads indicate the positive signal. SG, spermatogonia; SC, spermatocytes; ST, spermatids. (b) Expression of eEF1A1b, cyp11b2 and vasa in the eEF1A1b+/+ and eEF1A1b+/− XY fish at 90 dah by real-time PCR. (c) Serum 11-KT levels of the eEF1A1b+/− and eEF1A1b+/+ XY fish. (d) GSI of eEF1A1b+/− and eEF1A1b+/+ XY fish at 90 dah. Results were expressed as mean ± SD. Different letters indicate statistical differences at P < 0.05 as determined by one-way ANOVA followed by post hoc test.

Conserved synteny of eEF1A1a with kcnq5a and eEF1A1b with kcnq5b was observed in teleosts. Taken together, these results suggested that eEF1A1a and eEF1A1b were derived from the teleost-specific genome duplication.
In the present study, eEF1A1b deficiency resulted in spermatogenesis arrest and consequent infertility in F₀ XY fish. This was reflected by defective of meiosis, reduced GSI and abnormal spermiogenesis. Heterozygous mutants of eEF1A1b displayed a phenotype similar to that of the F₀ XY fish. The eEF1A1b+/- testis showed no and very few spermatocytes at 90 and 120 dah, respectively. Spermatocytes, spermatids and spermatozoa were remarkably reduced at 150 and 180 dah. Furthermore, most sperm from the eEF1A1b+/- XY fish were flagella-less, and therefore, incapable of fertilization. Anatomical examination of eEF1A1b+/- XY fish showed mostly normal organs and tissues, including the heart and brain, the organ with high eEF1A1b abundance (supplemental Fig. S7). Thus, our results indicated the impairment of testis development, and failure of fertilization in mutant fishes were probably not the result of defects in these tissues, but a specific effect of the eEF1A1b deficiency in testis. Both the eEF1A1b deficient and eEF1A1b+/- XY fish were infertile, clearly indicating that eEF1A1b is critical for spermatogenesis and fertility in male tilapia.

Of all eEF1A isoforms, only eEF1A1b was expressed in the spermatogonia in tilapia. Consequently, mutation of eEF1A1b caused spermatogenesis arrest and infertility. It is well documented that eEF1A is indispensable for protein synthesis. Recently, a report showed that genetic ablation of eEF4, which is a key quality-control factor in translation, causes testis-specific dysfunction in oxidative phosphorylation, leading to male infertility in mice20. Besides elongation factors, disruption of other genes expressed in the spermatogonia also blocks spermatogenesis27,29,37. Consistently, in the present study, the mRNA level of suz12a, aspm, and usp26, which were expressed in spermatogonia and critical for spermatogenesis28,37–39, were significantly down regulated in the eEF1A1b+/- testis. It is reasonable to believe that they were also down regulated in protein levels although we did not check the protein

Figure 6. Histological observations of testis from eEF1A1b+/- XY fish at 120, 150 and 180 dah. Abundant spermatocytes, spermatids and spermatozoa were observed in the testis of eEF1A1b+/- XY fish at 120, 150 and 180 dah (A-F), while just very few spermatocytes were observed at 120 dah, and a markedly reduced number of spermatocytes, spermatids and spermatozoa were observed at 150 and 180 dah in the testis of eEF1A1b+/- XY fish (G-L). D-F and J-L, Higher magnification of the A-C and G-I, respectively. SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa.
excellent model for studying human asthenozoospermia. Spermatogenesis and male fertility in the Nile tilapia. In addition, mutation of eEF1A1b in tilapia may provide an indication that the spermatogenesis arrest and infertility phenotypes observed in the eEF1A1b−/− testis it is exclusively expressed in the spermatogonia. Loss of function and transgenic overexpression studies indicated that eEF1A1b is a duplicated copy of eEF1A1. In summary, phylogenetic and syntenic analyses revealed that eEF1A1b is a duplicated copy of eEF1A1. In mammals, eEF1A1b is essential for spermatogenesis and male fertility. The phenotype of eEF1A1b−/− tests was rescued by eEF1A1b overexpression as reflected by the normal testis size and appearance of all types of male germ cells, including spermatocytes and spermatids, which were absent in the eEF1A1b−/− tests. Moreover, eEF1A1b transgene also rescued the expression of those genes involved in the spermatogenesis process and steroidogenesis altered in the eEF1A1b−/− tests. These results validated that the spermatogenesis arrest phenotype is exclusively caused by eEF1A1b haploinsufficiency rather than off-target effects.

Previous reports showed that asthenozoospermia is a common cause of human male infertility, diagnosed by shortened sperm flagella, reduced sperm concentration and motility similar to the infertile phenotype of eEF1A1b mutant XY fish. The exact cause of asthenozoospermia is unclear so far, and mutation of eEF1A1 might be lethal in mammals. Mutation of eEF1A1b in tilapia could be used as a model for investigating the possible involvement of eEF1A1 in asthenozoospermia.

In summary, phylogenetic and syntenic analyses revealed that eEF1A1b is a duplicated copy of eEF1A1.
Materials and Methods

Animals. Nile tilapia, Oreochromis niloticus, was kept in recirculating aerated freshwater tanks at 26 °C under a natural photoperiod. All-XX progenies were obtained by crossing a pseudomale (XX male, producing sperm after hormonal sex reversal) with a normal female (XX). All-XY progenies were obtained by crossing a supermale (YY) with a normal female. Animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

Phylogenetic and synteny analysis of eEF1A1b. A phylogenetic tree of eEF1A1s was constructed using tilapia 42Sp50 as the outgroup. Sequences were obtained from the NCBI (http://blast.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html) databases. The accession numbers of the sequences used are listed in the Supplemental Tab.S1. Full-length amino acid sequences were aligned using MEGA5.046. The credibility of the branching was tested using bootstrap resampling with 1000 pseudo-replicates.

For syntenic analysis, the location of eEF1A1 (including eEF1A1a and eEF1A1b) and their adjacent genes were determined for human, rat, chicken, green anole, clawed frog, Nile tilapia, medaka, fugu and zebrafish using the Ensembl genome browser (http://www.ensembl.org/index.html).

Tissue distribution of eEF1A1b by IHC. The various tissues obtained from XX and XY tilapia at 180 dah tilapia were fixed in Bouin’s solution for 12 hours at room temperature, dehydrated and embedded in paraffin. All tissue blocks were sectioned at 5 μm and used for IHC analysis, which was performed as described previously27. The antibody against eEF1A1b was diluted 1:1000 for use.

Production and characterization of eEF1A1b antibody. The peptide antigen (SGWNGDNMLEPSPNMT) of tilapia eEF1A1b was prepared and injected into rabbits. eEF1A1b antiserum (a-DTc) was collected and affinity purified. To confirm the specificity of the polyclonal antibody, total protein was extracted from XX and XY gonads of 30, 90, 120 and 300 dah tilapia. Western blots were carried out using the purified antibody at dilution of 1:500.

Expression profile of eEF1A1b in tilapia gonad by real-time PCR and Western blot. For ontogenic expression analysis, three parallel gonadal samples were prepared from the fish at 5, 10, 30, 40, 50, 60, 70, 90,
120, 150 and 180 dah. For sampling points from 5 to 60 dah, fish were dissected and viscera were removed under a stereoscopic microscope. RNAlater reagent (Ambion, TX) was poured on the coelomic epithelium to stabilize the RNA in the gonads, and then the gonads were removed using fine forceps. Gonads were pooled in a tube with RNAlater reagent and stored at −80 °C before RNA extraction.

Total RNAs (2.0 μg) were extracted and reverse transcribed into cDNA using PrimeScript RT Master Mix Perfect Real Time Kit according to the manufacturer's instructions (Takara, Japan). Real-time PCR was carried out on an ABI-7500 real-time PCR machine according to the protocol of SYBR® Premix Ex Taq II. The relative abundance of eEF1A1b mRNA transcripts was evaluated using the formula: 

\[ R = 2^{\Delta\Delta Ct} \]

The geometric mean of the copy numbers of the three reference genes (β-actin, gapdh and eEF1A1a) was used to normalize the expression of eEF1A1b. Primer sequences used for real-time PCR are listed in Supplemental Tab. S2. Data were expressed as the mean ± S.D. Statistical analysis was performed using GraphPad Prism4 software (GraphPad Software, USA). Significant differences between groups were tested by one-way ANOVA followed by post-hoc test. 

P < 0.05 was considered to be statistically significant.

Total proteins extracted from testes and ovaries of 30, 90, 120 and 300 dah tilapia were separated using 12% SDS-PAGE under reducing condition. Western blot was carried out as reported previously48 using the purified antibodies at dilution of 1:500.

**Cellular location of eEF1A1b in gonads analysis by ISH and IHC.** ISH was performed using tilapia gonads collected 180 dah from monosex (XX or XY) fish. Gonads were dissected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 4% PFA) at 4 °C overnight. After fixation, gonads were embedded in paraffin. Cross-sections of 5μm were cut with a sliding microtome and adhered to polylysine-treated slides with diethylpyrocarbonate-treated water. Slides were dried at 180°C for 4 hours to remove the RNase contamination and stored at 4 °C until use. Probes for both sense and antisense digoxigenin-labeled RNA strands were transcribed in vitro from a linearized GEM-Teasy-eEF1A1b cDNA clone using the RNA labeling kit (Roche, Germany). ISH was performed as described previously49.

For IHC analysis, the gonads of 5, 30, 90 and 180 dah monosex (XX and XY) fish were dissected, fixed in Bouin’s solution for 12 hours at room temperature, dehydrated and embedded in paraffin. All tissue blocks...
were sectioned at 5 μm for IHC analysis, which was performed as described previously. The antibody against eEF1A1b was diluted 1:1000 for use.

**Disruption of eEF1A1b by CRISPR/Cas9.** CRISPR/Cas9 was performed to knockout eEF1A1b in tilapia. The gRNA target site was selected from eEF1A1b sequences corresponding to GGN18NNG on the sense or antisense strand of DNA (http://zifit.partners.org/ZiFiT/). Candidate target sequences were compared with the entire tilapia genome using the Basic Local Alignment Search Tool to avoid cleavage of off-target sites. One-cell stage embryos were divided into two batches, one for microinjection and the other for control. The gRNA and Cas9 mRNA were co-injected into one-cell stage embryos with the optimal concentration of 150 ng/μl and 300 ng/μl, respectively. Twenty injected embryos were collected 72 hours after injection. Genomic DNA was extracted from pooled injected embryos and control embryos, and used to access the mutations. DNA fragments spanning the eEF1A1b target site was amplified using gene specific primers (Supplemental Tab. 2). The indels were confirmed by restriction enzyme digestion with BsaI and Sanger sequencing. In addition, the percentage of uncleaved band was measured by quantifying the band intensity using Quantity One Software (Bio-Rad, USA). The indel (insertion and deletion) frequency was calculated by dividing uncleaved band intensity to the total band intensity from single digestion experiment.

The remaining micro-injected fish were reared until sampling for phenotypic assays. To screen the mutant fish, a piece of tail fin was clipped from each individual, and genomic DNA was extracted as described above. The target genomic locus was amplified using the primers eEF1A1b-cas-F/R. Mutations were assessed by restriction enzyme digestion and Sanger sequencing. The genetic sex of the mutants was determined using a sex-linked DNA marker (marker5) reported previous by our group. The eEF1A1b-deficient fish were used for gonad histology, gene expression (real-time PCR, IHC, and Western blot), serum androgen (11-KT) analyses and fertility test at 90 and 180 dah.

**eEF1A1b deficiency on spermatogenesis and fertility.** Gonads of eEF1A1b deficient fish were sampled at 90, 120, 150 and 180 dah. Samples were fixed in Bouin's solution for 24 hours at room temperature, dehydrated and embedded in paraffin. Tissue blocks were sectioned at 5 μm and stained with hematoxylin and eosin (H.E) or used for IHC analysis. Vasa (germ cell marker) antibody was donated by Prof. Nagahama, the National Institute of Genetics, Japan. The specificity of the antibody has been analyzed previously. Antibodies against Sf-1 and Star1 were diluted 1:1000 and 1:200 for use, respectively. Sf-1 and Star1 antibodies were prepared by our laboratory. The specificity of Sf-1 and Star1 antibodies was checked previously. Antibodies against eEF1A1b, Vasa and Cyp11b2 were diluted 1:1000, 1:1000 and 1:500 for use, respectively. IHC analyses were performed as described above. Photographs were taken under Olympus BX51 light microscope.

Western blots were performed to confirm the expression change of eEF1A1b, Cyp11b2 and Vasa in the eEF1A1b deficient and the control tests at 180 dah.

Serum 11-KT level was measured using the ELISA kits (Cayman Chemical Co, USA) following the manufacturer's instructions. Blood samples were collected from the caudal vein of the eEF1A1b deficient and control XY fish at 90 dah and kept at 4 °C overnight. Serum was collected after centrifugation and stored at −20 °C until use.

Sperm collected from eEF1A1b deficient and the control XY fish at 180 dah were diluted 1:1000 with phosphate buffered saline. Examination of morphology and motility of sperm was performed under Olympus BX51 light microscope.

To test fertility, 1200 mature eggs from an XX mother fish were divided into six groups (each with 200 eggs), and artificial insemination was performed using sperm from eEF1A1b deficient and control XY fish (n = 3). 12 hours later, the number of gastrula-stage embryos was scored under a light microscope to calculate fertilization rate. Hatching rates were calculated at 1 dah (5 days post fertilization). The concentration of sperm from eEF1A1b deficient and control XY fish was calculated using cell count data from flow cytometry (n = 3).

**Production of F1.** The eEF1A1b heterozygous F1 offspring were obtained by mating the F0 female founder with a wild-type male. F1 fish were screened by restriction enzyme digestion of PCR amplifications, as described above. Mutate sequences were confirmed by Sanger sequencing.

**Heterozygous mutant of eEF1A1b on spermatogenesis and fertility.** The gonads of heterozygous mutant of eEF1A1b (eEF1A1b+/−) XY fish were sampled at 90, 120, 150 and 180 dah for H.E and IHC analysis. eEF1A1b, Oct 4 (spermatogonia maker) and Cyp11b2 antibodies were prepared by our laboratory. Phospho-histone h3 (spermatocytes maker) antibody was obtained from the Cell signaling Technology (Beverly, MA, USA). Antibodies against eEF1A1b, Oct4, Histone 3 and Cyp11b2 were diluted 1:1000, 1:1000, 1:1000 and 1:500 for use, respectively. SF-1 and Star1 antibodies were prepared by our laboratory. The specificity of SF-1 and Star1 antibodies was checked previously. Antibodies against SF-1 and Star1 were diluted 1:1000 and 1:200 for use, respectively. Serum 11-KT level of eEF1A1b+− and eEF1A1b+/+ fish at 90 and 180 dah were measured using the ELISA kits as described above.

Sperm of eEF1A1b+− and eEF1A1b+/+ XY fish was sampled 180 dah to assay sperm quality. Fertility tests were performed as described above. Sperm analysis, including sperm count, morphological abnormality, trajectory, flagellum beat frequency, motility and path velocity, were conducted using Sperm Quality Analyzer (ZKPACS-E) in The Ninth People's Hospital of Chongqing.

**Transcriptome analysis.** The transcriptomes of 180 dah control testes was sequenced previously by our group. Six testes from eEF1A1b+/− XY fish were used for transcriptome analyses. Total RNA was extracted from testes using the RNeasy Mini Kit (50) (Qiagen) according to the manufacturer's instructions. The extracted RNA was further treated with deoxyribonuclease 1 (ribonuclease free) to eliminate genomic DNA contamination. The
oligo (dT) bead-enriched mRNA was disrupted into short fragments (200–700 nt) using fragmentation buffer. These short fragments were used as templates for first- and second-strand cDNA synthesis using a DNA synthesis kit (Invitrogen). A QiaQuick PCR purification kit (Qiagen) was used to purify these fragments, and the elution buffer was used for end repair and addition of the poly (A) tail. Then, these short fragments were ligated to sequencing adapters. After agarose gel electrophoresis, fragments between 320 and 370 nt were cut from the gel for PCR amplification. cDNA libraries were constructed from the two samples and sequenced on an Illumina HiSeq2000 instrument. Clean reads from each library were aligned to the reference genome (Orenil1.0.0, http://www.ensembl.org/Oreochromis_niloticus/Info/Index) using Tophat with default parameters, and the reads and pk per million reads (RPKM) method was used to calculated gene expression level. The assembled transcripts were merged with the reference annotation (Oreochromis niloticus. Orenil1.0.78.gtf, downloaded from Ensembl) using Cuffmerge, and differential expression analysis was performed using Cuffdiff. Transcriptomes of control-XY and eEF1Ab+/- XY were used to analyze the gene expression profiles. The threshold for the p-value was determined using false discovery rate (FDR) set at 0.01. In this study, genes with RPKM < 1 in the transcriptome were considered to be background expression and were excluded from further analysis. Gonadal expressed genes were divided into non-differentially expressed genes between control and eEF1Ab+/- XY (NDGs), eEF1Ab+/- XY up-regulated genes (URGs), and eEF1Ab+/- XY down-regulated genes (DRGs). FDR > 10^-2 and |log2[(control-XY_RPKM/eEF1Ab+/- XY_RPKM)]| > 1 were used to identify NDGs, FDR > 10^-2 and log2(control-XY_RPKM/eEF1Ab+/- XY_RPKM) > 1 were used to identify DRGs, and FDR > 10^-2 and (control-XY_RPKM/eEF1Ab+/- XY_RPKM) < -1 were used to identify URGs. To study the biological pathways of the up/down-regulated genes involved, we mapped these differentially expressed genes to pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) using KOBASE web server (http://kobas.cbi.pku.edu.cn/).

Transgene mediated rescue of spermatogenesis in eEF1Ab+/- XY fish. In vivo transgenic overexpression of the eEF1Ab in eEF1Ab+/- XY individuals was performed according to the methods of our previous study. An expression plasmid was prepared as follows. The ORF of eEF1Ab was amplified by PCR with a primer set introducing the BamHI and EcoRI sites (Supplemental Tab. 2). The amplified fragment was ligated into the BamHI and EcoRI sites (Supplemental Tab. 2). The amplified fragment was digested by BamHI and EcoRI and ligated into the multiple cloning sites downstream of the cytomegalovirus (CMV) sequence of the pRES-hGFP-1a vector (Stratagene, La Jolla, CA). The prepared construct was microinjected into eEF1Ab+/- XY fertilized eggs at the one-cell stage. Genomic DNA was extracted from injected fish and used to screen transgenic fish at 90 dah. DNA fragments were amplified using gene specific primers (Supplemental Tab. 2). The tests of the injected fish was examined by monitoring the GFP signal, and later the testes were subjected to both histological and immunohistochemical analyses. Antibodies against Ph3, Vasa and Cyp11b2 were diluted as described above. Serum 11-KT level was measured using the EIA Assay kits as described above.

References
1. Mateyak, M. K. & Kinsey, T. G. eEF1A: thinking outside the ribosome. Journal of Biological Chemistry 285, 21209–21213 (2010).
2. Koji, N., Masataka, K., Shigekazu, N. & Yoshito, K. Structure of the two genes coding for polypeptide chain elongation factor 1α (EF-1α) from Saccharomyces cerevisiae. Gene 45, 265–273 (1986).
3. Hovemann, B., Richter, S., Walldorf, U. & Czeplech, C. Two genes encode related cytoplasmic elongation factors 1α (EF-1α) in Drosophila melanogaster with continuous and stage specific expression. Nucleic Acids Research 16, 3175–3194 (1988).
4. Danforth, B. N. & Ii, S. Elongation factor-1 alpha occurs as two copies in bees: implications for phylogenetic analysis of EF-1 alpha sequences in insects. Molecular biology and evolution 15, 225–235 (1998).
5. Gao, D., Li, Z., Murphy, T. & Sauerbier, W. Structure and transcription of the gene for translation elongation factor 1 subunit alpha (EF-1αalpha) (EF-1αalpha/EF-1αbeta) from Saccharomyces cerevisiae. J Biol Chem 265, 265–273 (1990).
6. Chambers, D. M., Peters, J. & Abbott, C. M. The lethal mutation of the mouse wasted (wst) is a deletion that abolishes expression of translation elongation factor 1α genes in the flatfish Senegalese sole (Solea senegalensis). Journal of Fish Biology 74, 1244–1262 (2009).
7. Nowell, M. A. et al. Cloning and expression of an elongation factor 1α isoform: Isolation and characterisation of a cDNA encoding a novel variant of human elongation-factor 1α isoform. Biochemistry 26, 6803–6809 (1987).
8. Gao, D., Li, Z., Murphy, T. & Sauerbier, W. Structure and transcription of the gene for translation elongation factor 1 subunit alpha (EF-1α) in Drosophila melanogaster with continuous and stage specific expression. Nucleic Acids Research 16, 3175–3194 (1988).
9. Infante, C., Asensio, E., Cañavate, J. P. & Manchado, M. Molecular characterization and expression analysis of five different elongation factor 1 alpha genes in the flatfish Senegalese sole (Solea senegalensis Kaup): differential gene expression and thyroid hormones dependence during metamorphosis. BMC molecular biology 9, 1 (2008).
10. Abadalla, B., HOURDY, J., Krieg, P. A., Denis, H. & Mazabraud, A. Germ cell-specific expression of a gene encoding eukaryotic translation elongation factor 1 alpha (eEF1Aalpha) and generation of eEF1A1 alpha retro pseudogenes in Xenopus laevis. Proceedings of the National Academy of Sciences 88, 9277–9281 (1991).
11. Deschamps, S. et al. Two forms of elongation factor 1 alpha (EF-1 alpha O and 42Sp50), present in oocytes, but absent in somatic cells of Xenopus laevis. The Journal of cell biology 114, 1109–1111 (1991).
12. Loeber, J., Claußen, M., Jahn, O. & Pieler, T. Interaction of 42Sp50 with the vegetal RNA localization machinery in Xenopus laevis oocytes. FEBS Journal 277, 4722–4731 (2010).
13. Lee, S., Francoeur, A.-M., Liu, S. & Wang, E. Tissue-specific expression in mammalian brain, heart, and muscle of S1, a member of the elongation factor-1 alpha gene family. Journal of Biological Chemistry 267, 24064–24068 (1992).
14. Knudsen, S. M., Frydenberg, J., Clark, B. F. & Leffers, H. Tissue-dependent variation in the expression of elongation factor 1α isoforms: Isolation and characterisation of a cDNA encoding a novel variant of human elongation-factor 1α. European Journal of Biochemistry 215, 549–553 (1993).
15. Lee, S., Wolfram, L. A. & Wang, E. Differential expression of S1 and elongation factor-1 alpha during rat development. Journal of Biological Chemistry 268, 24453–24459 (1993).
16. Lee, S., Lefblanc, A., Duttary, A. & Wang, E. Terminal differentiation-dependent alteration in the expression of translation elongation factor 1α and its sister gene, S1, in neurons. Experimental cell research 199, 389–397 (1991).
17. Chambers, D. M., Peters, J. & Abbott, C. M. The lethal mutation of the mouse wasted (wst) is a deletion that abolishes expression of a tissue-specific isoform of translation elongation factor 1α, encoded by the Eef1a2 gene. Proceedings of the National Academy of Sciences 95, 4463–4468 (1998).
18. Khalifa, A. et al. Characterization of elongation factor-1A (eEF1A-1) and eEF1A-2/S1 protein expression in normal and wasted mice. *Journal of Biological Chemistry* **276**, 22915–22922 (2001).

19. Newbery, H. et al. Translation elongation factor eEF1A2 is essential for post-weaning survival in mice. *Journal of Biological Chemistry* **282**, 28951–28959 (2007).

20. Gao, Y. et al. Mammalian elongation factor 4 regulates mitochondrial translation essential for spermatogenesis. *Nature structural & molecular biology* **23**, 441–449 (2016).

21. Shiina, N., Gotoh, Y., Kubomura, N., Iwamatsu, A. & Nishida, E. Microtubule severing by elongation factor 1 alpha. *Science* **266**, 282–285 (1994).

22. Yang, F., Demma, M., Warren, V., Dharmawardhane, S. & Coneeldies, J. Identification of an actin-binding protein from Dictyostelium as elongation factor 1a. *Nature* **347**, 494–496 (1990).

23. Abbas, W., Kumar, A. & Herbein, G. The eEF1A proteins: at the crossroads of oncogenesis, apoptosis, and viral infections. *Frontiers in oncology* **5** (2015).

24. Hotokezaka, Y. et al. Interaction of the eukaryotic elongation factor 1A with newly synthesized polypeptides. *Journal of Biological Chemistry* **277**, 18545–18551 (2002).

25. Chuang, S.-M. et al. Proteasome-mediated degradation of cotranslationally damaged proteins involves translation elongation factor 1A. *Molecular and cellular biology* **25**, 403–413 (2005).

26. Tash, I. S. et al. Gemendazole, an orally active indazole carboxylic acid male contraceptive agent, targets HSP90AB1 (HSP90BETA) and eEF1A1 (eEF1A), and stimulates liss1a transcription in rat Sertoli cells. *Biology of reproduction* **78**, 1139–1152 (2008).

27. Li, M. et al. Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. *Genetics* **197**, 591–599 (2014).

28. Wosnitzer, M. S. et al. Ubiquitin specific protease 26 (USP26) expression analysis in human testicular and extragonadal tissues indicates diverse action of USP26 in cell differentiation and tumorigenesis. *PloS one* **9**, e96638 (2014).

29. Wangelius, A. et al. Dnd knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. *Scientific reports* **6** (2016).

30. Wang, Y. et al. Prmt5 is required for germ cell survival during spermatogenesis in mice. *Scientific reports* **5** (2015).

31. Tao, W. et al. Characterization of gonadal transcriptomes from Nile tilapia (Oreochromis niloticus) reveals differentially expressed genes. *PloS one* **8**, e63604 (2013).

32. Sun, Y.-L. et al. Screening and characterization of sex-linked DNA markers and marker-assisted selection in the Nile tilapia (Oreochromis niloticus). *Aquaculture* **433**, 19–27 (2014).

33. Brawand, D. et al. The genomic substrate for adaptive radiation in African cichlid fish. *Nature* **513**, 375–381 (2014).

34. Taylor, J. S., Braasch, I., Frickey, T., Meyer, A. & Van de Peer, Y. Genome duplication, a trait shared by 22,000 species of ray-finned fish. *Genome research* **13**, 382–390 (2003).

35. Munshi, R. et al. Overexpression of translation elongation factor 1A affects the organization and function of the actin cytoskeleton in yeast. *Genetics* **157**, 1425–1436 (2001).

36. Gross, S. R. & Kinzy, T. G. Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology. *Nature structural & molecular biology* **12**, 772–778 (2005).

37. Mu, W., Starmer, J., Fedoriw, A. M., Yee, D. & Magnuson, T. Repression of the soma-specific transcriptome by Polycomb-repressive complex 2 promotes male germ cell development. *Genes & development* **28**, 2056–2069 (2014).

38. Zhong, B. et al. Establishment of a proteomic profile associated with gonocyte and spermatogonial stem cell maturation and differentiation in neonatal mice. *Proteomics* **14**, 274–285 (2014).

39. Pulvers, J. N. et al. Mutations in mouse Aspm (abnormal spindle-like microcephaly associated) cause not only microcephaly but also major defects in the germline. *Proceedings of the National Academy of Sciences* **107**, 16595–16600 (2010).

40. Miura, C., Miura, T., Yamashita, M., Yamauchi, K. & Nagahama, Y. Hormonal induction of all stages of spermatogenesis in germ-somatic cell cokulture from immature Japanese eel testis. *Development, growth & differentiation* **38**, 257–262 (1996).

41. M Escott, G., A da Rosa, L. & da Silveira Loss, E. Mechanisms of Hormonal Regulation of Sertoli Cell Development and Proliferation: A Key Process for Spermatogenesis. *Current molecular pharmacology* **7**, 96–108 (2014).

42. Manova, K., Nocka, K., Besmer, P. & Bachvarova, R. F. Gonadal expression of c-kit encoded at the W locus of the mouse. *Development* **110**, 1057–1069 (1990).

43. Rios-Rojas, C., Spiller, C., Bowles, J. & Koopman, P. Germ cells influence cord formation and leydig cell gene expression during mouse testis development. *Developmental Dynamics* (2015).

44. Quu, Y. et al. Flagr Favor Ovarian Differentiation by Antagonizing Spermatogenesis in a Teleosts, Nile Tilapia (Oreochromis niloticus). *PloS one* **10**, e0123900 (2015).

45. Garti, S. et al. Asthenozoospermia: analysis of a large population. *Archives of andrology* **49**, 343–349 (2003).

46. Tamura, K. et al. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution* **28**, 2731–2739 (2011).

47. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. *methods* **25**, 402–408 (2001).

48. Yu, X. et al. Characterization of two paralogue STAR genes in a teleost, Nile tilapia (Oreochromis niloticus). *Molecular and cellular endocrinology* **392**, 152–162 (2014).

49. Wang, D.-S. et al. FoxD2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. *Molecular Endocrinology* **21**, 712–725 (2007).

50. Ostyn, A., De Buyser, M. L., Guillier, F., Krys, S. & Hennekinne, J. A. Benefits of the combined use of immunological-and PCR-based methods for determination of staphylococcal enterotoxin food safety criteria in cheeses. *Food Analytical Methods* **5**, 173–178 (2012).

51. Kobayashi, T., Kajiura-Kobayashi, H. & Nagahama, Y. Two isoforms of vasa homologs in a teleost fish: their differential expression during germ cell differentiation. *Mechanisms of development* **111**, 167–171 (2002).

52. Zhang, X. et al. Isolation of doublesex- and Mab-3-related transcription factor 6 and its involvement in spermatogenesis in tilapia. *Biology of reproduction*, biopredrepr. 114,121418 (2014).

53. Xie, Q.-Y. et al. Haplinsufficiency of SF-1 Causes Female to Male Sex Reversal in Nile Tilapia, Oreochromis niloticus. *Endocrinology*, en, 2015–2049 (2016).

54. Sun, L.-N. et al. Transdifferentiation of differentiated ovary into functional testis by long-term treatment of aromatase inhibitor in Nile tilapia. *Endocrinology* **155**, 1476–1488 (2014).

55. Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L. & Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature methods* **5**, 621–628 (2008).

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Author Contributions
D.W. conceived and designed the experiments; J.C., D.J., D.T., Z.F. and Y.W. performed the experiments; D.W. and J.C. analyzed the data; D.J. and M.L. contributed reagents/materials/analysis tools. D.W. and J.C. wrote the manuscript. All authors read and approved the manuscript.

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