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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 F_0 XY fish. Consistently, heterozygous mutation of *eEF1A1b* (*eEF1A1b*^{+/-}) 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*^{+/-} XY fish were infertile due to abnormal spermiogenesis. Transcriptomic analyses of the *eEF1A1b*^{+/-} 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*^{+/-} 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 reaction¹. 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 cells². Fruit flies (*Drosophila melanogaster*) have two *eEF1A* genes, one of which is expressed only in certain life history stages^{3,4}. 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*)⁵⁻⁸. 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*)⁹. 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 oocytes¹⁰⁻¹². 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 tissues¹³⁻¹⁶. Despite this, homozygous mutation of mammalian *eEF1A2* is lethal during postnatal stages, possibly due to its critical function in translation elongation¹⁷⁻¹⁹.

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*²⁰. Besides its canonical function in translation elongation, *eEF1A* is also involved in other cellular processes, such as severing microtubule²¹, bundling F actin²², controlling cell apoptosis²³ and regulating protein degradation^{24,25}. To date, only one report showed that *eEF1A* genes might be involved in the

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gametogenesis 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-spermatids ectoplasmic specialization junctions²⁶. 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 gametogenesis^{27–30}. 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 *Xenopus*^{11,12}. 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 sequences³³. 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 *slc17a5*, its downstream gene *ddx43* and *kcnq5a* showed conserved synteny both in teleosts and tetrapods. *eEF1A1b* and its upstream gene *dtd1* 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 *eEF1A1a* and *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. ISH 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 site containing *BsajI* 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 assays. 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 efficiency over 90% (Supplemental Tab. S3). Twenty mutant fish were further confirmed by Sanger sequencing. In total, we analyzed 200 sequences and identified 14 mutation types, including 11 types of deletion mutation and 3 types of insertion mutation. The majority (67.5%) were frame-shift mutations, while 32.5% were in-frame mutations (Supplemental Fig. S4). F₁ offspring were obtained by outcross *eEF1A1b* chimeric XX with XY control fish, and two mutation types (4 and 13 bp deletion) were identified in F₁ offspring by Sanger sequencing (Fig. 3c,d and e).

Effects of eEF1A1b deficiency on spermatogenesis and fertility in F₀ fish. Morphologically, all kinds of germ cells including spermatogonia, spermatocytes, spermatids and spermatozoa were presented 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 spermatids and spermatozoa

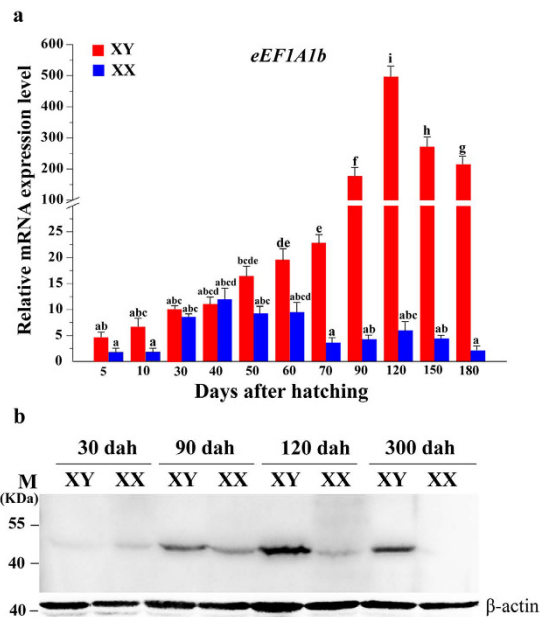


Figure 1. Expression patterns of eEF1A1b in developing gonads. (a) Ontogenetic expression of *eEF1A1b* in tilapia gonads analyzed by real-time PCR. Data are expressed as mean \pm SD of three different gonadal pools at each developmental stage. Different letters indicate statistical differences ($P < 0.05$) as determined by one-way ANOVA followed by post hoc test. (b) Expression of eEF1A1b in gonads of XX and XY fish at 30, 90, 120 and 300 dah by Western blot. Lanes 1, 3, 5 and 7, proteins extracted from testes; lanes 2, 4, 6 and 8, proteins extracted from ovaries. eEF1A1b was detected in both testis and ovary with significant higher expression in testis. 0.2 micrograms of protein were added per lane.

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 \times 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 $16.2 \pm 2.4 \mu\text{m}$, 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 $7.55 \pm 3.16 \mu\text{m}$, 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 F_0 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 F_1 fish. The heterozygous mutation of *eEF1A1b* (*eEF1A1b*^{+/-}) XY fish with a 4 bp deletion were employed for phenotype analyses. Consistent with the phenotype of F_0 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*^{+/-} XY testis at 90 dah (Fig. 5a). By IHC, eEF1A1b and Oct4 (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, Oct4 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.

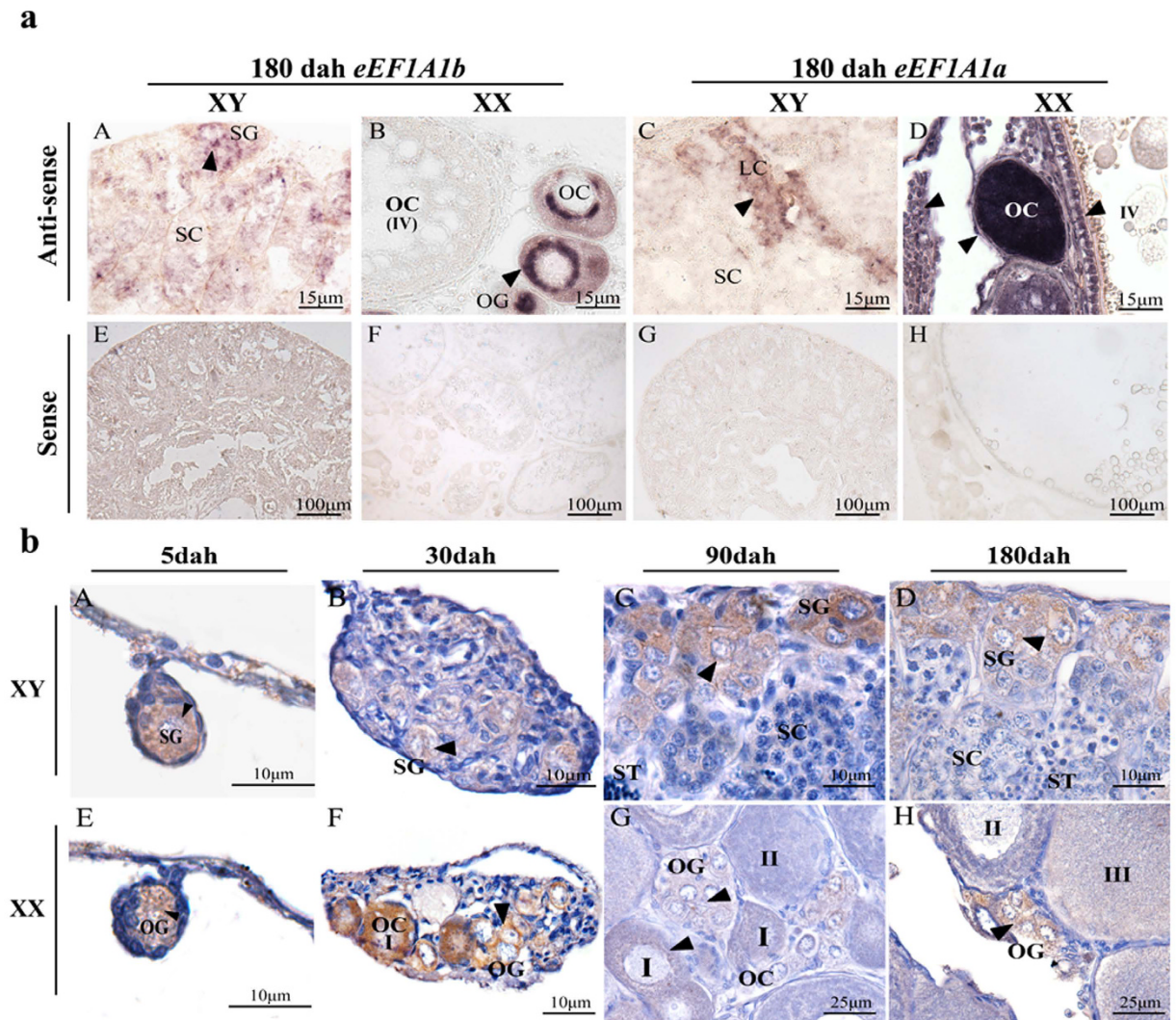


Figure 2. Cellular localization of *eEF1A1b* in tilapia testis and ovary at different developmental stages by ISH and IHC. (a) Cell type expressing *eEF1A1a* and *eEF1A1b* in tilapia gonads by ISH. *eEF1A1b* was detected in spermatogonia of the testis, while in the oogonia and phase I oocytes of the 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.

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). Consistently, the activity of sperm flagella was significantly lower in *eEF1A1b*^{+/-} XY fish than that in *eEF1A1b*^{+/+} XY fish (Fig. 7E). 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 group³¹ as a control. The total gene counts of the *eEF1A1b*^{+/-} XY fish (22,284) were slightly more than those of the

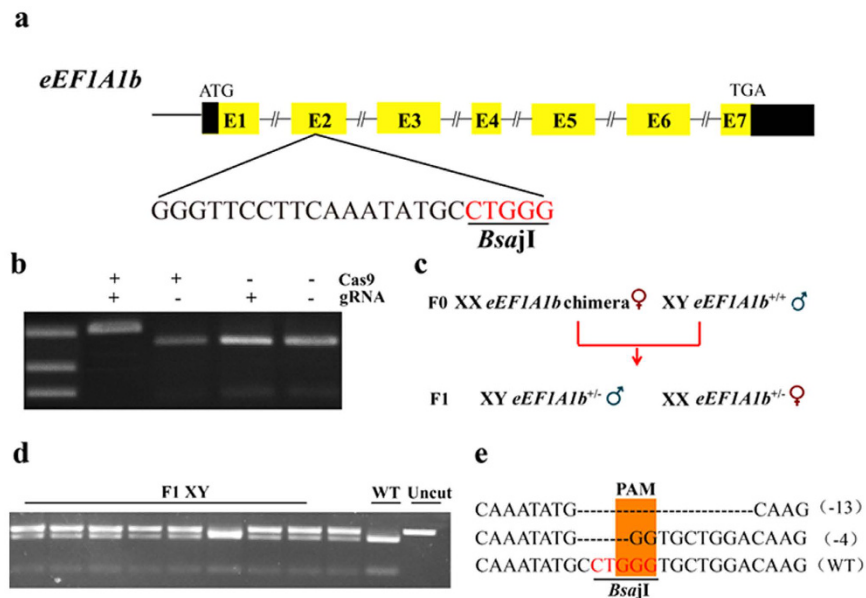


Figure 3. Design of CRISPR/Cas9 targeting *eEF1A1b* and establishment of the *eEF1A1b* mutation line. (a,b) Disruption of tilapia *eEF1A1b* by CRISPR/Cas9. Gene structure of *eEF1A1b* showing the target site and the *Bsa*JI restriction site. 300 ng/ μ l of Cas9 mRNA and 150 ng/ μ l of gRNA were co-injected into one-cell stage embryos. At 72 hours after injection, 20 embryos were randomly selected and pooled to extract their genomic DNA for PCR amplification. The indels were confirmed by two assays, restriction enzyme digestion and Sanger sequencing. The Cas9 mRNA and gRNA were added as indicated. Clear undigested band was detected in embryos injected with both Cas9 mRNA and gRNA compared with the control. (c) Schematic diagram showing the breeding plan of F₀ to F₁ fish. (d) *eEF1A1b*^{+/-} F₁ generation detected by restriction enzyme digestion. (e) Sanger sequencing results from the uncleaved bands were listed. Deletions are marked by dashes, and the PAM is marked in light orange. Numbers to the right of the sequences indicate the loss or gain of bases for each allele, with the number of bases inserted (+) or deleted (-) indicated in parentheses. WT, wild type.

eEF1A1b^{+/+} XY fish (21,365). Compared with the control testis, 1,522 genes were down regulated, 1,891 genes were up regulated, 11,554 genes were not differentially expressed and 7,317 genes were at background expression level in the *eEF1A1b*^{+/-} XY testis (Fig. 8a).

Further analysis revealed that of the 1522 down-regulated genes, 416 genes were involved in spermatogenesis process (e.g. *aspm*, *cdk16*, *suz12a*, *usp26* and *spo11*), 86 genes were involved in steroidogenesis (e.g. *sf-1*, *cyp11a1*, *star* and *cyp11b2*) and 19 genes were involved in sperm motility (e.g. *tuba1b*, *tuba3d* and *tuba8*) (Fig. 8b). Real-time PCR analyses showed that mRNA levels of *suz12a*, *spo11*, *usp26*, *sf-1*, *cyp11a1*, *star1* and *cyp11b2* were significantly down-regulated in the *eEF1A1b*^{+/-} testis compared with the control (Fig. 8c).

By IHC, eEF1A1b were observed in the spermatogonia, and Sf-1, Star1 and Cyp11b2 were observed in the Leydig cells of the *eEF1A1b*^{+/+} testis (Fig. 8d, A-D). In contrast, reduced eEF1A1b and Sf-1 expression and very sparse Star1 and Cyp11b2 were detected in the *eEF1A1b*^{+/-} testis (Fig. 8d, E-H). Consequently, the serum 11-KT level of the *eEF1A1b*^{+/-} XY fish was significantly lower than that of *eEF1A1b*^{+/+} XY fish (Fig. 8e).

Transgene mediated rescue of spermatogenesis in *eEF1A1b*^{+/-} XY fish. To rescue the spermatogenesis arrest phenotype of *eEF1A1b*^{+/-} XY fish, transgenic overexpression of *eEF1A1b* was conducted in the *eEF1A1b*^{+/-} XY fish using a CMV promoter-derived expression vector. A specific band of 720 bp was amplified from the genomic DNA of *eEF1A1b* transgenic (*eEF1A1b*^{+/-}^{TG}) fish (Fig. 9a). GFP signals were detected in the *eEF1A1b*^{+/-}^{TG} 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 detected in the Leydig cells of the *eEF1A1b*^{+/-}^{TG} testis (Fig. 9b, C-E). In contrast, reduced Vasa, and no Ph3 and Cyp11b2 were observed in the *eEF1A1b*^{+/-} testis (Fig. 9b, F-H).

By real-time PCR, the expression of *eEF1A1b*, *cyp11b2*, *vasa*, *suz12* and *usp26* in the *eEF1A1b*^{+/-}^{TG} testis showed no significant differences compared with the *eEF1A1b*^{+/+} testis, but significantly higher than that of the *eEF1A1b*^{+/-} testis (Fig. 9c). Consistently, the serum 11-KT level and GSI of the *eEF1A1b*^{+/-} XY fish showed no significant differences compared with the *eEF1A1b*^{+/+} testis, but significantly higher than those of the *eEF1A1b*^{+/-} testis (Fig. 9d and e)

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 duplication³⁴. *eEF1A1a* has retained the ancestral functions, while *eEF1A1b* has evolved novel function/expression patterns. Moreover,

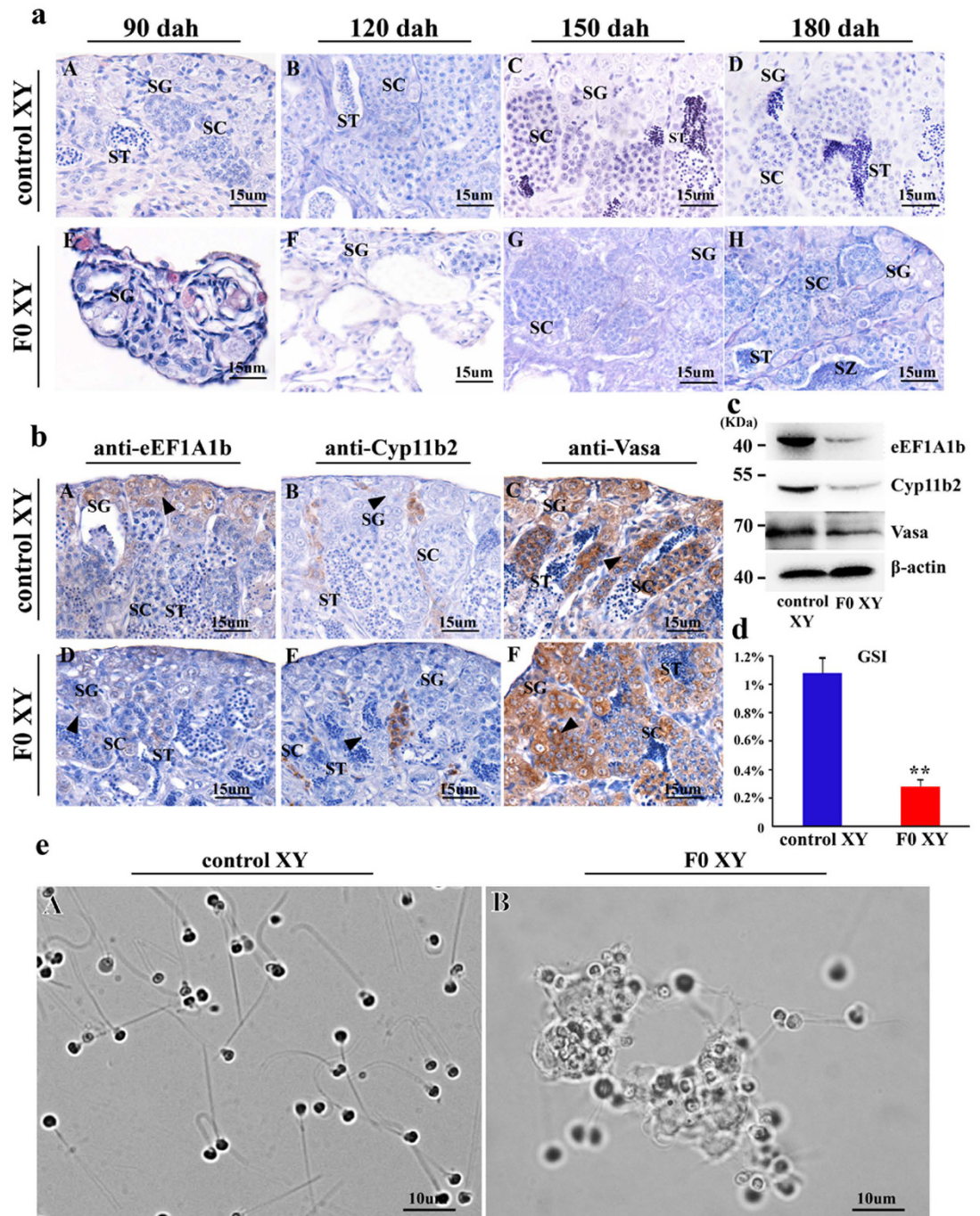


Figure 4. Effects of *eEF1A1b* deficiency on spermatogenesis and fertility in F_0 . (a) Histological observations of testis from F_0 and control XY fish at 90, 120, 150 and 180 dah. *eEF1A1b* deficiency resulted in spermatogenesis arrest. All kinds of germ cells including spermatogonia, spermatocytes, spermatids and spermatozoa were present at the control testis at 90, 120, 150 and 180 dah (A–D). In contrast, in the F_0 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 F_0 and control XY fish by IHC. By IHC, *eEF1A1b* was observed in the control testis (A) while almost undetectable in F_0 testis (D). Reduced *Cyp11b2* expression was observed in Leydig cells in the F_0 testis (B) compared with the control testis (E). *Vasa* positive signals were detected in both the control and F_0 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 F_0 and control XY fish by Western blot. (d) Gonadal somatic index (GSI) of the F_0 and control XY fish. (e) Morphology and motility of sperm from 180 dah F_0 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 F_0 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). F_0 , *eEF1A1b* deficiency.

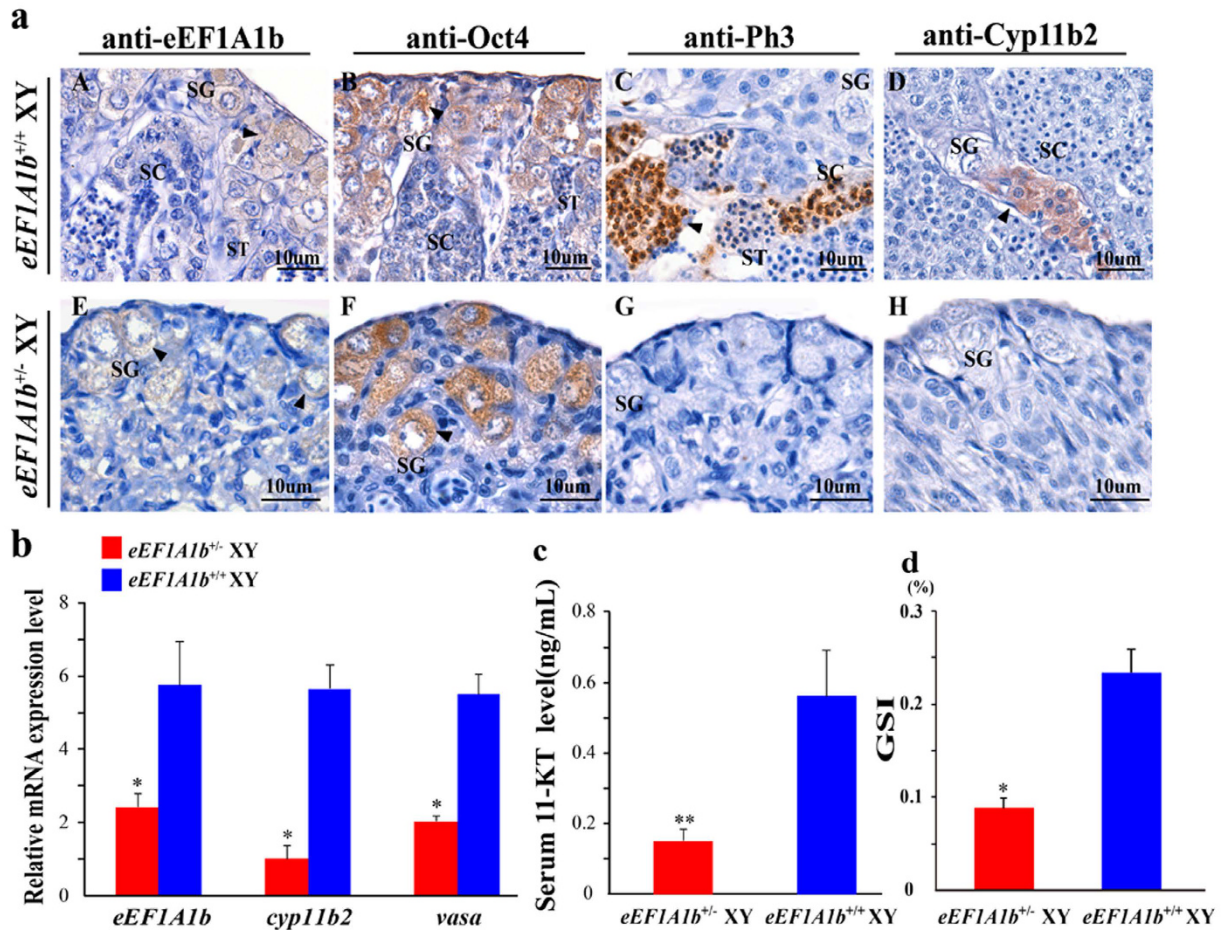


Figure 5. Effects of the heterozygous mutation of *eEF1A1b* on spermatogenesis and fertility. (a) IHC analyses of *eEF1A1b*, Oct4 (spermatogonia maker), Ph3 (spermatocyte maker) and *Cyp11b2* expression in the testis of *eEF1A1b*^{+/-} and *eEF1A1b*^{+/+} XY fish at 90 dah. *eEF1A1b* and Oct4, Ph3, *Cyp11b2* were observed in the spermatogonia, spermatocytes and Leydig cells, respectively, of the *eEF1A1b*^{+/+} testis. In contrast, Oct4 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.

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 testis from 90 dah onwards, correlated with the initiation of spermatogenesis and the appearance of spermatocytes. The expression of *eEF1A1b* reached its highest levels with the increase of the spermatogonia at 120 dah. Further, *eEF1A1b* was expressed exclusively in spermatogonia, while *eEF1A1a* was found to be mostly expressed in somatic cells of the testis. In the ovary, the expression of *eEF1A1b* was low and constant. *eEF1A1* is a housekeeping gene required for survival of all eukaryotic cells^{35,36}, and therefore, at least one *eEF1A* gene must be expressed in the germ cells and somatic cells of the gonad in vertebrates. In mammals, *eEF1A1* is a sex unbiased gene, and is expressed in the germ cell and somatic cell of the gonad¹³⁻¹⁶. In *Xenopus*, *eEF-1S* and *eEF-1O* are expressed in somatic cells and germ cells respectively, and *42sp50* is detected only in oocytes^{10,11}. In tilapia, *eEF1A1a* was expressed in the somatic cells of the testis and ovary, while *eEF1A1b* and *42Sp50* were expressed in the spermatogonia and oogonia/oocytes, respectively. Taken together, different elongation factor isoforms are used for male and female germ cells, as well as germ cells and somatic cells in lower vertebrates, while only one (*eEF1A1*) is used in both male and female germ cells and somatic cells of gonad in mammals.

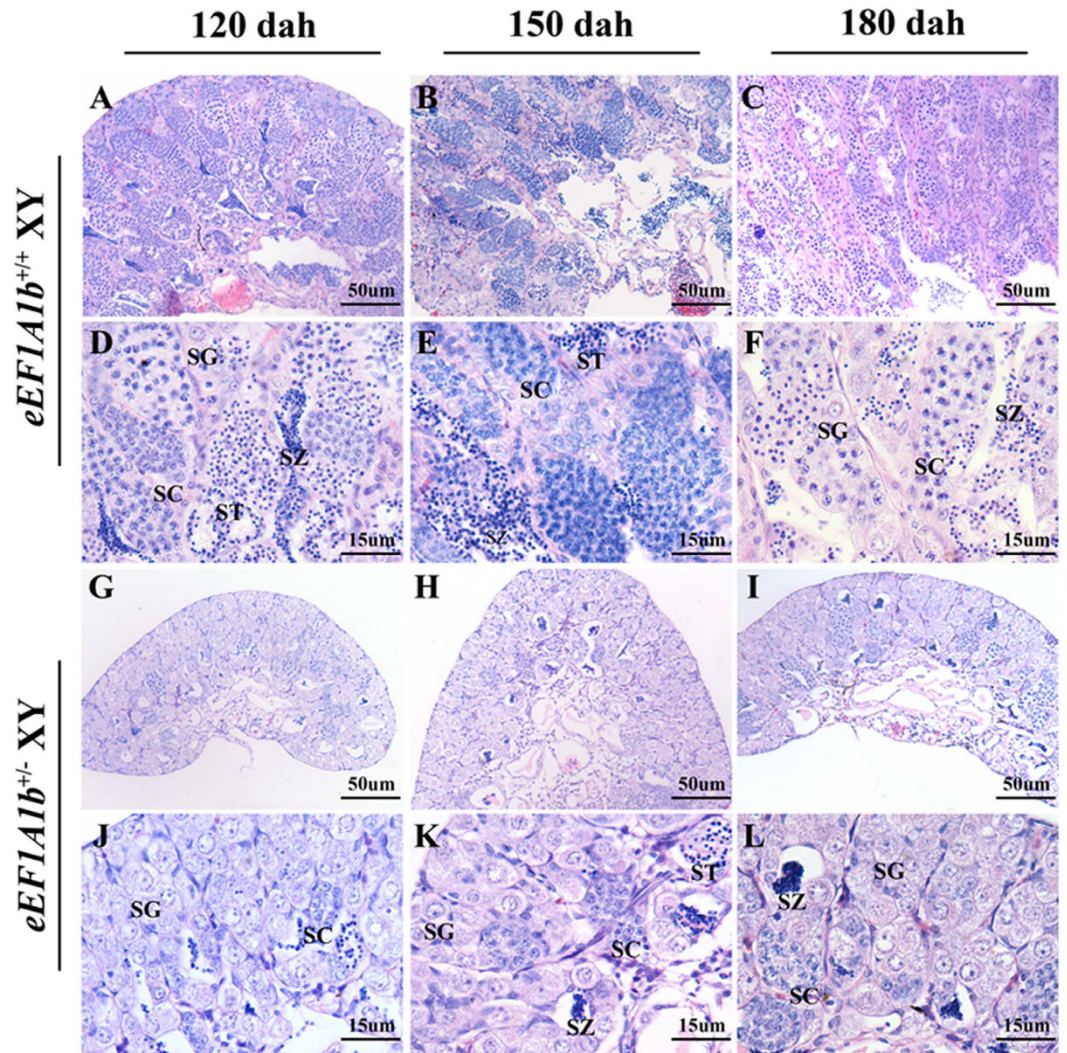


Figure 6. Histological observations of testis from *eEF1A1b*^{+/+} XY fish at 120, 150 and 180 dah. Abundant spermatocytes, spermatids and spermatozoa were observed in the testis of *eEF1A1b*^{+/+} XY fish at 120, 150 and 180 dah (A-F), while just very few spermatocytes were observed at 120 dah, and 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.

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. Further, 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 spermatogenesis^{27,29,37}. Consistently, in the present study, the mRNA level of *suz12a*, *aspm*, and *usp26*, which were expressed in spermatogonia and critical for spermatogenesis^{28,37-39}, 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

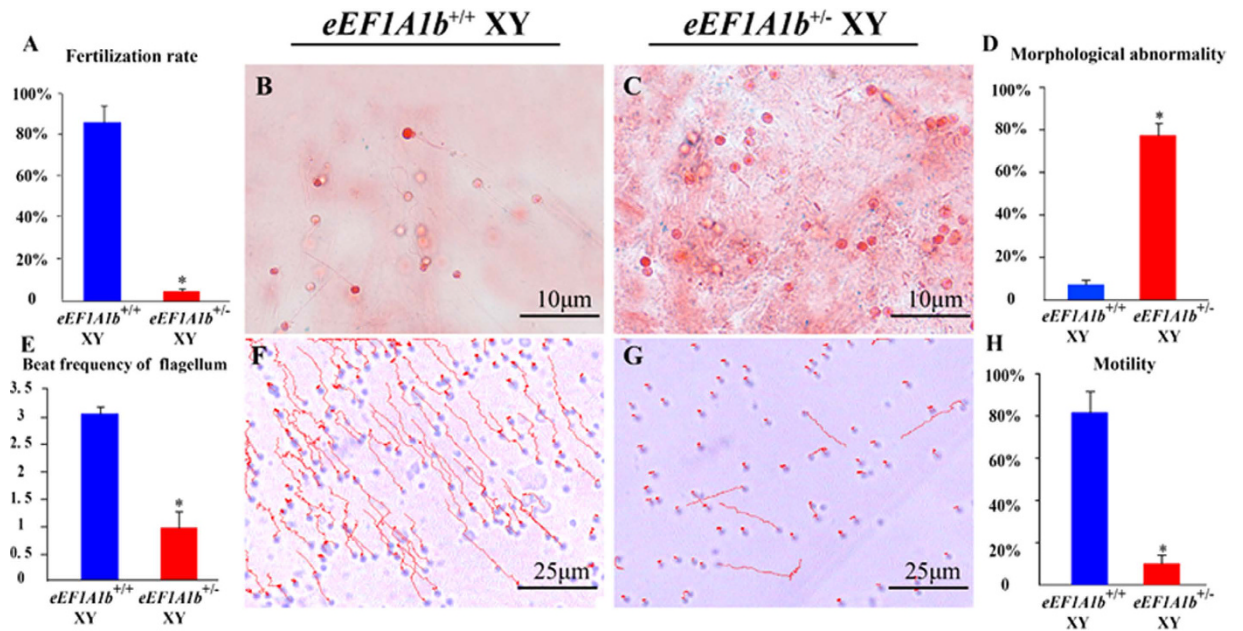


Figure 7. Sperm analyses of *eEF1A1b*^{+/-} XY fish at 180 dah. The fertilization rate of *eEF1A1b*^{+/-} XY fish was significantly lower than that of *eEF1A1b*^{+/+} XY fish (A). Sperm of *eEF1A1b*^{+/+} XY fish were with normal flagella, while most sperm of *eEF1A1b*^{+/-} XY fish were flagella-less, and the ratio of sperm with morphological abnormalities to the total sperm was higher in *eEF1A1b*^{+/-} XY fish than in *eEF1A1b*^{+/+} XY fish (B–D). The beat frequency of sperm flagella was higher in *eEF1A1b*^{+/+} XY fish than in *eEF1A1b*^{+/-} XY fish (E). And the ratio of forward motility sperm was higher in *eEF1A1b*^{+/+} XY fish than in *eEF1A1b*^{+/-} XY fish (F–H). The red line indicates the trajectories of sperm. 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.

levels of these genes due to lack of specific antibodies. On the other hand, *eEF1A1* might also be involved in spermatogenesis via other cellular processes²⁶. However, the non-canonical function of *eEF1A1b* in spermatogenesis of tilapia remains to be elucidated.

In addition, suppression of steroidogenesis was observed in *eEF1A1b* mutant XY fish, as reflected by the decrease of Leydig cells, down-regulation of the expression of Sf-1 and the steroidogenic enzyme StarI, StarII, Cyp11a1, Cyp11b2 and 11β-HSD and a decrease in serum 11-KT level. It is well known that 11-KT is essential for spermatogenesis in fish⁴⁰, and therefore, spermatogenesis arrest and infertility in *eEF1A1b* deficient and *eEF1A1b*^{+/-} XY fish might be the resulted of low levels of 11-KT. Nevertheless, it is an indirect effect of the *eEF1A1b* mutation as this gene is not expressed in the Leydig cells of tilapia. These results suggested that spermatogonia-derived signals might affect the Leydig cells directly or indirectly. It is widely accepted that, during the development of testis, male germ cells are influenced by signals from the surrounding somatic cells, but not vice versa⁴¹. But this conventional perspective was challenged recently. For example, mutation dominant-white spotting locus, which is expressed in spermatogonia of testis⁴², resulted in absence of germ cells, decreased Leydig cell population and disordered expression profiles of Leydig cells key markers (*cyp11a1*, *hsd3β* and *insl3*) in mice⁴³. Moreover, overexpression of *figla* resulted in up-regulation of 11-KT in male tilapia⁴⁴. Taken together, we speculate that the Leydig cells in the testis might be influenced by signals from the male germ cells. However, the mechanisms involved remain to be elucidated.

The phenotype of *eEF1A1b*^{+/-} testis 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*^{+/-} testis. Moreover, *eEF1A1b* transgene also rescued the expression of those genes involved in the spermatogenesis process and steroidogenesis altered in the *eEF1A1b*^{+/-} testis. 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 motility⁴⁵ 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*. In the testis it is exclusively expressed in the spermatogonia. Loss of function and transgenic overexpression studies indicated that the spermatogenesis arrest and infertility phenotypes observed in the *eEF1A1b*^{+/-} XY fish was indeed caused by *eEF1A1b* haploinsufficiency. Taken together, our results suggested that *eEF1A1b* is essential for spermatogenesis and male fertility in the Nile tilapia. In addition, mutation of *eEF1A1b* in tilapia may provide an excellent model for studying human asthenozoospermia.

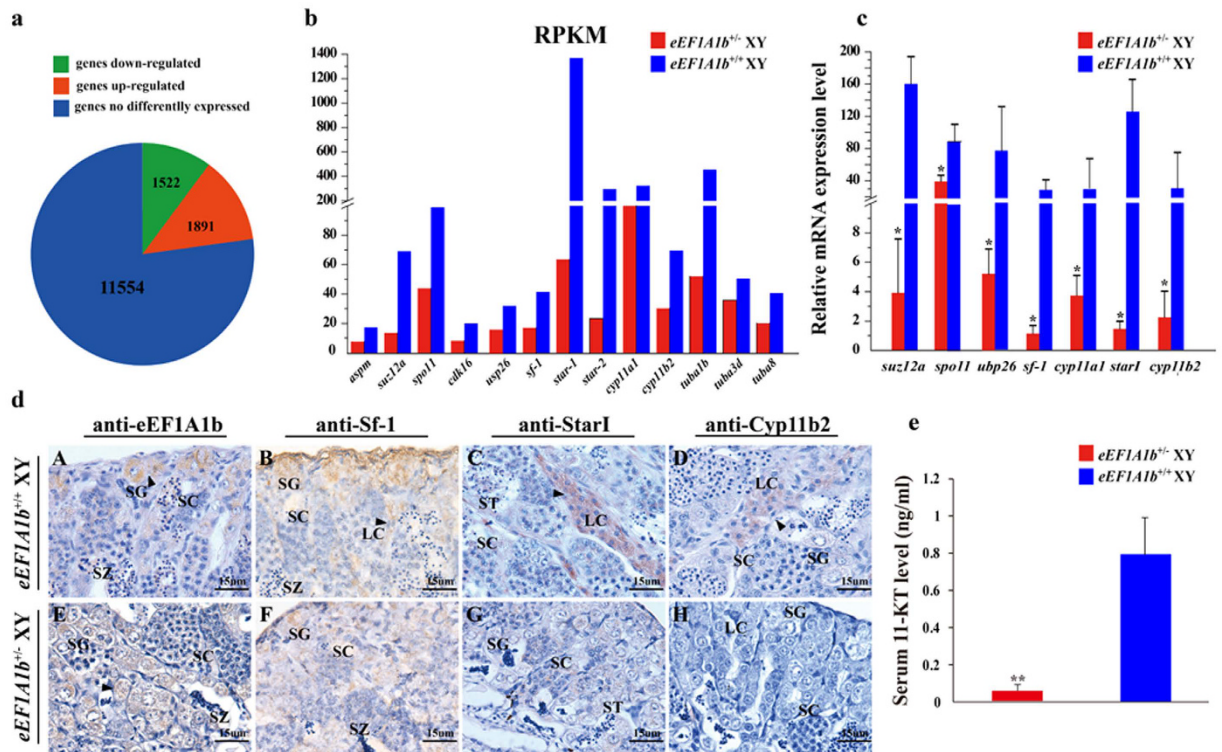


Figure 8. Transcriptomic analyses of gene expression profiles in *eEF1A1b*^{+/-} testis at 180 dah. (a) Testis expressed genes were divided into three parts: 1522 down-regulated genes, 1891 up-regulated genes, and 11554 non-differentially expressed genes. (b) Validation of genes with disrupted expression files from transcriptome data by real-time PCR. All examined genes displayed similar expression profiles to those from the transcriptome data. (c) Validation of genes with disrupted expression files from transcriptome data by IHC. Arrowheads indicate the positive signal. SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa; LC, Leydig cells. (d) Serum 11-KT levels of the *eEF1A1b*^{+/-} and *eEF1A1b*^{+/+} XY fish.

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 synteny 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,

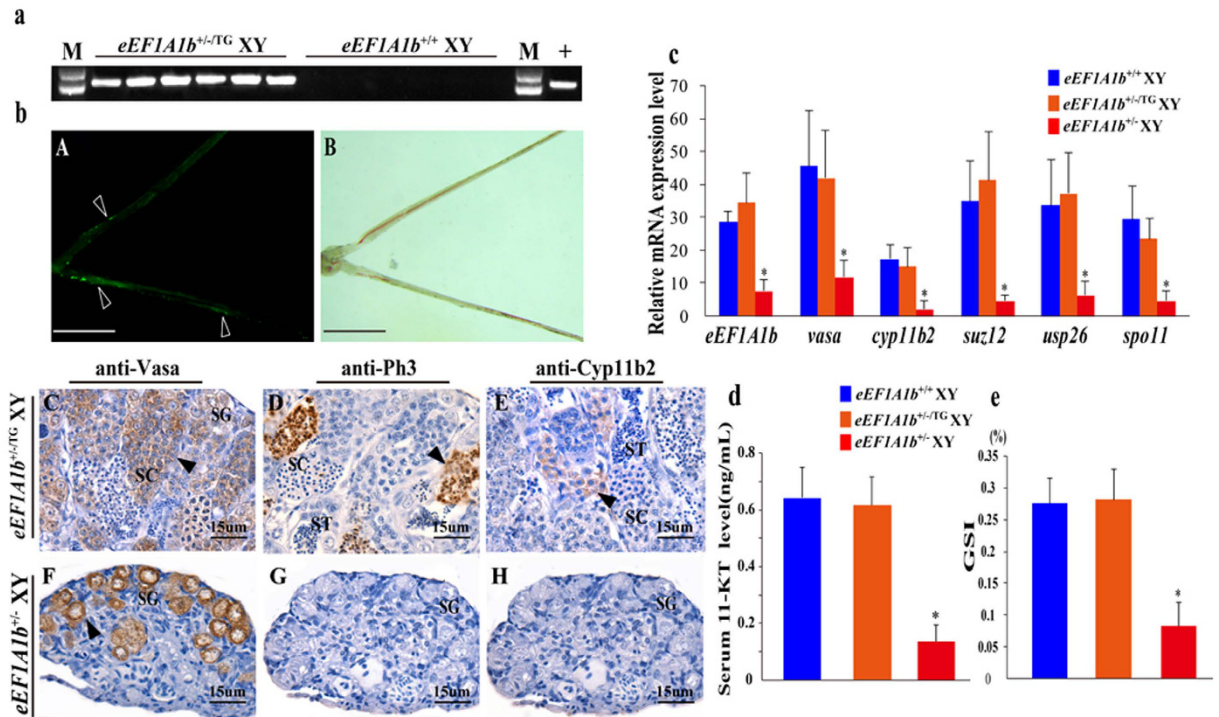


Figure 9. Transgene mediated rescue of spermatogenesis in *eEF1A1b*^{+/-} XY fish. (a) Confirmation of *eEF1A1b* transgene insertion by genomic PCR. Lane 1 and 14, DNA marker; Lane 2-7, XY fish carrying *eEF1A1b*-transgene; Lane 8-13, XY control fish; Lane 15, empty plasmid as template. (b) A, the GFP signals in the testis of *eEF1A1b*^{+/-TG} XY fish in a dark field; Arrowheads indicate the positive signal. B, bright field; C-H, IHC analyses of Vasa, PH3 and Cyp11b2 expression in the testis of *eEF1A1b*^{+/-TG} and *eEF1A1b*^{+/-} XY fish; SG, spermatogonia; SC, spermatocytes; ST, spermatids. (c) Expression of *eEF1A1b*, *cyp11b2*, *vasa*, *suz12*, *usp26* and *spo11* in the *eEF1A1b*^{+/-TG}, *eEF1A1b*^{+/-} and *eEF1A1b*^{+/+} XY fish by real-time PCR. (d) Serum 11-KT levels of the *eEF1A1b*^{+/-TG}, *eEF1A1b*^{+/-} and *eEF1A1b*^{+/+} XY fish. (e) GSI of *eEF1A1b*^{+/-TG}, *eEF1A1b*^{+/-} and *eEF1A1b*^{+/+} XY fish. Results were expressed as mean \pm SD. Different letters indicate statistical differences at $P < 0.05$ as determined by one-way ANOVA followed by post hoc test.

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: $R = 2^{-\Delta\Delta\text{Ct} 47}$. 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 \pm S.D. Statistical analysis was performed using GraphPad Prism4 software (GraphPad Software, USA). Significant differences between groups were tested by one-way ANOVA with 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 $5\mu\text{m}$ 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 pGEM-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 previously²⁷. The antibody against eEF1A1b was diluted 1:1000 for use.

Disruption of *eEF1A1b* by CRISPR/Cas9. CRISPR/Cas9 was performed to knockout *eEF1A1b* in tilapia²⁷. The gRNA target site was selected from *eEF1A1b* sequences corresponding to GGN18NGG on the sense or anti-sense 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 *Bsa*I and Sanger sequencing. In addition, the percentage of uncleaved band was measured by quantifying the band intensity using Quantity One Software (Bio-Rad, USA)⁵⁰. 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 group³². 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 Basic Biology, Okazaki, Japan. The specificity of the antibody has been analyzed previously⁵¹. eEF1A1b and Cyp11b2 (11 β -hydroxylase, the key enzyme for androgen 11-KT synthesis) antibody were prepared by our laboratory. The specificity of Cyp11b2 antibody was checked previously⁵². 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 testis at 180 dah.

Serum 11-KT level was measured using the EIA Assay kits (Cayman Chemical Co, USA) following the manufacturers' instructions. Blood samples were collected from the caudal vein of the eEF1A1b deficient and control XY fish at 90 dah and kept at 4 $^{\circ}\text{C}$ overnight. Serum was collected after centrifugation and stored at -20 $^{\circ}\text{C}$ 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 StarI antibodies were prepared by our laboratory. The specificity of Sf-1 and StarI antibodies was checked previously^{48,53}. Antibodies against Sf-1 and StarI 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 EIA Assay 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 group⁵⁴. 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 (Oreochromis niloticus, Orenil1.0, http://www.ensembl.org/Oreochromis_niloticus/Info/Index) using Tophat with default parameters, and the reads per kb per million reads (RPKM) method was used to calculate gene expression level⁵⁵. 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 *eEF1A1b*^{+/-}-XY were used to analyze the genes expression profiles. The threshold for the p-value was determined using false discovery rate (FDR) set at 0.01³¹. 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 *eEF1A1b*^{+/-}-XY (NDGs), *eEF1A1b*^{+/-}-XY up-regulated genes (URGs), and *eEF1A1b*^{+/-}-XY down-regulated genes (DRGs). 'FDR > 10⁻²' and ' $-1 < \log_2 |(\text{control-XY_RPKM}/eEF1A1b^{+/-}\text{-XY_RPKM})| < 1$ ' were used to identify NDGs, 'FDR > 10⁻²' and $\log_2(\text{control-XY_RPKM}/eEF1A1b^{+/-}\text{-XY_RPKM}) > 1$ were used to identify DRGs, and 'FDR > 10⁻²' and $(\text{control-XY_RPKM}/eEF1A1b^{+/-}\text{-XY_RPKM}) < -1$ 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 KOBAS web server (<http://kobas.cbi.pku.edu.cn/>).

Transgene mediated rescue of spermatogenesis in *eEF1A1b*^{+/-}-XY fish. *In vivo* transgenic overexpression of the *eEF1A1b* in *eEF1A1b*^{+/-}-XY individuals was performed according to the methods of our previous study⁴⁹. An expression plasmid for *eEF1A1b* was prepared as follows. Complementary DNA encoding the ORF of *eEF1A1b* was amplified by PCR with a primer set introducing the *Bam*HI and *Eco*RI sites (Supplemental Tab. 2). The amplified fragment was digested by *Bam*HI and *Eco*RI and ligated into the multiple cloning sites downstream of the cytomegalovirus (CMV) sequence of the pIRES-hrGFP-1a vector (Stratagene, La Jolla, CA). The prepared construct was microinjected into *eEF1A1b*^{+/-}-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 testis of the injected fish was examined by monitoring the GFP signal, and later the testis was 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.

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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.

Additional Information

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