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Disruption of Zar1 leads to arrested oogenesis by regulating polyadenylation *via* Cpeb1 in tilapia (*Oreochromis niloticus*)

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ABSTRACT

Oogenesis is a complex process regulated by precise coordination of multiple factors, including maternal genes. Zygote arrest 1 (*zar1*) has been identified as an ovary-specific maternal gene that is vital for oocyte-to-embryo transition and oogenesis in mouse and zebrafish. However, its function in other species remains to be elucidated. In the present study, *zar1* was identified with conserved C-terminal zinc finger domains in Nile tilapia. *zar1* was highly expressed in the ovary and specifically expressed in phase I and II oocytes. Disruption of *zar1* led to the failed transition from oogonia to phase I oocytes, with somatic cell apoptosis. Down-regulation and failed polyadenylation of *figla*, *gdf9*, *bmp15* and *wee2* mRNAs were observed in the ovaries of $zar1^{-/-}$ fish. Cpeb1, a gene essential for polyadenylation that interacts with Zar1, was down-regulated in $zar1^{-/-}$ fish. Moreover, decreased levels of serum estrogen and increased levels of androgen were observed in $zar1^{-/-}$ fish. Taken together, *zar1* seems to be essential for tilapia oogenesis by regulating polyadenylation and estrogen synthesis. Our study shows that Zar1 has different molecular functions during gonadal development by the similar signaling pathway in different species.

1. Introduction

Oogenesis refers to the formation, development and maturation of female gametes, including oogonial proliferation, oocyte growth and maturation. This process involves complex and coordinated interactions of many autocrine, paracrine and endocrine factors [1–4]. It has been reported that oogenesis of vertebrates is regulated by sex steroid hormones [5–9]. In addition, a variety of maternal effect genes, including *bmp15*, *gdf9*, *mater*, *nanos*, *vasa* and *zar1*, also affect the normal progress of oogenesis in vertebrates [10–13]. *zar1* was one of the earliest oocyte specific maternal effect genes discovered in mouse [14].

The zinc finger (Znf) domain at the C-terminus of the Zar1 protein contains an atypical plant homeodomain (PHD) that is highly conserved and potentially essential for transcriptional regulation [15,16]. ZAR1 expression was reported to be restricted to oocytes in mice, and to ovary and testis in human (*Homo sapiens*), pig (*Sus scrofa*) and cattle (*Bos taurus*) [14,17]. However, the expression of *zar1* was widely observed in ovary, lung and muscle, but not in testis of frog (*Xenopus laevis*) [15]. In addition to oocytes, *zar1* transcript was also observed in spleen, lung, uterus, heart, liver and kidney of New Zealand white rabbits (*Oryctolagus cuniculus*) [18]. In zebrafish (*Danio rerio*), *zar1* is exclusively expressed in the ovary, is highly expressed in phase I and II oocytes, and is required for very early oogenesis [12]. In rainbow trout (*Oncorhynchus mykiss*), abundant expression of *zar1* is detected in the ovary, and to a lesser extent also in testis [19]. In Japanese eel (*Anguilla japonica*), the mRNA expression level of *zar1* was higher in the ovary than in the testis, but tended to be elevated at later developmental stages rather than early ovarian development [20]. Taken together, spatio-temporal expression and molecular functions of *zar1* among different species are not highly conserved, although *zar1* plays an indispensable role in the ovarian development in many species.

Previous studies have shown that Zar1 plays an important role in oogenesis and maternal-zygotic transition in mouse and zebrafish [16,21,22]. *zar1*-deleted female mice were infertile. Although the

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ovarian development, oogenesis, and early stages of fertilization were normal in *zar1* knockout mice, the resulting embryos showed disorders of cell division and could only develop to the 1–2 cell stages, suggesting that *zar1* was important for early embryogenesis [14,22]. In zebrafish, *zar1* deletion led to early oocyte apoptosis, and the gonad of mutant fish undergoes sex reversal from female to male [22]. Additionally, several studies have indicated that sex steroid hormones, especially estrogen and cortisol, play an important role in ovarian development and oogenesis in fish [9,23,24]. Moreover, several genes exclusively expressed in oocytes have been reported to be essential for fish oogenesis, including *figla*, *bmp15*, *gdf9* and *foxh1*. *figla* deletion in zebrafish results in the arrest of oocyte development and ultimately the all-male phenotype [25]. Oocytes of zebrafish with deletion of *bmp15* degenerated during the juvenile stage and their ovaries were transformed to testis [26]. Additionally, *gdf9* deletion led to complete arrest of primary follicle development in zebrafish [27]. In tilapia, disruption of *foxh1* caused oogenesis arrest and thus infertility [28]. Furthermore, deletion of oocytes-specific genes in tilapia, including *bmp15* and *foxh1*, led to decreased estrogen levels and increased androgen levels [26,28]. It should be noted that sex of zebrafish is determined by the development of germ cells, and deletion of many of the genes expressed in germ cells leads to apoptosis of oocytes and a male-biased sex ratio [29,30]. Thus, the role of oocyte-specific genes including *zar1* in the gonadal development of other fish is worth studying.

Meiosis is indispensable for vertebrate oogenesis, and many genes have been reported to be involved in this process [31,32]. For example, *aldh1a2* and *cyp26a1*, the genes encoding retinoic acid (RA) synthase and catabolic enzyme, were involved in meiotic initiation [33–35]. *aldh1a2* deletion in tilapia caused delayed meiotic initiation, while *cyp26a1* deletion caused earlier meiotic initiation [35]. *sycp3* was participated in the formation of meiosis synaptonemal complex, and *sycp3* deleted mice showed defective meiotic pairing and synapsis [36]. Besides, disruption of *sycp3* in zebrafish led to germ cells apoptosis and sex reversal to males [37]. *spo11*, *rec8a* and *rec8b* were essential for homologous recombination during the meiosis I, and disruption of these genes in mouse and medaka resulted in recombination defects [31,38,39]. *tpx2* and *wee2*, important factors for oocyte meiosis maturation, were vital for spindle assembly and normal progress of the cell cycle, respectively [40,41]. The effect of *zar1* deletion on fish on meiosis-related genes is still mostly unknown.

Polyadenylation plays a key role in controlling mRNA stability [16]. It has been reported in mouse that *zar1/2* deletion would affect the normal progress of oocyte meiosis, as well as polyadenylation of certain mRNAs [22]. Cpeb1 is an RNA-binding protein that regulated the length of the poly(A) tail of mRNAs and controlled 3′-UTR alternative splicing, which played an indispensable role in vertebrate oogenesis [42,43]. Co-immunoprecipitation of Zar1 and the translation regulator Cpeb1 has been reported in zebrafish and African clawed frog [12,44]. However, whether *zar1* deletion affects *cpeb1* expression and thus mRNA polyadenylation remains unclear.

Nile tilapia (*Oreochromis niloticus*), in which sex is not determined by germ cell development, is an ideal model for studying oogenesis due to the well-established genome editing methods [45], available high-quality genome sequences [46], the breeding of monosex fish [47] and the ease of blood sample collection. In the present study, we established *zar1* mutation lines, explored the specific effects of *zar1* deletion on the early oogenesis and investigated the possible molecular mechanisms in tilapia.

2. Materials and methods

2.1. Animals

The Nile tilapia (*O. niloticus*) used in this study were derived from a line developed by Prof. Nagahama (Laboratory of Reproductive Biology, National Institute for Basic Biology, Japan). They were raised in 60-L fish tanks within a recirculating, aerated freshwater system with approximately pH 7.5 and dissolved oxygen 6.8 mg/L at 26 °C under a natural photoperiod (usually 12 h) in our lab. In total, 7 dah fish (400 individuals), 20 dah fish (240 individuals), 30 dah fish (200 individuals), 90 dah fish (100 individuals), 120 dah fish (20 individuals), 180 dah fish (20 individuals) and 240 dah fish (10 individuals) in glass tanks were used for analysis. Fish were fed 3 times a day with commercial feed (Shengsuo, Yantai, China). All XX progenies were obtained by mating an XX pseudo-male (XX female transformed into spermatogenic pseudo-male after hormone treatment) with a normal XX female, and all XY progenies were obtained by mating a YY supermale fish with a normal XX female. Animal experiments were carried out in strict accordance with the provisions of the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University (NO. IACUC-20181015-12).

2.2. Phylogenetic and syntenic analyses

The amino acid sequences of the Zar1 proteins of representative vertebrates were retrieved from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) and Ensembl Genome Browser (http://www.ensembl.org/). Sequences were aligned by ClustalW using the multiple alignment software BioEdit [48]. The phylogenetic tree was constructed by the neighbour-joining (NJ) method using the MEGA7.0 software with a bootstrap value of 1000 replicates to assess the confidence in the phylogeny [49]. The synteny map was conducted by comparing genomic regions that harbor *zar1* and its neighboring genes in tilapia with those in representative species.

2.3. Quantitative real time PCR (qRT-PCR)

In total, 400, 240, 200, 9 and 9 gonads of XX and XY fish were dissected and pooled for RNA extraction at 7, 20, 30, 90 and 180 dah (days after hatching), respectively. Three parallel ovary and testis samples with different number of gonads were prepared at each developmental stage. For the tissue distribution analysis, three parallel samples (each composed of ovaries from three individuals) at 180 dah were prepared to evaluate *zar1* expression. Briefly, total RNA was extracted from brain, pituitary, gill, heart, spleen, liver, intestine, ovary, testis, kidney, headkidney and muscle. In addition, total RNA was extracted from the ovaries of WT and *zar1*$^{-/-}$ XX fish at 90 dah to detect expression profile of selected genes.

RNA was extracted from each sample using RNAiso Plus (TaKaRa, Dalian, China) and subsequently treated with Dnase I (RNase-free, 5 U/mL) to eliminate genomic DNA contamination. Total RNA (1.0 μg) was reverse transcribed using PrimeScript RT Master Mix Perfect Real Time Kit according to the manufacturer's instructions (Takara, Dalian, China). The qRT-PCR was carried out according to the SYBR® Premix Ex TaqTM II protocol (Takara, Japan). The relative abundance of mRNA transcripts was evaluated by the formula $R = 2^{-\Delta\Delta Ct}$ [50]. *β-actin* was used as an endogenous control to normalize the expression values. Data were expressed as the mean ± SD of the triplicates. The primer sequences are shown in Supplementary Table 1.

2.4. Fluorescence immunohistochemistry (FIHC)

Six XX fish of each genetype (WT and *zar1*$^{-/-}$) were analyzed. Ovaries were dissected at 90, 180 and 240 dah, and then fixed in Bouin's solution for 24 h at room temperature, dehydrated and embedded in paraffin. Tissue blocks were sectioned at 5 μm thickness using the Leica microtome (Leica Microsystems, Wetzlar, Germany) for FIHC analysis, which was performed as described previously [51]. Zar1 polyclonal antibody was prepared by Suzhou Qiangyao Biological Technology (Qyaobio, Suzhou, China) to verify the cellular localization in tilapia ovary. The antibody was diluted at 1:1000 for use. Vasa and Cyp19a1a

(the key enzyme for estrogen synthesis) antibody were donated by Prof. Nagahama (National Institute for Basic Biology, Okazaki, Japan) [52,53]. Cyp11c1 (the key enzyme for androgen 11-KT synthesis) antibody was prepared by our laboratory [54]. Antibodies against Vasa, Cyp11c1 and Cyp19a1a were diluted at 1:1000, 1:500, 1:3000 for use, respectively. The secondary antibody Alexa Fluor 596-conjugated goat anti-rabbit (Invitrogen, Shanghai, China) for immunofluorescence staining was used to detect the primary antibodies. DAPI was used for DNA visualization (Invitrogen, Carlsbad, USA). Olympus BX51 light microscope (Olympus, Tokyo, Japan) and confocal microscopy (Olympus FV3000) was used to image the stained sections.

2.5. Establishment of *zar1* mutants by CRISPR/Cas9

The tilapia *zar1* mutants were generated by CRISPR/Cas9, as described previously [45]. Briefly, the guide RNA (gRNA) target site was designed in the first exon of *zar1* by an online website (https://crispr.dbcls.jp/). The fertilized eggs were randomly divided into two batches, one for control and the other for microinjection. The gRNA and Cas9 mRNA were co-injected into one-cell stage embryos at concentration of 500 ng/μL and 1000 ng/μL. We randomly collected 20 embryos from each group at 72 h after injection. The genomic DNA was extracted from pooled control and injected embryos for the mutation assays. DNA fragments spanning the *zar1* target site were amplified using the primers listed in Table S1. The mutated sequences were analyzed by restriction enzyme digestion and Sanger sequencing. Heterozygous F1 offspring were produced by mating F0 XY males with WT XX females. XY male and XX female siblings of the F1 generation that carried the same mutation (7 bp deletion) were mated to generate homozygous F2 mutants. The homozygous *zar1* mutant fish were screened by polyacrylamide gel electrophoresis (PAGE) as described previously [55] and subsequent Sanger sequencing.

2.6. Western blot

Ovaries of WT and *zar1*$^{-/-}$ females ($n = 5$/genotype) at 90 dah were dissected to extract total protein and diluted to the final concentration of 20 mg/mL. Western blots were performed as previously described [56]. Zar1 antibody was diluted at 1:1000 with skim milk powder blocking solution. α-Tubulin was used as a sample internal reference and diluted at a concentration of 1:1000. The horseradish peroxidase conjugated goat anti-rabbit antibody (Beyotime, Shanghai, China) was used as the secondary antibody at a concentration of 1:1000.

2.7. Histological analysis of ovaries from WT and *zar1*$^{-/-}$ XX fish

The gonad-somatic index (GSI) of WT and *zar1*$^{-/-}$ XX fish at 90 dah was calculated as (gonad weight/body weight) × 100 % ($n = 6$/genotype). Ovaries processed for histology at 90 dah were fixed in Bouin's solution (24 h, room temperature), and then dehydrated and embedded in paraffin. Tissue blocks were cut serially into slices of 5 μm thickness. The sections were stained with hematoxylin and eosin (H&E) as described previously [9]. Photographs were taken under Olympus BX51 light microscope (Olympus, Tokyo, Japan). Histological classification of oocytes was conducted as described previously [28,61]. Oocyte development has been divided into 5 stages in the ovaries of female tilapia including oogonia, phase I and II oocytes (primary growth stage), phase III and IV oocytes (vitellogenesis stage). Phase I oocytes are similar to but larger than oogonia in appearance and reside in nests with other oocytes (size range: 7–11 μm). Phase II oocytes contain several nucleoli and chromatin strands (size range: 36–240 μm). Phase III oocytes contain numerous vesicles close to oocytes periphery and small yolk granules appear (size range: 224–658 μm). Phase IV oocytes are with larger yolk granules and many apparently empty large vacuoles (size range: 428–1416 μm).

2.8. TUNEL assay

To detect apoptotic cells in the ovaries of WT and *zar1*$^{-/-}$ XX fish ($n = 6$/genotype) at 90 dah, terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay was carried out with *in situ* cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's protocol as previously described [60]. The images were captured under a Zeiss Axio Imager Z2 microscope (Zeiss, Jena, Germany).

2.9. Measurement of steroid hormones

Blood samples were collected from the caudal veins using 1 mL single-use sterile syringes (Yuekang, Changzhou, China) of WT and *zar1*$^{-/-}$ XX fish ($n = 7$/genotype) at 90 dah and stored at 4 °C overnight. The fish for the blood sampling were ranged from 5.6 to 6.5 cm in length (2.8 to 4.9 g in weight) ($n = 6$). The serum was collected after centrifugation (10,000 *g* for 5 min) and kept at −80 °C until use. Serum estradiol-17β (E2) and 11-ketotestosterone (11-KT) levels were measured using an enzyme-linked immunosorbent assay (EIA) kit (Cayman Chemical Co, USA) following the manufacturer's instructions [57,58].

2.10. Co-immunoprecipitation (Co-IP)

To test whether Zar1 interacted with Cpeb1, the pcDNA3.1 (+) expression plasmids encoding an N-/C-terminally HA-tagged Zar1 (pcDNA3.1-HA-Zar1) and N-terminally Flag-tagged Cpeb1 (pcDNA3.1-Flag- Cpeb1) were prepared as the initial step [59]. These Zar1 and Cpeb1 expression plasmids were used to co-transfect Human embryonic kidney (HEK)-293 T cells cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, USA) with 10 % fetal bovine serum with 5 % CO_2 at 37 °C. After 48 h of transfection, cells were lysed for 30 min on ice in a lysis buffer. The cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C and supernatants were used either for Western blot or Co-IP assay. After the supernatants were incubated overnight at 4 °C with anti-HA magnetic beads, the Co-IP assay was carried out using the immunoprecipitation kit (Thermo, USA) following the manufacturer's instructions. Then, Western blot was performed with rabbit anti-Flag antibody (1:1000).

2.11. Fluorescence in situ hybridization (FISH)

FISH was used to explore the cellular co-localization of *zar1* and *cpeb1* in tilapia ovaries at 120 dah (the ovaries from six WT individuals). FISH was performed as described in previous study [60]. Briefly, the coding sequences of Nile tilapia *zar1* and *cpeb1* were amplified using specific primers, and the amplified fragments were cloned into pGEM-T Easy Vector. The RNA probes for both sense and antisense labeled with digoxigenin (DIG) were transcribed *in vitro* from the linearized plasmids DNA of tilapia *zar1* using the RNA labeling kit (Roche, Mannheim, Germany). The ovaries of tilapia were isolated at 120 dah and fixed in 4 % paraformaldehyde in 0.85× PBS at 4 °C subsequently. All samples were dehydrated, embedded in paraffin, and serial sections of 5 μm thickness were cut. The sections were deparaffinized, hydrated and then hybridized with DIG-labeled RNA probes at 60 °C overnight. Then, the sections were incubated for 30 min at room temperature with a horseradish peroxidase (HRP)-con-jugated anti-DIG antibody (Roche, Basel, Switzerland), and the hybridization signals were amplified with the TSA™ Plus Tetramethylrhodamine (TMR) system (PerkinElmer, Boston, USA). In addition, FISH experiment was performed as previously described [60]. ImageJ software was used to quantify all FISH positive signals. The images of FISH were taken by confocal microscopy (Olympus FV3000) (Olympus, Tokyo, Japan).

2.12. 3′-RACE (rapid amplification of cDNA ends)

3′-RACE kit (Sangon Biotech, Shanghai, China) was used to amplify the 3′ end of genes related to oogenesis and meiosis in WT and *zar1*-deleted females ($n = 6$/genotype) to detect whether the deletion of *zar1* would affect the 3′ end polyadenylation and mRNAs alternative splicing of these genes. The poly (A) tail at the 3′ end of the mRNA of target gene was used as the binding site of the 3′ adaptor Primer, and the first cDNA strand was synthesized by reverse transcription. The second cDNA strand was synthesized using the gene specific primer F1 and the universal primer 3′-RACE Outer Primer. Subsequently, the gene specific primer F2 and the general primer 3′-RACE Inner Primer were used as a pair of primers for PCR amplification of the obtained cDNA strand, so as to obtain the 3′ end sequence of cDNA. The experiment was performed according to the manufacturer's instructions.

2.13. Data analyses

All data were based on biological repetitions of at least three independent experiments and presented as the mean ± SD. The data were statistically analyzed using the GraphPad Prism 8 (GraphPad Software, La Jolla, USA) software package. The Student's *t*-test was used to determine the statistically significant difference between the two groups. One way ANOVA and Tukey test were used to compare more than two groups. In all analyses, "*" represents statistical difference ($P < 0.05$), "**" represents significant difference ($P < 0.01$), "***" represents extremely significant difference ($P < 0.001$), and "ns" represents no significant difference.

3. Results

3.1. Characterization of zar1 expression in tilapia

A single copy of Zar1 with zinc finger domain at the C-terminal was identified in representative vertebrates (Fig. S1 and S2). Tilapia *zar1* gene is located on LG23 in a region of conserved synteny (identical gene content and order) to that on medaka (Fig. S3). As demonstrated by qRT-PCR, *zar1* was mainly expressed in the ovary of adult tilapia, and its expression level in the ovary was significantly higher than that in the testis of adult fish at 180 dah (Fig. 1A). In addition, the expression of *zar1* in the ovary increased gradually, while the expression in the testis remained at a low level from 7 to 180 dah (Fig. 1B). The results of FIHC showed that *zar1* was expressed in phase I and II oocytes, but absent in phase III and IV oocytes at 240 dah (Fig. 1C).

3.2. Establishment of zar1 homozygous mutants

The gRNA target site was located in the first exon of *zar1*, which contained a *Dde* I restriction site for mutation identification (Fig. 2A). The results of restriction digestion by *Dde* I showed that two bands (393 and 90 bp) were produced in three control groups, while an intact band (~483 bp) was detected in embryos co-injected with Cas9 mRNA and gRNA (Fig. 2B). The F0 *zar1* deficient fish were screened by *Dde* I digestion. The mutation types were detected by subclone sequencing (Fig. 2C). The mutation type of 7 bp deletion of *zar1* led to the premature termination of *zar1* translation before the zinc finger domain (Fig. 2D). After obtaining F1 mutant fish by crossing a F0 male fish with a WT

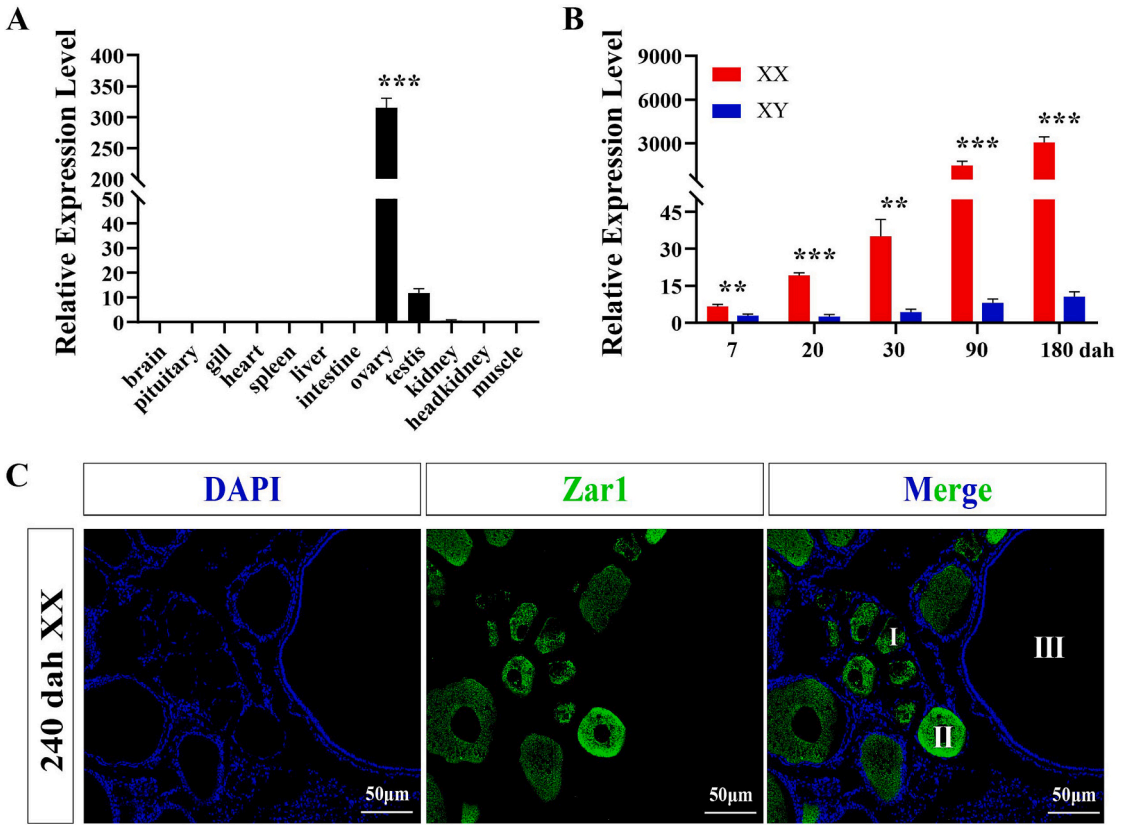

Fig. 1. Expression pattern and cellular localization of *zar1* in tilapia.
(A) The relative expression of *zar1* in twelve tissues of adult tilapia at 180 dah ($n = 9$/sex). *β-actin* was used as internal control. (B) The relative expression of *zar1* mRNA in gonads of tilapia at 7 dah (400 gonads pooled for each sex), 20 dah (240 gonads pooled for each sex), 30 dah (200 gonads pooled for each sex), 90 dah (9 gonads pooled for each sex) and 180 dah (9 gonads pooled for each sex). *β-actin* was used as internal control. Values in A and B are presented as the mean ± SD, "**" above the error bar indicates significant differences at $P < 0.01$ and "***" above the error bar indicates highly significant differences at $P < 0.001$ as determined by two-tailed Student's *t*-test. (C) Immunohistochemistry was used to verify the cellular localization of Zar1 in ovaries at 240 dah ($n = 6$). The blue fluorescence indicates the DAPI signal. The green marked cells indicates the positive signal of Zar1. dah, days after hatching. I to III, phase I to III oocyte.

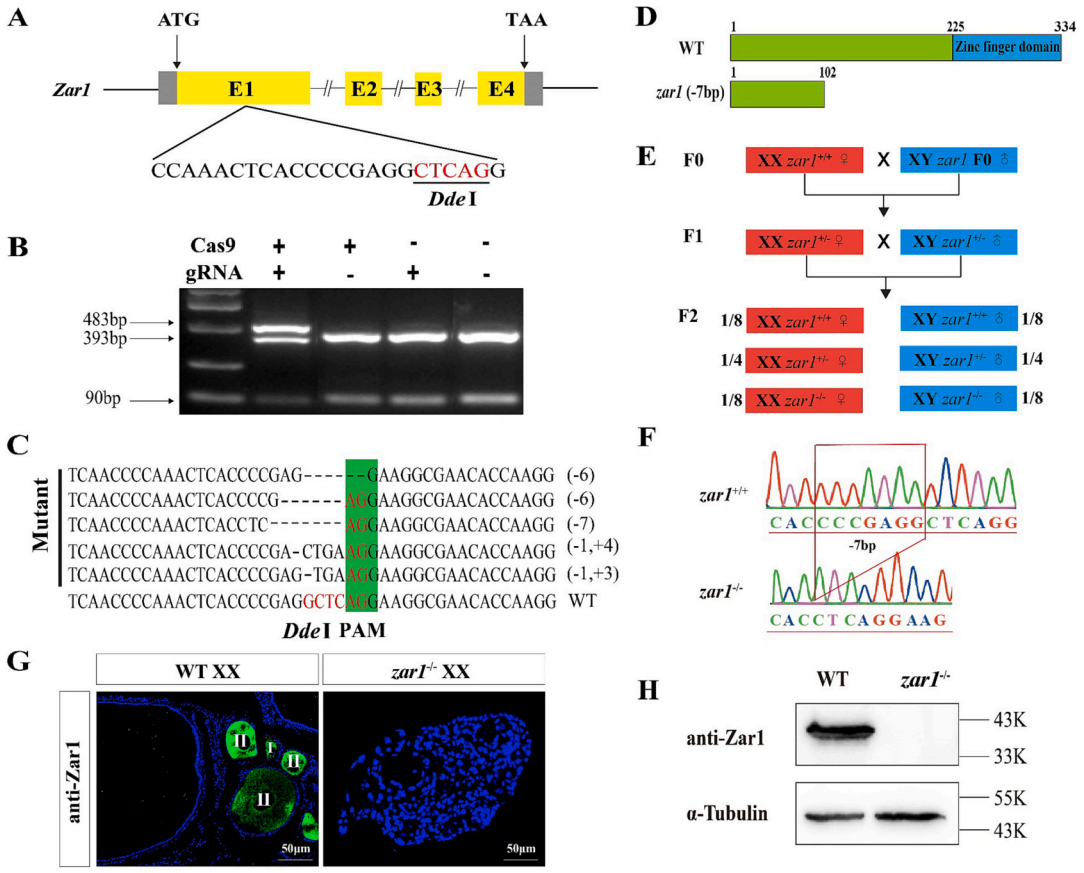

Fig. 2. Establishment of *zar1* mutant line by CRISPR/Cas9.
(A) Target design diagram of *zar1* gRNA. ATG and TAA indicate initiation codon and stop codon, respectively. Boxes indicate exons, grey regions indicate the position of the UTRs of gene, and yellow regions indicate the position of coding sequences of genes. The *Dde* I digestion site was underlined. (B) Enzymatic cleavage detection of mutations. (C) Analysis of mutation types by subclone sequencing. Dotted lines represent deleted bases, numbers to the right of the sequence indicate the number of deletion-insertion variant, and green regions indicate PAM structure. (D) Diagram of the structure of the truncated protein from *zar1* (−7 bp). (E) Construction of homozygous *zar1* mutants. (F) Sanger sequencing of *zar1* homozygous mutants. (G) Validation of *zar1* homozygous mutation by Immunofluorescence (n = 6/genotype). Blue fluorescence represents the DAPI signal. Cells with a green colour indicated the positive signal of Zar1. WT, wild type. I and II represent phase I and II oocytes. (H) Validation of *zar1* homozygous mutation by Western blot (n = 5/genotype). α-Tubulin served as an internal reference.

female fish, heterozygous *zar1* F1 with the 7 bp deletion were selected to breed the homozygous mutant F2 generation (Fig. 2E), and F2 homozygous mutants of the 7 bp deletion was confirmed by Sanger sequencing (Fig. 2F). No morphological difference of embryonic and juvenile development was observed between $zar1^{-/-}$ and $zar1^{+/+}$ fish. FIHC results showed that Zar1 specific signal was detected in the ovaries of WT females but not in the ovaries of *zar1* knockout females at 180 dah (Fig. 2G). No Zar1 protein was detected in the ovaries of the $zar1^{-/-}$ XX fish at 90 dah by Western blot (Fig. 2H). These data suggested that *zar1* was successfully knocked out.

3.3. Deletion of zar1 impaired oocyte development in XX tilapia

Histological analysis and FIHC showed only a few oogonia with obvious aberrant vesicles in the $zar1^{-/-}$ fish, while oogonia, phase I, and phase II oocytes were observed in the ovaries of WT fish at 90 dah (Fig. 3A-C). No significant differences of the GSI were found between $zar1^{-/-}$ XX fish and WT fish (Fig. 3D). Statistically, the number of oogonia, phase I, and phase II oocytes in the ovaries of $zar1^{-/-}$ fish was significantly decreased compared to WT fish (Fig. 3E). In addition, the results of FIHC and statistical data showed that Vasa (germ cell marker) positive signals were significantly decreased in ovaries of $zar1^{-/-}$ fish compared with the WT females at 90 dah (Fig. 3C and F). Consistent with this, the mRNA expression level of *vasa* was significantly decreased in the $zar1^{-/-}$ ovaries at 90 dah (Fig. 3G). These results indicated that deletion of *zar1* resulted in a significant decrease in the number of germ cells in female fish.

3.4. Deletion of zar1 impaired meiosis initiation and increased somatic apoptosis in XX tilapia

To further explore the effect of *zar1* deletion on meiosis, we quantified the expression levels of *aldh1a2*, *sycp3*, *cyp26a1*, *spo11*, *rec8a* and *rec8b* in the ovaries of WT and $zar1^{-/-}$ fish at 90 dah by qRT-PCR. Compared with the WT fish, the expression of *aldh1a2* and *sycp3* was significantly decreased, while the expression of *cyp26a1* was significantly up-regulated in the $zar1^{-/-}$ fish. However, there were no significant differences in the expression of *spo11*, *rec8a* or *rec8b* in ovary between the $zar1^{-/-}$ and WT fish (Fig. 4A). These results indicated that the deletion of *zar1* caused impaired germ cell development and meiosis initiation at early stage. The TUNEL assay showed that there were green positive signals on somatic cells in ovaries of female $zar1^{-/-}$ fish, while few apoptotic cells could be observed in the WT fish at 90 dah (Fig. 4B and C). Statistical analysis showed that the green fluorescence signals in the $zar1^{-/-}$ ovaries were significantly increased compared with that in the WT ovaries (Fig. 4D). Consistently, the expression of apoptosis-promoting genes *caspase3a*, *caspase3b*, *caspase8*, *caspase9*, *baxa* and *tp53* was significantly up-regulated, while the expression of the apoptosis-inhibiting gene *bcl2* was significantly decreased in ovaries of $zar1^{-/-}$ fish at 90 dah (Fig. 4E).

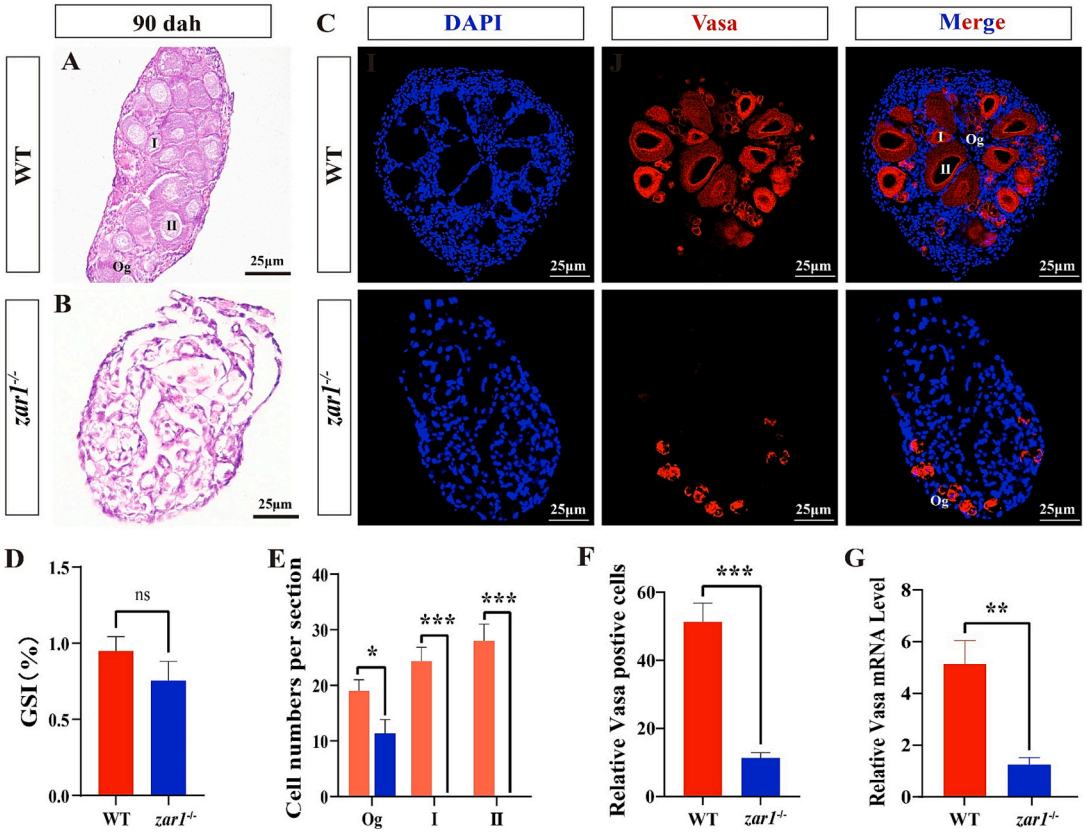

Fig. 3. Deletion of *zar1* resulted in abnormal oogenesis in XX tilapia at 90 dah.
(A-B) Histological observation of ovaries from 90 dah mutant fish and WT fish (n = 6/genotype). (C) Immunofluorescence analysis (n = 6/genotype). The Vasa positive germ cells were marked by red colour. Blue fluorescence represents the DAPI signal. (D) Gonadosomatic index from 90 dah mutant fish and WT fish (n = 6/genotype). (E) Statistical analysis of oocyte counting from 90 dah mutant fish and WT fish (n = 6/genotype, and five sections for per sample were counted). (F) Statistical analysis of the positive signals (n = 6/genotype, and five sections for per sample were counted). (G) Analysis of relative *vasa* mRNA expression levels (n = 6/genotype). WT, wild type. dah, days after hatching. Og, oogonia. I to II, phase I to phase II oocytes. Differences between the mutants and WT were tested by two-tailed unpaired Student's *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Results were presented as the mean ± SD in statistical chart.

3.5. Deletion of zar1 resulted in abnormality of estrogen and androgen biosynthesis

Genes specifically expressed in germ cells possibly maintain female ovary structure by regulating estrogen synthesis. To further investigate the effects of *zar1* deletion on tilapia oogenesis, we detected the expression of Cyp11c1 and Cyp19a1a in the ovaries of *zar1*$^{-/-}$ and WT fish at 90 dah by both FIHC and qRT-PCR. Compared with WT ovaries, the positive signal of Cyp11c1 significantly increased in the *zar1*$^{-/-}$ ovaries (Fig. 5A-D), while the positive signal of Cyp19a1a was significantly down regulated in the *zar1*$^{-/-}$ ovaries (Fig. 5E-H). The expression level of *cyp11c1* mRNA measured by qRT-PCR was significantly increased (Fig. 5I), while that of *cyp19a1a* mRNA was significantly decreased in the ovaries of *zar1*$^{-/-}$ fish at 90 dah (Fig. 5J). Moreover, examination of serum concentrations of sex steroid hormones showed that the level of 11-KT was significantly up-regulated (Fig. 5K), while the level of E2 was significantly down-regulated in *zar1*$^{-/-}$ fish at 90 dah (Fig. 5L).

3.6. Deletion of zar1 affected the expression and stability of genes related to oogenesis

The cytoplasmic polyadenylation of specific mRNAs in the cytoplasm is a crucial regulatory step in oogenesis and early development of vertebrates [62]. The significant polyadenylation of mRNA during the MI stage is a critical step in translational activation, which drives the meiotic process [22,63]. *figla*, *gdf9*, *bmp15*, *wee2* and *tpx2* are vital genes for oogenesis in animals. To further investigate the effects of *zar1* mutation on the maturation and polyadenylation of these genes, 3′-RACE assay and gel electrophoresis were performed. The results showed that the poly(A) tail elongation of *figla*, *gdf9*, *bmp15* and *wee2* mRNAs failed in the ovaries of *zar1*$^{-/-}$ fish compared with that of WT at 90 dah, with alternative splicing at the 3′ end (Fig. 6A-D). No obvious abnormities of polyadenylation and alternative splicing of *tpx2* mRNA were observed between *zar1*$^{-/-}$ and WT fish (Fig. 6E). qRT-PCR results showed that the expression of these genes was significantly down regulated (Fig. 6F-J).

CPEB controls the cytoplasmic polyadenylation and translational regulation, thus Co-IP assay was performed to test whether Zar1 interacts with Cpeb1. The specific bands of Zar1 (33.86 kDa) and Cpeb1 (63.45 kDa) could be detected in the total protein of HEK-293 T cells transiently transfected with pcDNA3.1-HA-Zar1 and pcDNA3.1-Flag-Cpeb1 plasmids, respectively, while no band was observed in the control group (Fig. 7A and B). Subsequently, HA-Zar1 and Flag-Cpeb1 vectors were co-transfected into HEK-293 T cells for Co-IP experiments. The results showed that Zar1 coprecipitated with Cpeb1 (Fig. 7C and D), indicating the interaction between Zar1 and Cpeb1 proteins *in vitro*. Interestingly, *zar1* and *cpeb1* co-localized in phase I and II oocytes of tilapia ovaries by FISH (Fig. 7E-H). Additionally, the expression of *cpeb1* was significantly decreased in ovaries of *zar1*$^{-/-}$ fish at 90 dah (Fig. 7I).

4. Discussion

zar1, initially identified as a maternal gene in mice, is later proved to

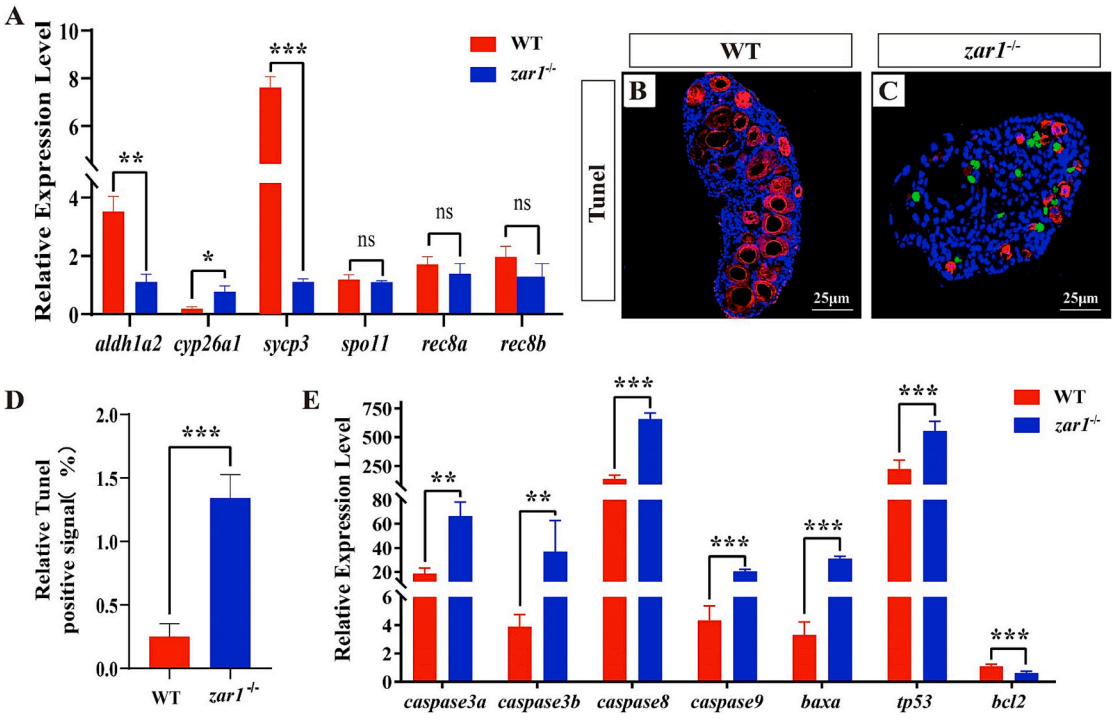

Fig. 4. Deletion of *zar1* resulted in impaired meiosis initiation and somatic cell apoptosis in XX tilapia at 90 dah.
(A) Analysis of relative mRNA expression levels of marker genes in meiosis stages (n = 6/genotype). (B-C) TUNEL analysis (n = 6/genotype). The Vasa positive germ cells were marked by red colour. Blue fluorescence represents the DAPI signal. Green fluorescence represents the TUNEL positive signal. (D) Statistical analysis of the TUNEL positive signals (n = 6/genotype, and five sections for per sample were counted). (E) Expression analysis of apoptosis-related genes (n = 6/genotype). Differences between the mutants and WT were tested by two-tailed unpaired Student's t-test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Results were presented as the mean ± SD in statistical chart.

play a role in embryogenesis and oogenesis in mammals. However, the function is not clear in fish based on the limited study in zebrafish [12,14]. In the present study, we identified the expression pattern and established *zar1* homozygous mutant lines using CRISPR/Cas9 in Nile tilapia. We found that disruption of *zar1* prevented transition from oogonia to oocytes, indicating the vital role of *zar1* in oogenesis.

Our sequence analysis confirmed that the amino acid sequences, particularly the sequences encoding the C-terminal zinc finger domain, are conserved in vertebrates [16,64,65]. Higher expression of *zar1* in ovaries than that in testis have been reported in several species [66–69]. However, the expression pattern is not so conserved among different species. In the present study, we found that the expression level of *zar1* in the ovary was significantly higher than that in the testis, and the expression gradually increased in the ovarian development at the early stages. FIHC showed that *zar1* was only expressed in phase I and II oocytes in the ovary of tilapia. Similarly, *zar1* mRNA is predominantly expressed in primary growth and cortical alveolus oocytes but absent in testis in zebrafish [12]. However, *zar1* was expressed in oocytes of later stages and required for later ovarian development rather than early stages in Japanese eels [20]. Interestingly, Zar1 protein was also detected in mature oocytes of zebrafish [70]. In *Xenopus*, the expression level of *zar1* reached a maximum in stages I-III and then declined through stages IV to VI oocytes [65]. It is noteworthy that *zar1* is not only expressed in gonadal tissues in other tetrapods, but also in other non-gonadal tissues including lung and muscle [18]. Moreover, recent studies have indicated that *zar1* was also expressed in cancer cells [71,72]. Therefore, the expression of *zar1* is not highly conserved, and its function needs to be investigated in more species.

Previous studies have proposed the role of *zar1* in early development of embryos and oogenesis by regulating translation of specific mRNAs [14,16]. In mouse, *zar1* mutants developed and laid eggs normally. However, loss of maternal *zar1* led to the arrested embryo development at the 1-cell or 2-cell stage, before zygotic genome activation [14]. The role of *zar1* in mouse oogenesis is not clear based on previous studies, potentially because ZAR2 can partially compensate for ZAR1 [14,16]. In zebrafish, disruption of *zar1* led to p53-dependent oocyte apoptosis at early stages and sex reversal from female to male [12]. However, the current functional studies of *zar1* in vertebrate oogenesis are limited to mouse and zebrafish [12,22], and showed that the function of *zar1* is not highly conserved. In the present study, no obvious abnormality was observed in the development of embryos and juvenile tilapia, similar to that in zebrafish [12]. Previous study in cows indicates that the embryos are transcriptionally and translationally active at the 1- and 2-cell stages, which shows *zar1* is indispensable for its embryo development [73,74]. Thus, the maintenance of maternal *zar1* in zebrafish and tilapia safeguard the onset of the major embryonic genome activation.

It should be noted that disruption of *zar1* in tilapia led to abnormal oogenesis, with a significant decrease of the germ cell number and massive somatic cells apoptosis. In zebrafish, *zar1* deletion induced upregulation of proteins involved in endoplasmic reticulum (ER) and subsequently ER stress, thereby early oogenesis arrest and eventual female-to-male sex reversal. p53-mediated germ cell apoptosis is involved gonad transformation in zebrafish, and loss of p53 restores the aberrant ovarian development caused by *zar1* deficiency [12]. In tilapia, deletion of *zar1* resulted in obvious apoptosis of somatic cells at 90 dah. In fact, if sampled at earlier stages, germ cell apoptosis would possibly be observed due to the significantly decreased number of oogonia and oocytes in $zar1^{-/-}$ tilapia. Germ cell apoptosis induces sex reversal in $zar1^{-/-}$ zebrafish [75], while no signs of sex reversal has been observed in $zar1^{-/-}$ tilapia with aberrant levels of sex steroids. The important roles of estrogen in fish oogenesis have been widely reported [23,76]. The significantly decreased level of serum E2 and increased serum 11-KT level have been observed in $zar1^{-/-}$ tilapia. Considering the specific expression of *zar1* in phase I and II oocytes, we propose that the

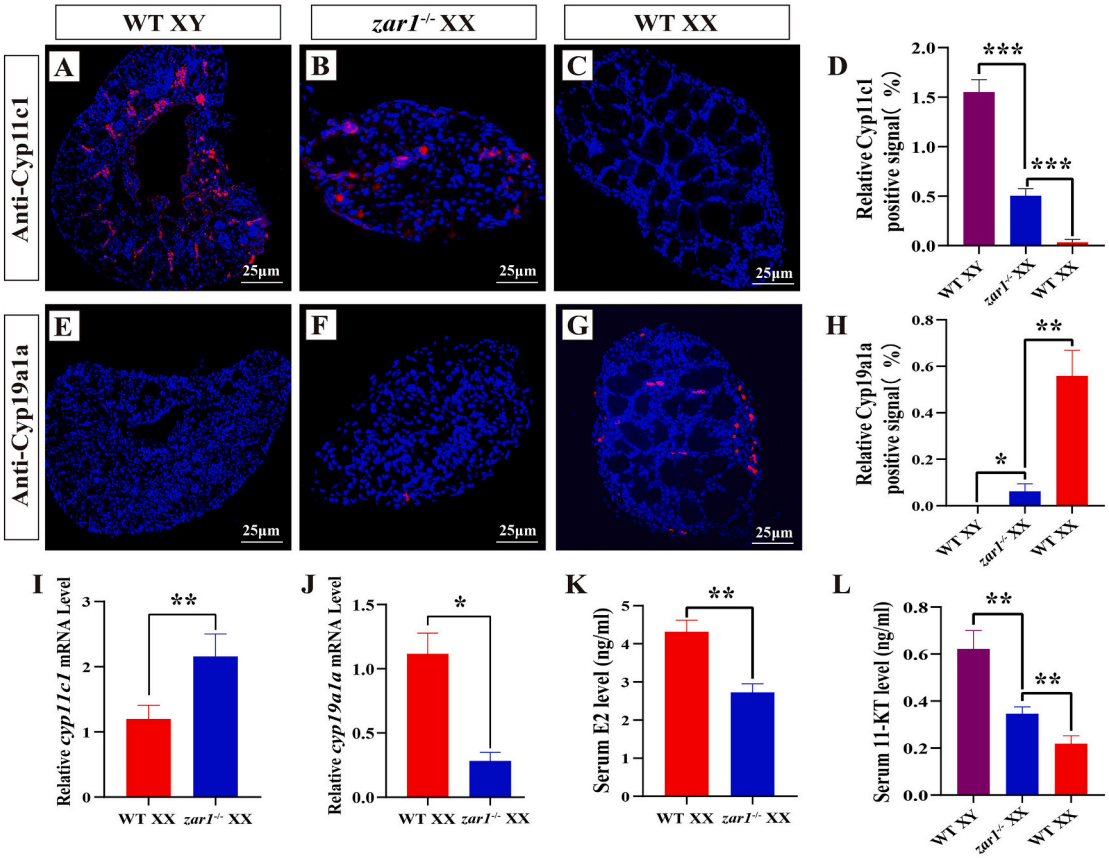

Fig. 5. Deletion of *zar1* resulted in impairment of estrogen and androgen biosynthesis in XX tilapia at 90 dah.
(A-H) Immunofluorescence analysis and statistical analysis of Cyp11c1 and Cyp19a1a. The Cyp11c1 positive cells and Cyp19a1a positive cells were marked by red colour. Blue fluorescence represents the DAPI signal (n = 6/genotype, and five sections for per sample were counted). (I-J) Analysis of relative mRNA expression levels of the genes encoding steroid synthesizing enzymes *cyp11c1* and *cyp19a1a* (n = 6/genotype). (K-L) EIA analysis of E2 and 11-KT in serum ($n = 7$/genotype). Differences between the WT and mutants were tested by two-tailed unpaired Student's t-test, * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$. Results are presented as the mean ± SD in the statistical charts.

disruption of *zar1* results in germ cell apoptosis first, and later abnormal development of steroidogenic cells, which further results in the obstruction of estrogen synthesis. A previous study has showed that the down-regulation of endogenous estrogen has led to the inhibition of female pathway genes and the activation of male pathway genes [77], which may be the reason for the up-regulation of 11-KT level in *zar1*$^{-/-}$ tilapia. Estrogen administration has been shown to suppress apoptosis and restore the ovarian development in *zar1*$^{-/-}$ zebrafish possibly through suppression of stress-related genes [12]. Thus, normal *zar1* expression is essential for maintaining estrogen levels, which is conserved in different fish species.

A key process of early oogenesis is the transition from mitotic oogonia to the entry in meiosis, which is regulated by a complex gene regulatory network [78]. RA homeostasis mediates meiotic entry in Nile tilapia through the genes encoding RA synthase *aldh1a2,* and catabolic enzyme *cyp26a1* [35]. Interestingly, the expression of *aldh1a2* was significantly down-regulated, while the expression of *cyp26a1* was significantly up-regulated, indicating that *zar1* deletion led to aberrant meiosis initiation in tilapia, as reported in mouse [22]. Consistently, decreased expression level of *sycp3* was also observed.

In addition, *wee2* was associated with the progression of the meiosis cell cycle [79]. *figla*, *bmp15* and *gdf9* are key genes responsible for oogenesis in zebrafish [25–27]. It is noteworthy that disruption of *zar1* led to significantly shortened poly(A) tail and aberrant alternative splicing at the 3′ end of *figla*, *gdf9*, *bmp15*, *wee2* mRNAs compared with WT fish, which may lead to failure of translational activation, ultimately affecting oocyte meiotic maturation [22]. *tpx2* was essential for meiotic spindle formation during oocyte maturation [80,81], while its poly(A) tails have not been shortened based on the present data. Besides, the expression of all these genes were also significantly down-regulated. In addition to direct RNA binding for regulation [12], Zar1 could also interact with other RNA-binding proteins to jointly regulate the stability of the transcriptome in oocyte [22]. Cpeb1 has been reported to be a component of the cytoplasmic polyadenylation machinery [42,82–85], and interfering with Cpeb1 prevents the translational activation of mRNAs that are vital for oocyte maturation. Consistent with that in zebrafish and *Xenopus*, Cpeb1 interacts with Zar1 in tilapia, and Cpeb1 was colocalized with Zar1 in phase I and II oocytes in tilapia. Thus, the decreased expression of *cpeb1* is responsible for the shortened poly(A) tails and aberrant alternative splicing of *figla*, *gdf9*, *bmp15* and *wee2* mRNAs in *zar1*$^{-/-}$ tilapia. Therefore, further study needs to be performed to examine how Zar1 regulates the stability of the ovarian transcriptome during tilapia oogenesis.

In conclusion, our results demonstrated that *zar1* deletion led to arrested oogenesis in tilapia. Disruption of *zar1* resulted in down-regulation and failed polyadenylation of *figla, gdf9*, *bmp15* and *wee2* mRNAs, impaired meiotic initiation, failed transition from oogonia to phase I oocytes and increased somatic apoptosis. Zar1 could interact with Cpeb1, and may co-regulate polyadenylation and alternative splicing of mRNAs associated with oogenesis, to maintain mRNA stability of early oocytes and ensure their entry into meiosis I, thereby maintaining the proper progression of early oogenesis in tilapia. Taken together, these results demonstrated that Zar1 plays an essential role in oogenesis by regulating polyadenylation *via* Cpeb1 in tilapia (Fig. 8).

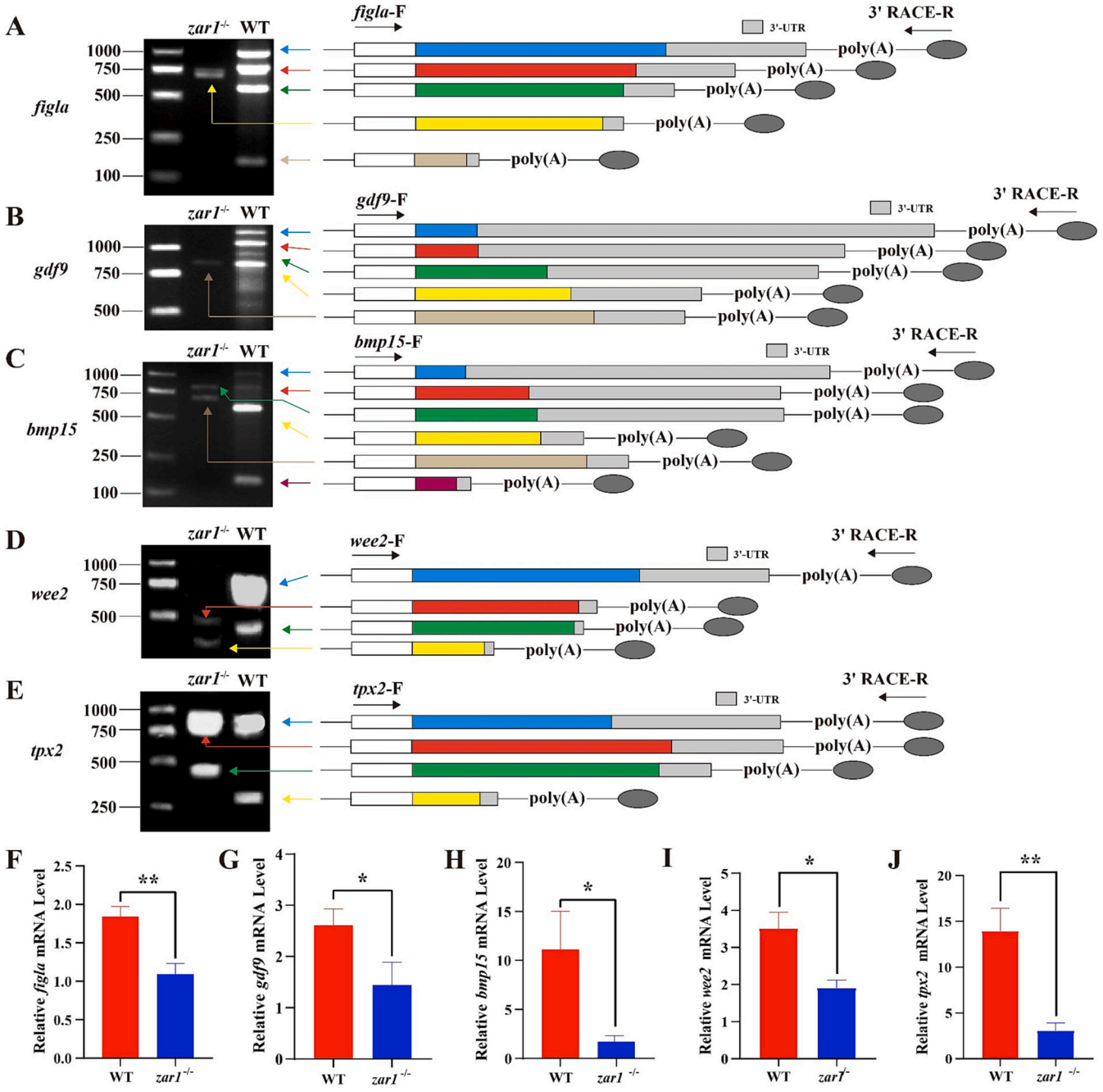

Fig. 6. 3′ end polyadenylation, alternative splicing and expression analysis of *figla*, *gdf9*, *bmp15, wee2* and *tpx2* in *zar1*$^{-/-}$ ovaries at 90 dah.
(A-E) 3′ end polyadenylation analysis of *figla*, *gdf9*, *bmp15, wee2* and *tpx2* mRNAs in *zar1*$^{-/-}$ ovaries (n = 6/genotype). The 3′ end sequences of *figla*, *gdf9*, *bmp15, wee2* and *tpx2* mRNAs were amplified by 3'-RACE and analyzed for polyadenylation changes at their ends. F-specific primers were designed on the mRNA of the respective genes, and 3'-RACE-R was the universal primer. Poly (A) indicates polyadenylation. (F-J) Analysis of *figla*, *gdf9*, *bmp15*, *cpeb1, wee2* and *tpx2* mRNAs relative expression levels (n = 6/genotype). Differences between the WT and mutants were tested by two-tailed unpaired Student's t-test, * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$. Results were presented as the mean ± SD in statistical chart.

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Ethics statement

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, China.

Disclosure summary

The authors have nothing to disclose.

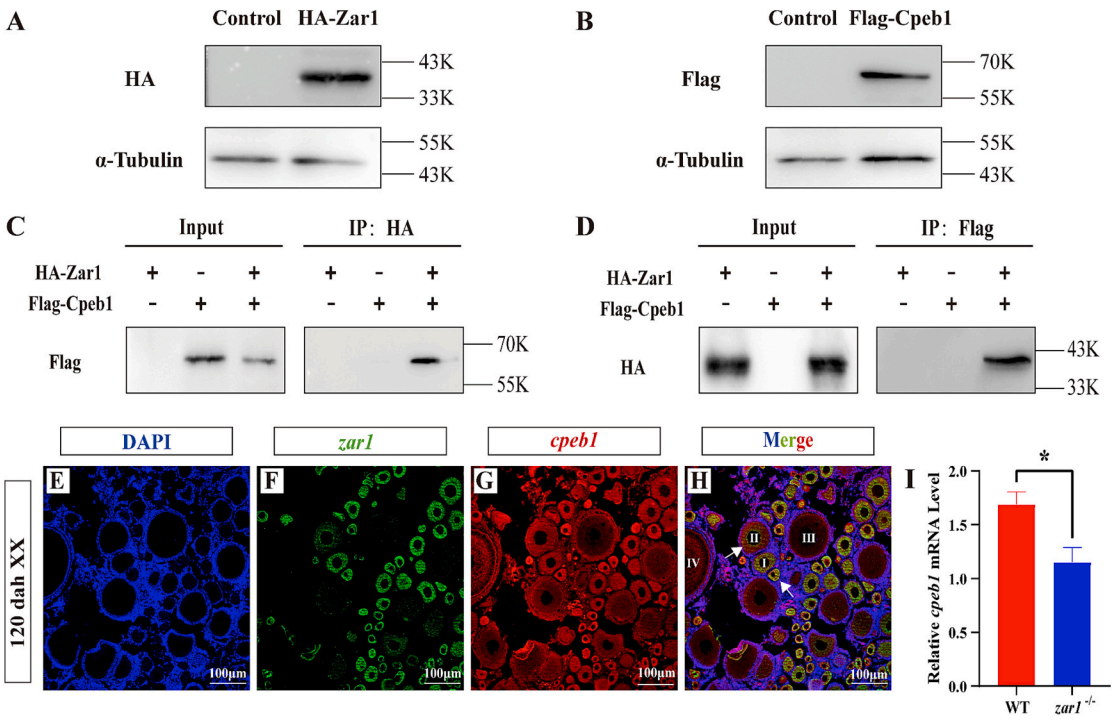

Fig. 7. Interaction between tilapia Zar1 and Cpeb1.
(A-B) Validation of Zar1 and Cpeb1 recombinant protein expression. pcDNA3.1-HA-Zar1 and pcDNA3.1-Flag-Cpeb1 were separately transfected in HEK-293 T cells. Control is the empty pcDNA3.1 vector. Tubulin was used as an internal reference. (C-D) Co-immunoprecipitation validated Zar1 and Cpeb1 interaction. HEK-293 T cells were transfected with overexpression vectors for HA-Zar1 and Flag-Cpeb1. The negative controls were transfected with vectors for pcDNA3.1 and HA-Zar1 or Flag-Cpeb1. Input indicates total protein extracted from cell lysate. IP indicates immunoprecipitation. (E-H) Verification of Zar1 and Cpeb1 co-location. *zar1* positive cells were labeled with green fluorescence, blue fluorescence indicates DAPI signal and red fluorescence indicates *cpeb1* signal, n = 6, and five sections for per sample were counted. (I) Analysis of *cpeb1* mRNA relative expression levels (n = 6/genotype). Differences between the WT and mutants were tested by two-tailed unpaired Student's t-test, * $P < 0.05$. Results were presented as the mean ± SD in statistical chart.

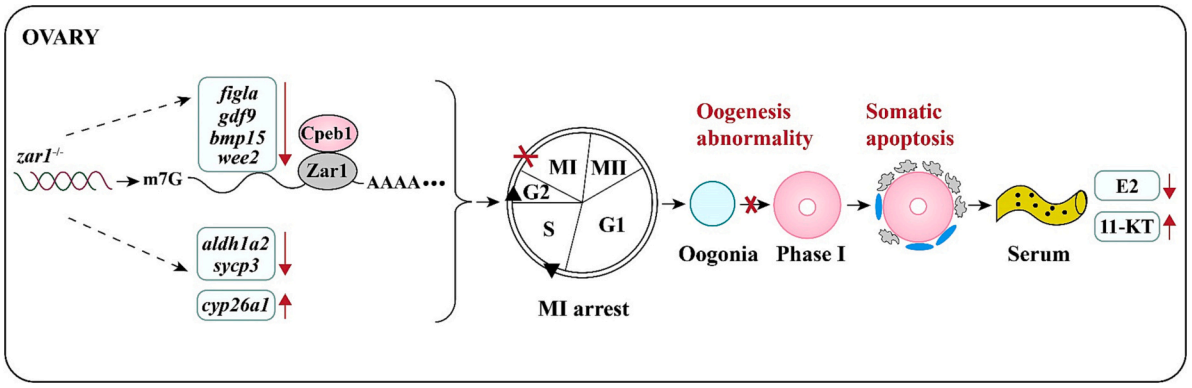

Fig. 8. A hypothetical model of *zar1* function on early oogenesis in tilapia.
zar1 deletion led to early oogenesis abnormalities in tilapia. Zar1 could interact with Cpeb1, and may co-regulate the transcription of *figla, gdf9*, *bmp15* and *wee2* genes. Disruption of *zar1* led to down-regulation and failed polyadenylation of *figla, gdf9*, *bmp15* and *wee2* mRNAs. Besides, *zar1* deletion also resulted in the expression of *aldh1a2* and *sycp3* decreased while the expression of *cyp26a1* increased, which together impaired meiotic initiation, further resulted in failed transition from oogonia to phase I oocytes, with somatic cell apoptosis, decreased serum E2 levels and increased 11-KT levels.

CRediT authorship contribution statement

Miao Yu: Data curation, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. **Shiyi Zhang:** Investigation, Methodology, Software, Validation, Visualization. **Zhisheng Ma:** Investigation, Methodology, Visualization. **Jun Qiang:** Investigation, Methodology, Visualization. **Jing Wei:** Methodology, Supervision. **Lina Sun:** Methodology, Supervision. **Thomas D. Kocher:** Writing – review & editing. **Deshou Wang:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision. **Wenjing Tao:** Conceptualization, Funding acquisition, Methodology, Resources, Software, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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