Functional Divergence of Multiple Duplicated Foxl2 Homeologs and Alleles in a Recurrent Polyploid Fish

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Abstract

Evolutionary fates of duplicated genes have been widely investigated in many polyploid plants and animals, but research is scarce in recurrent polyploids. In this study, we focused on foxl2, a central player in ovary, and elaborated the functional divergence in gibel carp (Carassius gibelio), a recurrent auto-allo-hexaploid fish. First, we identified three divergent foxl2 homeologs (Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B), each of them possessing three highly conserved alleles and revealed their biased retention/loss. Then, their abundant sexual dimorphism and biased expression were uncovered in hypothalamic–pituitary–gonadal axis. Significantly, granulosa cells and three subpopulations of thecal cells were distinguished by cellular localization of CgFoxl2a and CgFoxl2b, and the functional roles and the involved process were traced in folliculogenesis. Finally, we successfully edited multiple foxl2 homeologs and/or alleles by using CRISPR/Cas9. Cgfoxl2a-B deficiency led to ovary development arrest or complete sex reversal, whereas complete disruption of Cgfoxl2b-A and Cgfoxl2b-B resulted in the depletion of germ cells. Taken together, the detailed cellular localization and functional differences indicate that Cgfoxl2a and Cgfoxl2b have subfunctionalized and cooperated to regulate folliculogenesis and gonad differentiation, and Cgfoxl2b has evolved a new function in oogenesis. Therefore, the current study provides a typical case of homeolog/allele diversification, retention/loss, biased expression, and sub-/neofunctionalization in the evolution of duplicated genes driven by polyploidy and subsequent diploidization from the recurrent polyploid fish.

Key words: polyploid, gibel carp, gynogenesis, ovary development, foxl2.

Introduction

Genome polyploidy has been thought as an evolutionary driving force (Otto 2007; Gui and Zhou 2010; Soltis PS and Soltis DE 2016; Zhou and Gui 2017), and its significance and implications on trait innovation and ecological adaption have been supported from whole-genome sequencing and comparative genomic analyses (Van de Peer et al. 2009, 2017; Soltis et al. 2014; Soltis PS and Soltis DE 2016; Cheng et al. 2018). It might result in the complexity, variability, and diversity of organisms, especially leading to an increased short-term adaptative potential (Van de Peer et al. 2017) and a long-term evolvability (Cheng et al. 2018). Therefore, polyploidy is considered as a main driver of speciation nowadays and shapes almost every aspect of species evolution (Sessa 2019). All plants and most of extant vertebrates have evolved from polyploid ancestors. Two rounds (2R) of whole-genome duplication (WGD) were proposed to trigger the species explosion of vertebrates, and a teleost-specific third WGD (Ts3R) was believed to lead to dramatic rise and rapid radiation of teleosts (Amores et al. 1998; Meyer and Van de Peer 2005). The neopolyploid might firstly undergo a chaos of “genomic shock” (McClintock 1984; Ng et al. 2012), subsequently occur a series of complicated non-Mendelian changes to shape a novel genome, and finally evolve into a paleopolyploid or a new diploid (Comai 2005; Otto 2007; Soltis et al. 2014; Zhou and Gui 2017). These processes are usually referred to as diploidization, during which duplicated genes undergo homeolog or allele diversification, fractionation, sub- or neofunctionalization under relaxed purifying selection (Liu et al. 2009; Cheng et al. 2018). Although the fates of duplicated genes have been well documented in paleopolyploids, the research is scarce in recurrent polyploids with complex genomes.

In addition to the 3R of WGD shared by all teleosts, polyploidy happened repeatedly in some taxonomic fish orders (Leggatt and Iwama 2003; Taylor 2003; Van de Peer et al. 2003; Gui and Zhou 2010; Zhou and Gui 2017; Lu et al. 2021). For
example, a salmonid-specific fourth WGD (Ss4R) and an allopolyploidy event were shown to have occurred in the common ancestor of salmonids (Berthelot et al. 2014; Lien et al. 2016) or common carp (Cyprinus carpio) (Xu et al. 2014), respectively. Polyploid Carassius species complex, widely distributing across the Eurasian continent (Liu, Jiang, et al. 2017, Liu, Li, et al. 2017), include allo-tetraploid crucian carp (C. auratus) with 100 chromosomes and auto-allo-hexaploid gibel carp (C. gibelio) with over 150 chromosomes (Zhou and Gui 2002; Zhu et al. 2006; Yu et al. 2021). Two extra rounds of polyploidy, including an early allotetraploidy (4R) and a late autotriploidy (5R), were estimated to have undergone during the evolution of gibel carp (Li, Zhang, et al. 2014). Interestingly, gibel carp is able to reproduce by unisexual gynogenesis (Gui and Zhou 2010; Liu et al. 2015; Zhang et al. 2015). Compared with other unisexual vertebrates (Neaves and Baumann 2011), 1.2–26.5% male individuals were found in gibel carp natural populations (jiang et al. 2013; Li et al. 2018), which is determined by extra microchromosomes and is affected by rearing temperature (Li et al. 2016; Li and Gui 2018; Li et al. 2018). A few key genes, such as dsx- and mab-3-related transcription factor 1 (Dmr1) (Li, Li, et al. 2014) and a novel Setdm (SET domain-containing male-specific gene) located on extra microchromosomes (Li et al. 2017), show predominant or specific expression in gibel carp testis, but it is unclear which might serve as the initiator or master for ovary development in the all-female gynogenetic fish.

Foxl2, belonging to Forkhead (FKH) box (Fox) protein subfamily FoxL, was identified as the earliest key marker of mammalian ovarian differentiation (Cocquet et al. 2002; Schmidt et al. 2004; Kocer et al. 2008; Uhlenhaut et al. 2009). Its predominant expression in ovary has been confirmed in some bisexual fishes (Loffler et al. 2003; Nakamoto et al. 2006; Alam et al. 2008; Iijiri et al. 2008; Bertho et al. 2016; Bhat et al. 2016; Yang et al. 2017) and its pivotal roles in ovarian differentiation have been revealed in zebrafish (Danio rerio) (Yang et al. 2017), Nile tilapia (Oreochromis niloticus) (Zhang et al. 2017), and rainbow trout (Oncorhynchus mykiss) (Bertho et al. 2018). Although foxl2 is a conserved transcription factor in metazoa, its evolution in teleosts is complicated (Bertho et al. 2016). Zebrafish has two foxl2 paralogs (foxl2a and foxl2b) resulted from Ts3R. They sequentially and cooperatively regulate zebrafish ovary development and maintenance, and foxl2b plays a dominant role during the process (Yang et al. 2017). In rainbow trout, foxl2a was lost after Ts3R whereas foxl2b was further duplicated into two orthologs (foxl2b1 and foxl2b2) following Ss4R, which carried the salmonid master sex-determining gene SdY into the nucleus to prevent female differentiation (Bertho et al. 2018). Nile tilapia retained only one foxl2 gene and XX foxl2-/- mutant occurred female-to-male sex reversal (Zhang et al. 2017). Here, we chose gibel carp and foxl2, a recurrent hexaploid and master gene in ovary development, as a unique model to reveal the evolutionary fates of duplicated genes. We first investigated the diversification, evolution, and biased expression of foxl2 homeologs and alleles in auto-allo-hexaploid gibel carp. Then, we tried to knockout multiple foxl2 homeologs and/or alleles by using CRISPR/Cas9. Finally, we compared the sex ratio, gonadal histological structure, and dynamic changes of gonadal development-related gene expression among these mutants with wild-types (WTs) during oogenesis.

**Results**

**Diversification and Characterization of foxl2 Homeologs and Alleles**

A total of 12 Cgfoxl2 transcripts were cloned from hexaploid gibel carp ovary by homology-based cloning strategy (supplementary fig. S1, Supplementary Material online) and mapped onto the assembled genome of gibel carp (fig. 1A). Multiple nucleotide alignments and phylogenetic analysis showed that six transcripts were annotated as foxl2a and others belonged to foxl2b. The six Cgfoxl2 transcripts were clustered into two groups (three Cgfoxl2b-A and three Cgfoxl2b-Bs) (fig. 1B). The average identities among three Cgfoxl2b-As or Cgfoxl2b-Bs were about 99.7 ± 0.2% and 99.3 ± 0.1% respectively, whereas the average identity between Cgfoxl2b-As and Cgfoxl2b-Bs was about 88.6 ± 0.4%. These data suggest that Cgfoxl2b-A and Cgfoxl2b-B might be a pair of homeologs derived from early allotetraploidy (4R), and they both have three alleles because of late autotriploidy (5R). The major differences between Cgfoxl2b-As and Cgfoxl2b-Bs homeologs existed in the 5’ untranslated region (UTR) and the 3’ UTR. Thus, the transcripts of Cgfoxl2b-As and Cgfoxl2b-Bs were predicted to encode one Cgfoxl2b-A protein (Cgfoxl2b-A1/A2/A3) and two Cgfoxl2b-B proteins (Cgfoxl2b-B1/B2 and Cgfoxl2b-B3), and there was only one amino acid difference between Cgfoxl2b-B1/B2 and Cgfoxl2b-B3. Cgfoxl2b-A and Cgfoxl2b-B had about 95.0% amino acid identity (supplementary fig. S2, Supplementary Material online).

Unlike Cgfoxl2b, all six Cgfoxl2a transcripts were aligned to same position in the chromosome CgB15 (fig. 1C). We could not find any other Cgfoxl2a gene in the assembled genome of gibel carp (Wang Y, Li XY, Wu B, Xu M, Wang ZW, Li Z, Zhang XJ, Yin Z, Yang YL, Miao LJ, et al., unpublished data) or clone any other Cgfoxl2a transcripts from ovary. Thus, these Cgfoxl2a transcripts were named as Cgfoxl2a-B and Cgfoxl2a-A gene might be lost during evolution. According to the differences of 5’ UTR, Cgfoxl2a-B transcripts were also clustered into two classes (Cgfoxl2a-B1/B2/B3-a and Cgfoxl2a-B1/B2/B3-b), which came from the different transcriptional start sites at first and second exon of Cgfoxl2a-B genes, respectively (fig. 1A). Each of them also had three alleles, among which three and two single nucleotide polymorphism (SNP) existed in the open reading frame (ORF) and 3’ UTR, respectively. All Cgfoxl2a-B transcripts were predicted to encode the same Cgfoxl2a-B protein (Cgfoxl2a-B1/B2/B3).

Cgfoxl2a-B had 71.6% and 68.7% amino acid identities with Cgfoxl2b-A and Cgfoxl2b-B, respectively. As a comparative control, eight Cgfoxl2 transcripts were cloned from tetraploid crucian carp, which were predicted to encode two proteins of Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B, respectively. We also could not find a region containing foxl2a-A in the assembled genome of crucian carp (Wang Y, Li XY, Wu B, Xu M, Wang ZW, Li Z, Zhang XJ, Yin Z, Yang YL, Miao LJ, et al., unpublished data) and gold fish (Luo et al. 2020) by using GMAP (Wu and
Watanabe 2005) or clone any of Cgfoxl2a-A transcripts, which suggested that foxl2a-A gene might have been lost in the ancestor of Carassius complex after 4R. Phylogenetic analysis showed that Carassius complex Fox2a and Fox2b were clustered with zebrafish and channel catfish (Ictalurus punctatus) Fox2a and Fox2b, respectively (fig. 1B). Carassius complex Fox2b-A and Fox2b-B were grouped into two independent branches, indicating foxl2b-A and foxl2b-B are duplicated in the ancestor of gibel carp and crucian carp. Rainbow trout Fox2b1 and Fox2b2, Nile tilapia Fox2, spotted gar (Lepisosteus oculatus) Fox2, and coelacanth (Latimeria chalumnae) Fox2 were first grouped together and then clustered with other teleost Fox2b. Finally, all fish Fox2 clustered with tetrapod Fox2.

Subsequently, the genomic structure and syntenic alignment were also analyzed (fig. 1A and C). All gibel carp fox2 genes have a biconic structure same as zebrafish fox2 (Yang et al. 2017). Cgfox2a-B, Cgfox2b-A, and Cgfox2b-B are located on chromosome CgB15, CgA2, and CgB2 of gibel carp, respectively (fig. 1C), and three alleles of each gene in the three homologous chromosomes were confirmed by fluorescent in situ hybridization (FISH) (fig. 1D). For example, three green Cgfox2b-A signals were observed from three homologous chromosomes, and three red Cgfox2b-B signals were located on another three homologous chromosomes when simultaneously using Cgfox2b-A-BAC-DNA and Cgfox2b-B-BAC-DNA probe labeled with DIG, and the chromosomes with red signals (indicated by arrowheads) were stained by Cgfox2b-B-BAC-DNA probe labeled with Biotin. All metaphase chromosomes (blue) were counterstained with DAPI.

Divergent and Dynamic Expression Patterns in Brain–Pituitary–Gonad Axis

First, the distribution of foxl2as and foxl2bs mRNAs in adult tissues was analyzed by quantitative polymerase chain reaction

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**Fig. 1.** Molecular characterization of gibel carp foxl2a and foxl2b. (A) Genomic structure and transcript sketch of foxl2a and foxl2b. Exons and introns are shown by rectangle boxes and thick lines, respectively, and their sizes (bp) are indicated upon or below. ORFs and FKH domain are highlighted by black and red boxes, respectively. (B) Phylogenetic tree of vertebrate Foxl2. (C) Gene synteny of vertebrate foxl2. Gibel carp foxl2a-B, foxl2b-A, and foxl2b-B are located on chromosome CgB15, CgA2, and CgB2, respectively. Conserved gene blocks are represented in matching colors, and transcription orientations are indicated by arrows. (D) Localization on metaphase chromosomes of gibel carp Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B. The chromosomes with green signals (indicated by arrows) were stained by Cgfox2a-B-BAC-DNA or Cgfox2b-B-BAC-DNA probe labeled with DIG, and the chromosomes with red signals (indicated by arrowheads) were stained by Cgfox2b-B-BAC-DNA probe labeled with Biotin. All metaphase chromosomes (blue) were counterstained with DAPI.
(qPCR). Due to the extremely high identities among three alleles of each foxl2 gene (supplementary fig S1, Supplementary Material online), four specific pairs of primers (supplementary table S1, Supplementary Material online) were designed to amplify Cgfoxl2a-Bs-a, Cgfoxl2a-Bs-b, Cgfoxl2b-As, and Cgfoxl2b-Bs, respectively. Compared with other analyzed tissues, abundant gibel carp foxl2as and foxl2bs transcripts were amplified from brain–pituitary–gonad axis (fig. 2A), indicating their important and conserved roles in reproductive regulation. In hypothalamus and other brains, Cgfoxl2a-Bs-b and Cgfoxl2b-Bs were both remarkably higher than those of Cgfoxl2a-Bs-a and Cgfoxl2b-As. However, only Cgfoxl2a-Bs-a and Cgfoxl2a-Bs-b were highly expressed in the pituitary. Interestingly, Cgfoxl2b-As and Cgfoxl2b-Bs exhibited sexually dimorphic pattern with higher expression in ovary compared with testis, whereas Cgfoxl2a-Bs-a expression was almost absent in gonads and Cgfoxl2a-Bs-b showed similar expression levels between ovary and testis. The divergent expression patterns of gibel carp foxl2s imply significantly biased expression of them in adult tissues.

Subsequently, we analyzed their dynamic expression changes in unfertilized eggs, 50% epiboly, hatching fry, and developing ovaries from 10 days posthatching (dph) to 270 dph and mature ovary (fig. 2B). Maternal foxl2as and foxl2bs transcripts were detected in unfertilized eggs and their zygotic products are synthesized in hatching fry. During ovary differentiation and maturation, only a few of Cgfoxl2a-Bs-a transcripts were detected. Cgfoxl2a-Bs-b, Cgfoxl2b-As, and Cgfoxl2b-Bs were all abundantly expressed at 25 dph, quickly reached peak values at 35 dph, and then gradually decreased to the lowest values at 190 dph. From 25 to 35 dph, the expression level of Cgfoxl2a-Bs-b was higher (2.5–7.1 fold) than those of Cgfoxl2b-As and Cgfoxl2b-Bs. Along with the maturing of ovary, their expression all mildly increased, and more transcripts of Cgfoxl2b-As and Cgfoxl2b-Bs were detected than Cgfoxl2a-Bs-b.

According to the specific SNPs in three alleles of each foxl2 genes, we tried to present the expression differences among three alleles through sequencing the amplified fragments (fig. 2C). In the developing ovary at 45 dph, more than 92.6% of sequencing clones on average (about 20–30 clones each individual, n = 3) belonged to Cgfoxl2a-B2 and Cgfoxl2a-B3, indicating that the expression levels of Cgfoxl2a-B2 and Cgfoxl2a-B3 were much higher than that of Cgfoxl2a-B1. Although Cgfoxl2a-B1-a still kept low expression, Cgfoxl2a-B1-b seemed to raise about 4-folds of its expression, making up about a quarter of total Cgfoxl2a-B1/B2/B3-b expression in the maturing ovary at 270 dph. The biased expression pattern in developing testis was similar to developing ovary (45 dph), whereas the pattern in maturing testis was remarkably different from those in ovary. Cgfoxl2a-B2 appeared to play a dominant role, occupying two-thirds of Cgfoxl2a-B1/B2/B3-b expression in maturing testis. The three alleles of Cgfoxl2b-A and Cgfoxl2b-B did not show obviously biased expression in ovary and presented similar expression levels, respectively. In maturing testis, the expression of Cgfoxl2b-B1 was much more abundant than those of other alleles.

Distinct Cellular Localization of Cgfoxl2a and Cgfoxl2b during Ovary and Oocyte Development

Two polyclonal antibodies specific for Cgfoxl2a and Cgfoxl2b were produced and confirmed by Western blot (supplementary fig S3, Supplementary Material online). Anti-Cgfoxl2a antibody can specifically recognize the overexpressing HA-tagged Cgfoxl2a-B protein (HA-Cgfoxl2a-B) in 293T cells and not detect HA-Cgfoxl2b-A or HA-Cgfoxl2b-B proteins and vice versa for anti-Cgfoxl2b antibody. The cellular localization of Cgfoxl2a and Cgfoxl2b proteins were assessed in ovaries from 20 dph to adult by immunofluorescence analysis (fig. 2D). At 20 dph, a few of primordial germ cells (PGCs) scattered in the gonadal primordia and were surrounded by somatic cells. Punctate Cgfoxl2b immunofluorescence signals were observed in the perinuclear region of PGCs. Three kinds of somatic cells with positive signals in nuclei were distinguished. The first was strongly marked by both CgFoxl2a and CgFoxl2b (indicated by arrowheads), and the second had intensely CgFoxl2a positive signals in the fusiform nucleus (indicated by asterisks). The third had elliptical nuclei with dispersive and star-like signals of CgFoxl2a (indicated by trapezoid). At 45 dph, oogonia massively proliferated and cysts started to form. A few of oogonia differentiated into primary oocytes (PO, I). In the perinuclear region of oogonia and PO, much punctate CgFoxl2b positive signals were observed. At 50 dph, more oogonia differentiated into PO, where CgFoxl2b localized in the perinuclear region and close to chromatins. Several somatic cells scattered and surrounded PO, implying the initiation of follicular lay formation. PO was first surrounded by granulosa cells (indicated by arrowheads) which were coexpressed CgFoxl2a and CgFoxl2b, and then by theca cells (indicated by asterisks) emitted only CgFoxl2a immunofluorescence signals. Along with oogonia differentiation and PO growth (60-89 dph), CgFoxl2b proteins in peripheral area of PO nucleus interspersed into ooplasm, and granulosa cells (indicated by arrowheads) coexpressed CgFoxl2a and CgFoxl2b proteins began to proliferate. Merged imagination clearly discriminated three subpopulations of thecal cells, expressing CgFoxl2a (indicated by asterisks), coexpressing CgFoxl2a/B (indicated by hexagons) and not expressing CgFoxl2a/B cell (indicated by pentagons). In maturing ovary, two successive follicular layers, granulosa lay and thecal lay, formed and enclosed vitelligenic oocytes (III) and maturing oocytes (IV). As oocytes grew, CgFoxl2a immunofluorescence signals became weak and CgFoxl2b persisted abundant expression in granulosa cells. In thecal layer surrounding vitelligenic oocytes, about 26.1 ± 7.4%, 8.0 ± 3.0%, and 65.9 ± 8.9% thecal cells were expressed only CgFoxl2a, coexpressed CgFoxl2a and CgFoxl2b, or expressed neither CgFoxl2a nor CgFoxl2b, respectively. In addition, many CgFoxl2a-expressed cells were observed in the stromal layer throughout ovary development. In testis, very faint CgFoxl2a and CgFoxl2b immunofluorescence signals were observed only in the spermatogonia (SPG).
CgFoxl2a-B Deficiency Led to Ovary Development Arrest and Sex Reversal in a Dosage-Dependent Manner

The dynamic expression and distinct cellular localization of CgFoxl2a and CgFoxl2b suggest that their functions might have been diverged. To explore this, we generated Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B singly or simultaneously disrupted gibel carps using CRISPR/Cas9. The targeting site of Cgfoxl2a-B was chosen in the second exon to disrupt the FKH domain (supplementary fig. S4A, Supplementary Material online). Due to polyploidy nature of gibel carp, a lot of complicatedly mutated genotypes modified at the target sites were detected in the F0 individuals. All of F0 individuals developed normally and grew to fertile females, similar to WT. Two individuals, named as F0-1 and F0-2, were chosen to proliferate by gynogenesis. The sequencing results showed that the mutations of these two F0 individuals both caused a reading frame shift (supplementary fig. S4C and D, Supplementary Material online).
To exclude the influences of environmental factors on gibel carp sex differentiation, we raised WT individuals and \textit{Cgfoxl2a-B} mutated F1 gynogenetic offspring together to adult in the same cage. Gibel carp became sex mature after raising 1 year. A total of 212 individuals were randomly sampled to identify their genotypes and to analyze their gross morphology and histology of gonads, of which 140 individuals were identified as \textit{Cgfoxl2a-B} mutants with different mutated genotypes. According to the specific SNPs in three alleles of \textit{Cgfoxl2a-B}, the 61 F1 gynogenetic offspring of F0-1 were classified into three mutated genotypes \((\text{Cgfoxl2a-B}^{\Delta A7/\Delta 7+11/\Delta 4+5} \text{[n=5]}, \text{Cgfoxl2a-B}^{\Delta 47/\Delta 7+12/\Delta 2+1} \text{[n=4]}, \text{and} \text{Cgfoxl2a-B}^{\Delta 55/\Delta 54/\Delta 58+8} \text{[n=52]}), \text{and} the 79 F1 gynogenetic offspring of F0-2 were clustered into six mutated genotypes \((\text{Cgfoxl2a-B}^{\Delta 55/\Delta 54/\Delta 58+8} \text{[n=16]}, \text{Cgfoxl2a-B}^{\Delta 54/\Delta 42+1/\Delta 18+12} \text{[n=12]}, \text{Cgfoxl2a-B}^{\Delta 54/\Delta 64+2/\Delta 18+12} \text{[n=38]}, \text{Cgfoxl2a-B}^{\Delta 11+26/\Delta 47+26/\Delta 15+1} \text{[n=2]})\), and \text{Cgfoxl2a-B}^{\Delta 36/\Delta 56/\Delta 5} \text{[n=3]}, \text{respectively} (\text{fig. 3A and B}).

The vast majority (95.8\%) of WT gibel carp were fertile females (\text{fig. 3A}), with ovaries containing a lot of maturing oocytes (IV), vitellogenic oocytes (III), growth stage oocytes (II), and PO (I) (\text{fig. 3C}). According to the edited efficiency of three alleles, the mutated genotypes were classified into three groups. The first group (I) included \text{Cgfoxl2a-B}^{\Delta 47/\Delta 7+11/\Delta 4+5} and \text{Cgfoxl2a-B}^{\Delta 47/\Delta 7+11/\Delta 4+5} \text{in which the three alleles all created a premature stop codon and lost FKHD domain} (\text{fig. 3B}). About two-thirds of mutated individuals arrested ovary development at primary oocyte growth stage and the rest developed to males. Compared with WT mature ovary, the arrested ovary is small and translucent with a lot of PO and growth stage oocytes, and only a few of oocytes developed to cortical alveous stage. The mutated males had normal testis, filling numerous SPG, spermatocytes (SPC), and spermatids (SPD) into testicular lobules (\text{fig. 3C}). To confirm the knockout effects of \textit{CgFoxl2a-B}, the individuals of \text{Cgfoxl2a-B}^{\Delta 47/\Delta 7+11/\Delta 4+5} were sampled to perform immunofluorescence analysis (\text{fig. 4}). No \textit{CgFoxl2a} immunofluorescence signal was observed, implying the disruption of \text{CgFoxl2a-B} protein expression in ovary and testis of \text{Cgfoxl2a-B}^{\Delta 47/\Delta 7+11/\Delta 4+5} (\text{fig. 4}). Similar to WT, \textit{CgFoxl2b} proteins were localized in the perinuclear region of PO, nuclei of all granulosa cells and a minority of thecal cells, and SPG. The second group (II) included five mutated genotypes, in which one or two alleles lost FKHD domain and the rest alleles presented sense mutation and deletion between N-terminal and FKHD domain (\text{fig. 3B and C}). Among 120 adult individuals, about 45.0\% individuals were males and 11.7\% individuals had arrested ovary, whereas others had normal mature ovaries (\text{fig. 3A}). All mutated males had normal testis. The fertilization rates and hatching rates were similar to those in control groups (\text{supplementary table S2, Supplementary Material online}).

Immunofluorescence analysis also confirmed the loss of \textit{CgFoxl2a} signal (\text{fig. 4}). The third group (III) consisted of the rest two mutated genotypes \((\text{Cgfoxl2a-B}^{\Delta 39/\Delta 10} \text{and} \text{Cgfoxl2a-B}^{\Delta 36/\Delta 8})\), possessing WT \text{Cgfoxl2a-B1} allele, mutated \text{Cgfoxl2a-B2} allele with sense mutation and deletion between N-terminal and FKHD domain and mutated \text{Cgfoxl2a-B3} allele with premature stop codon (\text{fig. 3B}). Two individual of \text{Cgfoxl2a-B}^{\Delta 36/\Delta 8} arrested ovary development and the other developed mature females. Among eight individuals of \text{Cgfoxl2a-B}^{\Delta 39/\Delta 10} one appears to have arrested ovary, the rest developed to mature males (\text{fig. 3A}). Compared with WT ovary, obviously less immunofluorescence \textit{CgFoxl2a} signals were detected in the arrested ovary of \text{Cgfoxl2a-B}^{\Delta 36/\Delta 8}.

\textbf{Mutated Cgfoxl2a-B Allele with an Intact FKH Domain Can Activate Cgcytp191Aa-1A Expression}

Due to clinical heterogeneity and haploinsufficiency of human FOXL2 mutations (Verdin and De Baere 2012; Takahashi et al. 2013), we tried to compare the effects of different \textit{Cgfoxl2a-B} mutated alleles on ovary and testis at oogonia or SPG proliferation stage (\text{fig. 5} and \text{supplementary fig. S5, Supplementary Material online}). We chose \text{Cgfoxl2a-B}^{\Delta 55/\Delta 54/\Delta 58+8}, a mutated genotype having most F1 individuals, to perform further analysis. First, one pair of primers located in the no mutation site was designed to detect the expression of all \textit{Cgfoxl2a-B} alleles. Consistent with above result (\text{fig. 2B}), only a few of \text{Cgfoxl2a-B5s-a} transcripts were detected in the WT and \text{Cgfoxl2a-B} mutated ovary and testis. \text{Cgfoxl2a-B}^{\Delta 55/\Delta 54/\Delta 58+8} ovary showed about half \text{Cgfoxl2a-B1/b2/b3-b} expression level of WT ovary. Although overregulated \text{Cgfoxl2a-B1/b2/b3-b} expression (7.5-fold) was observed in \text{Cgfoxl2a-B}^{\Delta 55/\Delta 54/\Delta 58+8} testis when it was compared with WT testis, mutated testis had significantly lower \text{Cgfoxl2a-B1/b2/b3-b} expression (6.3-fold) than that of WT ovary (\text{fig. 5A}). The above results indicate that the mutated \text{Cgfoxl2a-B} alleles exhibited differential expression levels between ovary and testis at early gonadal development stage.

Subsequently, we first compared the subcellular localization of WT and mutated \textit{CgFoxl2a-B} proteins in order to analyze whether or not the mutated \textit{Cgfoxl2a-B} alleles still have function. WT \text{Cgfoxl2a-B2}, \text{Cgfoxl2a-B1}^{\Delta 55}, \text{Cgfoxl2a-B2}^{\Delta 54}, and \text{Cgfoxl2a-B3}^{\Delta 52+8} were linked to green fluorescent protein (GFP) and transfected into HEK293T cells, respectively (\text{fig. 5B}). Similar to WT \text{Cgfoxl2a-B2} protein, the mutated \text{Cgfoxl2a-B} proteins containing an intact FKHD domain encoded by \text{Cgfoxl2a-B2}^{\Delta 54} localized in the nucleus, implying that they may keep transactivation capacity. The truncated \text{Cgfoxl2a-B} protein without FKHD domain encoded by \text{Cgfoxl2a-B1}^{\Delta 55} and \text{Cgfoxl2a-B3}^{\Delta 52+8} diffusely distributed in the cytoplasm and nucleus.

A lot of studies have revealed the regulative roles of Foxl2 on cyp19a1 expression (Wang et al. 2007; Bertho et al. 2018). Subsequently, we performed luciferase assays to evaluate the expression of \text{Ccgpyp191Aa} by co-transfection of \text{Ccgpyp191Aa} promoter with WT \text{Cgfoxl2a-B2}, \text{Cgfoxl2a-B1}^{\Delta 55}, \text{Cgfoxl2a-B2}^{\Delta 54}, and \text{Cgfoxl2a-B3}^{\Delta 52+8}, respectively (\text{fig. 5C and supplementary fig. S6, Supplementary Material online}). Compared with the control empty vector, overexpression of WT \text{Cgfoxl2a-B2} alone could not trigger \text{Ccgpyp191Aa-1A} transcription, but coexpression of WT \text{Cgfoxl2a-B2} and \text{Cgsf1a-B} significantly activated \text{Ccgpyp191Aa-1A} transcription in Crucian carp blastula embryonic cells (CAB) (\text{fig. 5C}) and Epithelioma papulosum cyprinid cells (EPC) (\text{supplementary fig. S6, Supplementary Material online}). The results imply that
Cgfoxl2a-B2 can enhance SF1-activated Cgcyp19a1a-A expression. Although Cgfoxl2a-B1 $D_{55}$ and Cgfoxl2a-B3 $D_{25} + 8$ had no effect, overexpression of Cgfoxl2a-B2 $D_{54}$ and Cgsf1a-B can activate Cgcyp19a1a-A expression. In addition, overexpression of Cgsf1a-B significantly inhibited Cgcyp19a1a-B expression, which can be rescued by coexpression with WT Cgfoxl2a-B2 or Cgfoxl2a-B2 $D_{54}$. The results indicate that Cgfoxl2a-B2 $D_{54}$ possesses transcriptional activation capacity. Consistent with sexually dimorphic expression of Cgfoxl2a-Bs-b, Cgcyp19a1as also had higher expression in ovary than in testis at oogonia or SPG proliferation stage. Meantime, Cgdmrt1s was downregulated in ovari (fig. 5A). Taken together the above results, we speculate that the downregulated expression of Cgfoxl2a-Bs-b in some gonad may result in lower expression of Cgcyp19a1as, which is not sufficient to initiate ovary development.

We also compared the dynamic expression changes of a dozen of important genes involved in gonadal development, including germ cell markers (vasa and dnd) (Xu et al. 2005; Liu et al. 2015), testis differentiation-related genes (dmt1 and sox9b) (Forconi et al. 2013; Li, Li, et al. 2014), oocyte-derived factor (gdf9) (Liu et al. 2012), and genes in steroidogenic pathway (sf1a, cyp11a1, cyp17a1, cyp17a2, cyp19a1a, and srd5a1) (Payne and Hales 2004; Park and Jameson 2005; Vizziano et al. 2007; Kayampilly et al. 2010; Yang et al. 2017) in WT ovary, mutated ovary and testis (fig. 5D). Except cyp17a1 and cyp17a2, all other genes have two divergent homeologs, which showed significantly biased expression (supplementary fig. S7, Supplementary Material online). Compared with their expression in ovaries (WT and Cgfoxl2a-Bs $D_{55}/D_{54}/D_{25} + 8$ ovaries), dmrts, sox9bs, sf1as, cyp11a1s, vasa, cyp17a1-B, and cyp17a2-B remarkably increased, but dnd, cyp19a1a-A, gdf9s, and srd5a1s...
At oogonia proliferation and differentiation stage (45–60 dph, stage I), WT ovaries enlarged, and oogonia rapidly proliferated and started to differentiate to PO. Among 16 F0-mutated Cgfoxl2b-A/Cgfoxl2b-B individuals, seven, eight, and one individuals had delayed ovary, gonad without germ cell and testis. In comparison with normal ovary, the delayed ovary still retained in oogonia proliferation stage. The gonads without germ cells remained thin thread-like shape, and no PGCs and oogonia were found in the histological sections (fig. 6C), which were confirmed by no positive immunofluorescent signal of Vasa detected in their gonad sections (fig. 6D). Along with the oocyte growth (80–110 dph, stage II), overwhelming majority of WT gonads (95.5%, n = 22) developed into typical ovaries with a lot of PO (I) and growth stage oocytes (II). However, the ratios of delayed ovary, germ cell-depleted gonad, ovotestis, and testis in mutants were 13:4:1:0. The delayed ovaries were still at the oogonia proliferation stage. Gonadal somatic cells proliferated a little in germ cell-depleted gonads, and a huge number of PO (I), growth stage oocyte (II), and spermatogenic cysts containing SPG, SPC, and SPD were mixed in the ovotestis. None of Cgfoxl2b-A and Cgfoxl2b-B mutants developed mature female and spawned eggs (180–360 dph, stage III). The ratios of arrested ovary, germ cell-depleted gonad, ovotestis, and testis were 16:2:5:2. The results of F0-mutated Cgfoxl2b-A/Cgfoxl2b-B individuals are distinct from the normal ovary development in the F0-mutated Cgfoxl2a-B individuals, which indicates that CgFoxl2b might play a dominant role in gibel carp oogenesis.

Due to no fertile female obtained in this study, we can only randomly select three F0 individuals with different gonad phenotypes to analyze their genotypes. We also discriminated three alleles of Cgfoxl2b-A and Cgfoxl2b-B according to their specific SNPs. The mutation rates of F0 individuals with germ cell-depleted gonad were the highest, sequentially followed by F0 individuals having testis, ovotestis, and delayed ovaries, which seemed to positively associate with the severity of oocyte defects or changes (fig. 6E and supplementary table S3 and figs. S9–S12, Supplementary Material online). For example, no WT allele of Cgfoxl2b-B was found in the three mutated individuals possessing germ cell-depleted gonad, and their vast majority of sequencing clones (97.1–100.0%) of Cgfoxl2b-A were identified as mutated types (supplementary fig. S10, Supplementary Material online). In addition, all of mutated Cgfoxl2b-A and Cgfoxl2b-B alleles contain frameshift mutations in FKH domain. The results indicate that complete disruption of Cgfoxl2b-A and Cgfoxl2b-B led to the depletion of germ cell in gibel carp. The mutated individuals having testis also showed highly mutation rates. However, compared with mutated genotypes in germ cell-depleted gonad, a little more WT Cgfoxl2b-A allele or some mutated Cgfoxl2b-A alleles with intact FKH domain were detected in these mutated individuals with testis (supplementary fig. S12, Supplementary Material online). The average mutation rates of F0 individuals with ovotestis were 76.5 ± 1.3% of Cgfoxl2b-A alleles and 54.3 ± 14.0% of Cgfoxl2b-B alleles, which were lower than those of mutants with germ cell-depleted gonad.

Complete Disruption of Cgfoxl2b-A and Cgfoxl2b-B Resulted in Germ Cell Depletion

We also chose conserved sites in the second exon as targeting sites of CRISPR/Cas9 to simultaneously disrupt Cgfoxl2b-A and Cgfoxl2b-B (supplementary fig. S8A, Supplementary Material online). The F0-mutated Cgfoxl2b-A and Cgfoxl2b-B individuals were cocultured with WT in the same cage and randomly sampled at each stage from 45 dph to mature adult. Compared with WT gibel carp (97.6% of individuals were females, n = 84), all of Cgfoxl2b-A and Cgfoxl2b-B double-mutated F0 individuals (n = 59) seemed to have abnormal ovary development, possessing delayed ovary (n = 36), germ cell-depleted gonad (n = 14), ovotestis (n = 6), and testis (n = 3) (fig. 6A and B).

No or little Cgfoxl2a immunofluorescence signal was observed, and Cgfoxl2b immunofluorescence signals were observed in oogonia and PO (indicated by rhombuses), granulosa cell (indicated by arrowheads), thecal cells (indicated by hexagons), and SPG (indicated by arrows). Bars are shown at bottom-right of the images.

Decreased in Cgfoxl2b−Δ55/Δ54/Δ25 + 8 testis at 89 dph. In addition, Cgfoxl2b-A and Cgfoxl2b-B showed similar expression between WT and Cgfoxl2a-BΔ55/Δ54/Δ25 + 8 gonads (fig. 5D).

Fig. 4. Immunofluorescence localization of CgFoxl2a and CgFoxl2b proteins in gonads of WT, Cgfoxl2a−Δ14/Δ17 + 1/Δ11/Δ4 + 5, Cgfoxl2a−Δ55/Δ54/Δ25 + 8, Cgfoxl2a−Δ14/Δ36/Δ17, and Cgfoxl2a−Δ14/Δ39/Δ10−mutated individuals. No or little CgFoxl2a immunofluorescence signal was observed, and CgFoxl2b immunofluorescence signals were observed in oogonia and PO (indicated by arrowheads), granulosa cell (indicated by arrowheads), thecal cells (indicated by hexagons), and SPG (indicated by arrows). Bars are shown at bottom-right of the images.
or testis but higher than those of mutants with delayed ovary (64.2 ± 19.4% of $Cgfoxl2b$-A alleles and 51.5 ± 2.4% of $Cgfoxl2b$-B alleles) (supplementary figs. S9–S12, Supplementary Material online).

Similarly, immunofluorescence localization of $CgFoxl2a$ and $CgFoxl2b$ proteins were analyzed in the F0 individuals with different gonad phenotypes to confirm the knockout effects of $Cgfoxl2b$ (fig. 7). Compared with WT ovaries, no $CgFoxl2b$ immunofluorescence signal was observed in the somatic cells of delayed ovary, germ cell-depleted gonad, ovotestis, and testis. However, punctate $CgFoxl2b$ immunofluorescence signals still retained in the oogonia and PO perinuclear region of delayed ovary and ovotestis.

**Compensation of $Cgfoxl2a$ in Germ Cell-Depleted Gonad of F0 Mutated $Cgfoxl2b$-A/$Cgfoxl2b$-B Individuals Could Not Rescue the Defects**

We also analyzed the dynamic expression changes of above genes in F0-mutated $Cgfoxl2b$-A/$Cgfoxl2b$-B individuals (fig. 8). We first focused on their dynamic changes in germ cell-depleted gonad, which was induced by the complete disruption of $Cgfoxl2b$-A and $Cgfoxl2b$-B (supplementary table S3 and fig. S10, Supplementary Material online). None of vasa and dnd5 transcripts was detected in the germ cell-depleted gonad (fig. 8). Taking together no Vasa protein detected by immunofluorescent analysis (fig. 6D), the results confirmed the complete depletion of germ cell. Compared with WT ovary, delayed ovary and ovotestis, the five testis differentiation-related or androgen-producing genes ($dmrt1s$, $sox9bs$, $sf1as$, $cyp11a1s$, and $cyp17a1$-B) all showed dramatic
upregulation expression, whereas the female-biased genes (gdf9s and srd5a1s) were remarkably downregulated expression in germ cell-depleted gonad similar to those in testis. The results implied that the germ cell-depleted gonad developed testis-like gonads without spermatogenic cells, consistent with our previous study in which germ cells was depleted by dnd morpholino-mediated knockdown approach (Liu et al. 2015). Additionally, dmr1ts, sox9b-B, sf1as, cyp11a1-B, and cyp17a1-B raised their expression in germ cell-depleted gonad along with gonad development. cyp19a1as were remarkably upregulated their expression at oogonia proliferation and differentiation stage (I). Significantly, much more abundant Cgfoxl2a-Bs transcripts (3.3–25.3-folds vs. WT ovary) were detected, indicating the compensatory increase of Cgfoxl2a-Bs in the germ cell-depleted gonad (fig. 8).

The normal ovary of WT and delayed ovary of F0 mutated Cgfoxl2b-A/Cgfoxl2b-B individuals showed similar expression patterns of all analyzed genes. The expression levels of testis

Fig. 6. Complete disruption of Cgfoxl2b-A and Cgfoxl2b-B led to the depletion of germ cell in gibel carp. (A) Ratios of normal ovary, delayed ovary, germ cell-depleted gonad, ovotestis, and testis in WT and F0 Cgfoxl2b-A/Cgfoxl2b-B-mutated individuals. (B) Gross morphology and histological examination of adult gonads in WT and F0 Cgfoxl2b-A/Cgfoxl2b-B-mutated individuals. (C) Histological examination of gonads in WT and Cgfoxl2b-A/Cgfoxl2b-B-mutated individuals at three different stages (45–60, 80–110, and 180–360 dph). I, primary oocyte; II, growth stage oocyte; III, vitellogenic oocyte; IV, maturing oocyte. Bars are shown at bottom-right of the images. (D) Histological examination of gonads in WT and mutated Cgfoxl2b-A/Cgfoxl2b-B individuals at 45 dph. Green and blue fluorescences are stained by Vasa antisemurum and DAPI, respectively. (E) Mutated ratios of F0 Cgfoxl2b-A/Cgfoxl2b-B individuals with delayed ovary, germ cell-depleted gonad, ovotestis, and testis.
differentiation-related or androgen-producing genes in ovo-testis lied halfway in between those of testis and ovary (fig. 8).

More Serious Defects Observed in the Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B Triple-Mutated Individuals

The F0 individuals simultaneously mutated Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B were also obtained by coinjection their specific sgRNAs with Cas9 mRNA. Similar to the Cgfoxl2b-A and Cgfoxl2b-B double mutants, all of Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B triple mutants (n = 59) had aberrant ovary development (supplementary fig. S13, Supplementary Material online). The ratios of mutated individuals with delayed ovary, germ cell-depleted gonad, ovotestis, and testis were 50.8, 20.3, 11.9, and 17.0%, respectively. Compared with Cgfoxl2b-A and Cgfoxl2b-B mutated individuals (15.3% with ovotestis and testis), more F0 individuals with mutated Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B (28.9%) occurred partial or complete ovary to testis sex reversal. Especially, the ratio of complete sex reversal in F0-mutated Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B individuals (17.0%) was significantly higher than that of Cgfoxl2b-A and Cgfoxl2b-B individuals (5.1%). The latter is a little higher than that of WT (2.4%). Similar to F0-mutated Cgfoxl2b-A and Cgfoxl2b-B individuals, no fertile mutated Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B female was obtained. We analyzed the mutation rates of F0 individuals. The mutation rates of F0-mutated Cgfoxl2a-B, Cgfoxl2b-A, and Cgfoxl2b-B also appeared to positively correlate with the severity of ovary defects or changes (supplementary table S4 and figs. S14–S17, Supplementary Material online). The disruption of
CgFoxl2a and CgFoxl2b expression were also confirmed by immunofluorescence analysis (supplementary fig. S18, Supplementary Material online). No CgFoxl2a and CgFoxl2b immunofluorescence signal was observed in germ cell-depleted gonad. However, faint CgFoxl2a and CgFoxl2b positive signals were detected in delayed ovary and ovotestis. All analyzed genes showed similar dynamic expression changes in the simultaneous disruption of Cgfoxl2a-B/Cgfoxl2b-A/Cgfoxl2b-B

Fig. 8. qPCR analyses of key genes involved in gonadal development in WT ovaries and delayed ovary, germ cell-depleted gonad, ovotestis, and testis of F0 Cgfoxl2b-A- and Cgfoxl2b-B-mutated individuals at three different stages (45–60, 80–110, and 180–360 dph). eef1a1I was used as the normalizer. Gene expression levels are relative to that of WT ovary at stage II. Each bar represents mean ± SD. Asterisks indicate the significant differences (**P < 0.01).
F0-mutated individuals to F0-mutated Cgfox12b-A/Cgfox12b-B individuals (supplementary fig. S19, Supplementary Material online).

Discussion

With the explosion of genome sequencing, the importance of polyploidy and postpolyploid diploidization (PPD) in increasing genome complexity, variability, and diversity has been widely discussed later (Van de Peer et al. 2009, 2017; Cheng et al. 2018; Clark and Donoghue 2018; Mandakova and Lysak 2018). In this study, we first identified 12 gibel carp foxl2 cDNAs, belonging to three foxl2 genes (Cgfox12a-B, Cgfox12b-A, and Cgfox12b-B) located on three chromosomes of CgB15, CgA2, and CgB2, respectively (fig. 1). Thus, here we presented a typical case to show the diversification of homeologs and alleles in a recurrent hexaploid animal. In addition, most of genes analyzed in this study (figs. 5 and 8 and supplementary fig. S19, Supplementary Material online) and our previous study (Mou et al. 2018) have two divergent homeologs, indicating an allo-polyploidy event occurred in the evolution of gibel carp. Consistent with our previous studies about the identification of bmp15 and nanos2 (Jiang et al. 2020; Zhang et al. 2020), three alleles with high identities (≥99.0%) of each foxl2 gene were identified in this study, implying an auto-polyploidy event occurred in the evolution of gibel carp.

The complex process of PPD, including chromosomal loss/rearrangement, biased gene retention/loss, gene conversion/chimera/fusion, gene sub-/neofunctionalization, biased gene expression and coexpression networks, transposon reactivation, and epigenetic reprogramming, might generate evolutionarily advantageous genomic changes to shape a novel genome of paleopolyploid or new diploid (Doyle et al. 2008; Clark and Donoghue 2018; Mandakova and Lysak 2018). In this study, we identified 12 gibel carp foxl2 genes, belonging to three foxl2 genes (Cgfox12a-B, Cgfox12b-A, and Cgfox12b-B) located on three chromosomes of CgB15, CgA2, and CgB2, respectively (fig. 1). Thus, here we presented a typical case to show the diversification of homeologs and alleles in a recurrent hexaploid animal. In addition, most of genes analyzed in this study (figs. 5 and 8 and supplementary fig. S19, Supplementary Material online) and our previous study (Mou et al. 2018) have two divergent homeologs, indicating an allo-polyploidy event occurred in the evolution of gibel carp.

The biased gene expression was observed not only between Cgfox12a and Cgfox12b but also between Cgfox12b-A and Cgfox12b-B. Using qPCR, we also presented biased expression pattern of three alleles. Similar to zebrafish foxl2a and foxl2b (Yang et al. 2017), Cgfox12a and Cgfox12b showed sequential expression (fig. 2B and D). Altogether with other studies, foxl2 female-biased and predominant in granulosa expression is conserved and incontrovertible throughout vertebrate (Bertho et al. 2016). Besides granulosa cells, whether thecal cells, oocytes, or other somatic cells express Foxl2 or not seems to be species specific (Goverou et al. 2004; Pisarska et al. 2004). Gibel carp Foxl2a and Foxl2b proteins showed distinct cellular localization from zebrafish and medaka (Oryzias latipes) (fig. 2D). Medaka has only one copy of foxl2 gene and their foxl2b copy was lost during evolution. Both zebrafish Foxl2a and medaka Foxl2 were detected in the follicular cells surrounding previtellogenic oocytes and vitellogenic oocytes, not in early oocytes (Herpin et al. 2013; Cauaret et al. 2015). Cgfox12a and/or Cgfox12b are expressed in the nuclei of granulosa cells and theca cells, and somatic cells in stromal layer at the initial stage of follicular layer formation, implying their important roles in folliculogenesis. Interestingly, three subpopulations of thecal cells were identified. In addition, we observed abundant CgFox12b proteins distributed in oogonia and PO, which is similar to medaka Foxl2 proteins (Herpin et al. 2013). The expression of Foxl2 proteins in oogonia and PO implies their direct roles in early oogenesis, independent to their roles in cell communication between oocytes and follicular cells. Therefore, our study provides a clear example to show biased and divergent expression and cellular localization of two teleost paralogs. The results also indicate that CgFox12a and CgFox12b might display function partition during gibel carp oogenesis.

During ovary development, granulosa cells and theca cells form two layers of ovarian follicle. The interactions among granulosa cells, theca cells, and oocytes are pivotal to folliculogenesis, steroidogenesis, and female oogenesis (Jagarlaludhi and Rajkovic 2012; Richards et al. 2018). Foxl2 is one of the earliest known markers of granulosa cell differentiation (Cocquet et al. 2002; Schmidt et al. 2004). Although a few reports described morphological folliculogenesis in teleost species through histological and ultrastructural observation (Grier 2000, 2012; Grier et al. 2009; Quagio-Grassiotto et al. 2011), the studies on the process of folliculogenesis traced by granulosa cell or theca cell marker genes were scarce. By using two antibodies specific for CgFox12a and CgFox12b, respectively, we can precisely identify granulosa cells, thecal cells, and their precursor cells. When oogonia differentiate into PO, a few of granulosa cells coexpressed CgFox12a and CgFox12b directly attach to meiotic oocytes. Meanwhile, theca cells expressed CgFox12a are first recruited to the edge of cysts with oogonia and PO and then encompass granulosa cells. Interestingly, the somatic cells in the stromal layer have elliptical nuclei with star-like signals of CgFox12a, whereas theca cells are expressed more abundant CgFox12a proteins in their fusiform nuclei. The results suggest that thecal cells recruited from the stromal layer to thecal layer seem to undergo morphological differentiation of nuclei. Along with oocytes grow, granulosa cells proliferate and form a regular and successive monolayer, in which CgFox12b proteins persist abundant and CgFox12a expression decrease. Simultaneously, three subpopulations of thecal cells, expressing CgFox12a, coexpressing CgFox12a/2b, and not expressing CgFox12a/2b cells, recruit from the stromal layer and form successive thecal lay. Thereafter, granulosa cells and theca cells develop two layers of follicular cells (fig. 9A).

The complicated and crucial roles of Foxl2 in ovary development and maintenance have been well documented in mammals (Crisponi et al. 2001; Uhlenhaut et al. 2009; Boulanger et al. 2014) and three teleost fishes (Yang et al. 2017; Zhang et al. 2017; Bertho et al. 2018). Gibel carp is able to reproduce all female offspring by gynogenesis. Thus, we
speculate that foxl2 may act as dominant female-determining transcription factor in unisexual species. In this study, we successfully edited multiple foxl2 homeologs and/or alleles in gibel carp by using CRISPR/Cas9. Although sex of gibel carp is determined by genetic factors, it is also affected by rearing temperature (Li et al. 2016; Li and Gui 2018; Li et al. 2018). Therefore, very low proportion (<3.4%) of males were observed in the WT gynogenetic offspring (figs. 3A and 6A and supplementary fig. S13A, Supplementary Material online). Our results indicate that CgFoxl2a-B deficiency leads to ovary development arrest and complete female to male sex reversal (fig. 3). The phenomenon is different from the defects of premature ovarian failure observed in zebrafish foxl2a<sup>−/−</sup> females (Yang et al. 2017). Along with sex reversal, the testis differentiation-related or androgen-producing genes remarkably increased their expression, but female-biased genes sharply decreased in Cgfoxl2a-B<sup>Δ55/Δ54/Δ25+8</sup> testis (fig. 5),

![Diagram of folliculogenesis and cooperative regulation](image-url)

**Fig. 9.** Schematic diagram of folliculogenesis (A) and cooperative regulation between Cgfoxl2a and Cgfoxl2b (B) in gibel carp ovary development. Different granulosa cells, thecal cells, and their precursor cells are distinguished by expression of CgFoxl2a (green) and CgFoxl2b (red) proteins, respectively. Different types of gonads, including WT ovary, delayed ovary, germ cell-depleted gonad, ovotestis, and testis, different gonadal development-related genes and their upregulation and downregulation expressions, are indicated in detail.
which was consistent with previous studies (Yang et al. 2017; Zhang et al. 2017; Bertho et al. 2018).

In blepharophimosis-ptosis-epicanthus inversus syndrome patients, more than 200 different FOXL2 mutations have been found, which resulted in pleiotropic effects and clinical heterogeneity (Caburet et al. 2012; Verdin and De Baere 2012; Takahashi et al. 2013). In addition, the loss-of-function of one allele lead to FOXL2 haploinsufficiency (Prueitt and Zinn 2001), which emphasize critical role of its correct gene dosage for normal development. Due to the varied mutations in three Foxl2a-B alleles, we also observed the pleiotropy and haploinsufficiency effects of gonad development in this study. When in-frame mutations were produced in all Foxl2a-B alleles and resulted in truncated Cgfoxl2a-B1/B2/B3 protein-lacking FKH domain, the Cgfoxl2a-B1/-/- individuals arrested ovary development at primary oocyte growth stage or developed to males. We also detected two mutated genotypes possessing WT Cgfoxl2a-B1 allele and mutated Cgfoxl2a-B2 and Cgfoxl2a-B3 alleles. However, the WT Cgfoxl2a-B1 protein is not sufficient to keep normal ovarian development, which might be due to very low expression level of Cgfoxl2a-B1 allele in ovary at 45 dph (fig. 2C). Interestingly, about half of Cgfoxl2a-B mutated individuals, which were predicted to possess two truncated Cgfoxl2a-B proteins without FKH domain and one mutated Cgfoxl2a-B protein containing an intact FKH domain, developed to normal females (fig. 3). Subcellular localization showed that the mutated Cgfoxl2a-B proteins with an intact FKH domain, such as Cgfoxl2a-B2A54, keep tranactivation capacity (fig. 5B). Importantly, Cgfoxl2a-B2A54 can enhance SF1-activated Cgcyp19a1a-A expression (fig. 5C) similar to the previous study in Tilapia (Wang et al. 2007). We also found higher expression of mutated Cgfoxl2a-B alleles in ovary than that in testis at oogonia or SPG proliferation stage, which may lead to upregulated Cgcyp19a1as expression and downregulated Cgdmrt1s expression in ovary (fig. 5A). It is commonly considered that cyp19a1a upregulation is essential for triggering fish ovarian differentiation and its downregulation will lead to testicular differentiation (Guignen et al. 2010). Recently, Wu et al. found that cyp19a1a drive ovarian differentiation by suppressing dmrt1 (Wu et al. 2020). Therefore, this is probably the reason why about half of these mutated individuals were females, whereas the other half developed to males. Even though the regulative mechanism of Cgfoxl2a-B2A54 differential expression in gibel carp early gonad development is not clear as of now, our results indicate that correct gene dosage of Foxl2a-B is critical for gibel carp ovary development.

Consistent with abundant CgFoxl2b proteins distributed in oogonia and PO, complete disruption of Cgfoxl2b-A and Cgfoxl2b-B resulted in the depletion of germ cell in gibel carp (figs. 6 and 7 and supplementary table S3, Supplementary Material online). This defect was not observed in the other foxl2 disrupted animals, implying more important roles or other function of foxl2b in gibel carp early oogenesis although we could not exclude the indirect function of foxl2b expressed in somatic cells. The dynamic expression changes of analyzed genes in germ cell depleted gonad were similar to those in testis, but opposite to those in ovary (fig. 8). The results implied that the germ cell-depleted gonad developed testis-like gonads without spermatogenic cells, consistent with our previous study in which germ cells was depleted by dnd morpholino-mediated knockdown approach (Liu et al. 2015). However, the compensatory increase of Cgfoxl2a-Bs transcripts could not rescue the defect of germ cell depletion (fig. 8). Taken together, the detailed cellular localization and function differences indicate that Cgfoxl2a and Cgfoxl2b synergize but differentially govern gibel carp folliculogenesis and gonad differentiation. As shown in figure 9B, Cgfoxl2a is expressed in follicular cells and complete disruption of Cgfoxl2a alleles leads to the upregulated expression of testis differentiation-related and androgen-producing genes, which thereby triggers testis development. Cgfoxl2b evolves a new function in oogenesis. In addition to expression in follicular cells, CgFoxl2b is also localized in oogonia and PO, and complete deficiency of Cgfoxl2b-A and Cgfoxl2b-B alleles results in depletion of germ cells.

In total, we performed an extensive and detailed study on the origin and evolution of three foxl2 homeologs and their alleles in the auto-allo-hexaploid gibel carp. We revealed their functional divergence by editing multiple foxl2 homeologs and/or alleles by using CRISPR/Cas9. In conclusion, the current study covers almost all ranges of complex evolutionary scenarios associated with duplicated genes, including homeolog/allele diversification, retention/loss, biased expression, and sub-/neofunctionalization. Although theoretical presumption of these scenarios and their disparate cases have already been reported, our work has elaborated the evolutionary mechanisms involved in the duplicated foxl2 gene.

Materials and Methods

Maintenance of Fish

Gibel carp clone F was raised and collected from the National Aquatic Biological Resource Center, NABRC. Artificial fertilization, larval hatching, and fry nursing were performed as previously described (Zhu et al. 2018). In order to eliminate the influence of environmental factors on sex, each group consisting of 100–150 WT and 100–150 foxl2 mutated gibel carp was reared in the same cage. All sampled fish were anesthetized with MS-222 (Sigma, USA). All procedures were performed with the approval of the Animal Care and Use Committee of the Institute of Hydrobiology, Chinese Academy of Sciences.

Cloning and Sequence Analysis

According to the genome sequences of gibel carp clone F (CI01000363_00413629_00414546 and ENSONIP00000026093-D2), foxl2a and foxl2b cDNAs were amplified from gibel carp ovary cDNA library by 3’ and 5’ rapid amplification of cDNA ends (supplementary table S1, Supplementary Material online). The complete cDNA sequences of 12 foxl2 transcripts were deposited in GenBank (accession numbers from MT468195 to MT468206). Multiple amino acid sequence alignment and phylogenetic analysis were performed with DNAMAN version 7.0 and Molecular Evolutionary Genetics Analysis (MEGA 7) (Kumar et al. 2016). All the amino acid sequences used in this
study were obtained from GenBank (http://www.ncbi.nlm.nih.gov/, last accessed January 23, 2022). The accession numbers are as following: *Homo sapiens* FOXL2, NP_075555; *Mus musculus* FOXL2, NP_36150; *Gallus gallus* FOXL2, NP_001012630; *Lepisosteus osulatus* FOxl2, XP_006637658; *Latimeria chalumnae* FOxl2, XP_006001344; *Oncorhynchus mykiss* FOxl2b1, NP_001117957; *Oncorhynchus mykiss* FOxl2b2, XP_02437171; *Oreochromis niloticus* FOxl2, NP_001266707; *Ictalurus punctatus* FOxl2a, XP_017350481; *Ictalurus punctatus* FOxl2b, XP_017351917; *D. rerio* FOxl2a, NP_001038717; *D. rerio* FOxl2b, NP_001304690; *Carassius auratus* Fxol2a-B1, MT468189; *Carassius auratus* Fxol2a-B2, MT468190; *Carassius auratus* Fxol2b-A1, MT468191; *Carassius auratus* Fxol2b-A2, MT468192; *Carassius auratus* Fxol2b-A3, MT468193; and *Carassius auratus* Fxol2b-A4, MT468194. The exon–intron structure was determined by the aligning mRNA and genomic sequences of gibel carp *foxl2s*. Syntenic analyses were conducted by comparing the chromosomal regions around *foxl2s* genes in gibel carp chromosomes (Cg*foxl2a-B*, Cg*foxl2a-A*, and Cg*foxl2b-B*) and crucian carp (*C. auratus*) chromosomes (Ca*A2*, Ca*A1*, and Ca*B1*) with corresponding regions in *H. sapiens* chromosome 3, *M. musculus* chromosome 9, *G. gallus* chromosome 9, *Xenopus tropicalis* chromosome 5, and *D. rerio* chromosomes 2 and 15. The latter information was obtained from the Ensembl database (http://www.ensembl.org, last accessed January 23, 2021).

**Quantitative Reverse Transcription PCR**

Tissues, including heart, liver, spleen, kidney, pituitary, hypothalamus, other brains, ovary, and testis, were sampled from 5-month gibel carp females and males. Unfertilized eggs, 50% epiboly, hatching fry, gonads from WT, and *foxl2* mutants at different stages from 10 dpf to 1 year old were randomly sampled. qPCR was performed as described (Yang et al. 2017), and specific primers for each gene were listed in supplementary table S1, Supplementary Material online. Eukaryotic translation elongation factor 1 alpha 1, like 1 (*ef1a1l1*) was selected as the optimal reference gene according to our previous study (Mou et al. 2018). The relative gene expression levels were calculated with $2^{-\Delta\DeltaCT}$ method (Livak and Schmittgen 2001). All the samples were analyzed in triplicates. For statistical analysis, Tukey’s test was calculated with SPSS software (SPSS Inc.). A probability ($P$) of <0.05 was considered statistically significant.

In order to analyze the expression difference among three alleles of each *foxl2* genes, the qPCR products amplified from ovary (45 and 270 dph) and testis (45 and 150 dph) (n = 3) were purified and cloned into Trans5x Chemically Competent Cell. About 20–30 clones of each sample were sequenced and classified according to the specific SNPs in three alleles. The average ratios of sequencing clone numbers were used to show the expression levels.

**Subcellular Localization**

WT *Cgfoxl2a-B2*, *Cgfoxl2a-B2*Δ4, *Cgfoxl2a-B1*Δ5, and *Cgfoxl2a-B3*Δ25 were subcloned into *XhoI*KpnI sites of pEGFP-N3 vector (BD Biosciences Clontech). All the plasmids were verified by sequence analysis. One microgram plasmids were transfected into HEK293T cells. The cell culture, transfection, fixation, and nuclear staining with 4′, 6-diamidino-2-phenylindole (DAPI) were performed as previously described (Lu et al. 2018). The results were acquired by Carl Zeiss upright fluorescence microscope Axio imager M2 (Analytical and Testing Center, IHB, CAS, respectively).

**Plasmid Constructs**

For luciferase assays, expression plasmids were generated by cloning the full length ORF of WT *Cgfoxl2a-B2*, *Cgfoxl2a-B2*Δ4, *Cgfoxl2a-B1*Δ5, and *Cgfoxl2a-B3*Δ25 into KpnI*XhoI* sites of pcDNA3.1(+) vector. *Cgfl1a-B* was subcloned into *NheI*HindIII sites of pcDNA3.1(+) vector. For promoter activity analysis, 1,329 and 1,140 bp 5′-flanking regulatory region of *Cgcyp19α1a-A* promoter (GenBank accession number MW160449) and *Cgcyp19α1a-B* promoter
Transfection and Luciferase Activity Assays

CAb and EPC were seeded overnight in 24-well plates and transfected using FuGENE HD Transfection Reagent (Promega) with various plasmids at a ratio of 10:1 (200 ng expression plasmid: 200 ng Cgcypt19a1a-A pro-luc or Cgcypt19a1a-B pro-luc plasmid: 20 ng Renilla luciferase plasmid pRL-TK). Empty vector (pcDNA3.1) was adjusted to ensure DNA concentration in total among wells. Transient transfections and luciferase activity assays were performed as described (Lu, Zhou, et al. 2020). Luciferase activities were measured by a Junior LB9509 luminometer (Berthold, Pforzheim, Germany) and normalized to that of Renilla luciferase activity. All experiments were performed in triplicate and the significant differences were calculated by SPSS software (SPSS Inc.).

Supplementary Material

Supplementary data are available at Molecular Biology and Evolution online.

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Author Contributions

J.-F.G., L.Z., R.-H.G. designed the study. R.-H.G., Z.L., Z.-X.Y., X.-Y.L., J.-F.T., and X.-J.Z. prepared the samples and carried out the experiments. R.-H.G., L.Z., J.-F.G., and Y.W. analyzed and discussed the results. L.Z., J.-F.G., Y.W., and R.-H.G. wrote the paper.

Data Availability

The DNA sequences have been deposited at National Center for Biotechnology Information (NCBI), accession numbers MW160449, MW160450, and MT468189–MT468206. All other data are available from the corresponding authors upon reasonable request.

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