Mei4 deficiency leads to sexual dimorphism of early meiosis and occurrence of unreduced eggs in zebrafish

Li-Jun Miao[1,2], Li Zhou[1,2], Yan Chen[1,2], Zhi Li[1], Xiao Han[1,2], Fang Peng[1,2], Lei Yang[1,2], Yu-Die Li[1,2], Xiao-Juan Zhang[1,2], Meng Lu[1,2], Zhong-Wei Wang[1,2], Xi-Yin Li[1,2], Jian-Fang Gui[1,2,*] and Yang Wang [1,2,*]

[1] *State Key Laboratory of Breeding Biotechnology and Sustainable Aquaculture, Hubei Hongshan Laboratory, the Innovation Academy of Seed Design, Institute of Hydrobiology, Chinese Academy of Sciences Wuhan 430072, China*

[2] *University of Chinese Academy of Sciences, Beijing, 100049 China*

* Corresponding Authors, E-mail: wangyang@ihb.ac.cn; jfgui@ihb.ac.cn

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ABSTRACT

Polyploids typically arise from meiotic catastrophes, and homologous chromosome mis-segregation, resulting from defects in programmed DNA double-strand breaks (DSBs) formation, is a major cause. Mei4 is known to recruit the Spo11 nuclease onto chromosome axes to catalyze DSB formation in yeast and mice, but its function in fish remains unexplored. Here, we generated *mei4*$^{-/-}$ zebrafish mutants and observed severe defects in synapsis, DSB formation, homologous repair, and crossover during meiosis in both male and female germ cells. Significantly, distinct sexual dimorphism in responses to Mei4 deficiency was observed in meiotic prophase I between males and females. And, *mei4*$^{-/-}$ males were sterile, while females produced a lot of aneuploid oocytes and a few of unreduced eggs due to defective chromosome segregation. Strikingly, a considerable number of viable triploid offspring from *mei4*$^{-/-}$ females × wild type males were produced. Our findings demonstrate the conserved role of Mei4 in DSB formation across vertebrates, provide new insights into sex-specific meiotic behaviors in this vertebrate model, and highlight the critical role of meiotic DSB-based crossover defects in polyploidy occurrence.

Keywords: polyploidy, meiosis, Mei4, DSB, sexual dimorphism, unreduced eggs

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INTRODUCTION

Gamete formation requires faithful segregation of homologous chromosomes that depends on the formation and repair of DNA double-strand breaks (DSBs). DSBs mediate homolog pairing and formation of synaptonemal complex (SC), a ladder-like structure that juxtaposes homologous chromosomes by connecting two lateral elements (LEs) with a central region (CR) for each homologous pair (Gao and Colaiacovo, 2018; Mercier et al., 2015; Zickler and Kleckner, 2015). SCs serve a scaffold for efficient DSB repair that generates at least one crossover (CO) per homologous pair (Hunter, 2015; Zickler and Kleckner, 2015). COs provide physical connections for ensuring the proper segregation of homologous chromosomes at meiosis I, manifesting cytologically as chiasmata (Hunter, 2015; Petronczki et al., 2003; Zickler and Kleckner, 2015). To ensure sufficient but not excessive DSBs to support homolog pairing and recombination, DSB formation is under tight spatiotemporal control in meiosis (Dereli et al., 2021; Keeney et al., 2014; Thacker et al., 2014).

In many eukaryotes, meiotic DSB formation is catalyzed by the topoisomerase-like enzyme SPO11 and its co-factor subunit B of TopoVI DNA topoisomerase-like (TOPOVIBL) (Bergerat et al., 1997; Keeney et al., 1997; Robert et al., 2016; Vrielynck et al., 2016). This process is facilitated by auxiliary pro-DSB proteins, including meiotic double-stranded break formation protein 4 (MEI4) (Bouuaert et al., 2021; Kumar et al., 2010; Kumar et al., 2015; Lam and Keeney, 2015). MEI4 is first identified and studied in yeast, which recruits SPO11 nuclease to catalyze DSB and then induce meiosis recombination (Arora et al., 2004; Keeney, 2001; Maleki et al., 2007; Menees and Roeder, 1989; Menees et al., 1992; Prieler et al., 2005). However, due to weak sequence conservation of Mei4, the homolog in mice is not identified until MEI4 protein is demonstrated to recruit SPO11 (De Muyt et al., 2009; Keeney, 2008; Kumar et al., 2010; Kumar et al., 2015; Lam and Keeney, 2015; Richard et al., 2005). Depletion of Mei4 also results in the failures of meiotic DSB formation and synapsis in mouse, and ultimately

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contributes to spermatogenesis and oogenesis arrests during the early stages of meiotic prophase (Kumar et al., 2010; Kumar et al., 2015). However, its role in fish remains unknown. Although the functions of pro-DSB proteins may be conserved, most of them (e.g. MEI4 and its partner REC114) are weakly conserved in sequences from yeast to plant to mammal, confirming the rapid divergence of "DSB proteins" among eukaryotes (Daccache et al., 2023; De Muyt et al., 2009; Keeney, 2008; Laroussi et al., 2023; Richard et al., 2005).

Polyploids generally arise from the unreduced gametes owing to rare mitotic or meiotic catastrophes (Comai, 2005). In fish, different ploidy polyploids have been identified, but the molecular pathways and cytological mechanisms that drive polyploid formation remain largely unknown (Burgess, 2015; Gui et al., 2022; Ou et al., 2024; Van De Peer et al., 2017). Recently, comparative genome anatomy has revealed that amphitriploid *Carassius gibelio* generates unreduced eggs via an alternative ameiotic pathway, in which a suite of meiosis-related genes might be involved (Gui, 2024; Wang et al., 2022). Here, we chose Mei4 to investigate the functional roles and the resulted consequence in zebrafish by gene editing knockout. We first characterized the early meiosis defects of Mei4 deficiency in synapsis, DSB formation, homologous repair, and crossover, and observed significant sexual dimorphism between males and females. Then, we found that the *mei4*$^{-/-}$ males were sterile, while females produced a lot of aneuploid oocytes and a few of unreduced eggs. Finally, we obtained a considerable number of triploid offspring from the *mei4*$^{-/-}$ females × wild type males.

MATERIALS AND METHODS

Zebrafish (*Danio rerio*) lines and maintenance

Zebrafish (*D. rerio*) strain AB used in this study were cultured in a recirculating water facility at the National Aquatic Biological Resource Center (NABRC). The breeding, maintenance, reproduction and staging of zebrafish were performed according to standard protocols (Kimmel

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et al., 1995). The experimental plan using zebrafish was approved by the Institutional Animal Care and Use Committee of Institute of Hydrobiology, Chinese Academy of Sciences under protocol number 2016-001.

Generation of *mei4* mutant zebrafish

The *mei4* mutant lines were generated by CRISPR-Cas9 mutagenesis protocols as described (Hwang et al., 2013). *mei4* guide RNA (gRNA) was designed using ZiFiT Targeter website (http://zifit.partners.org/ZiFiT/CSquare9Nuclease.aspx), and gRNA was synthesized *in vitro* with the Transcript Aid T7 High-Yield Transcription Kit (Thermo Fisher Scientific, Waltham, MA). The Cas9 mRNA was transcribed using *XbaI*-digested pCS2-Cas9 expression vector and the mMESSAGE mMACHINE T7 ULTRA kit (Ambion). The primers for amplifying *mei4* gRNA template were 5′-GTAATACGACTCACTATAGGAGGATCTGTTGAAGGAGAGTTTTAGAGCTAGAAATAGC-3′ and 5′-AAAAGCACCGACTCGGTGCC-3′.

A mixture of Cas9 mRNA (500 ng/μL) and gRNA (50 ng/μL) was injected into 1- or 2-cell stage zebrafish embryos of the AB wild-type (WT) background. Injected founder fish were outcrossed with WT zebrafish (strain AB), and their offspring were genotyped for mutations. The primers used for genotyping were 5′-TGGTTTGGGATTCAGTAGTGC-3′ and 5′-ATTTGCCTTGGTGATGTTGG-3′. Heterozygous *mei4* knockout fishes were obtained by crossing heterozygous offspring carrying either a Δ2 bp or +20Δ2 bp frameshift mutation in exon 3 (Figure 1B). Homozygous mutants were observed at the expected frequency of 25% (data not shown) and exhibited normal external morphology. Fertilization and hatching rates were assessed as previously described (Brion et al., 2004). *mei4* mutant females were crossed with WT males in the morning. The unfertilized eggs and gastrulae embryos were counted for 6-hour postfertilization (hpf) at 28°C, and then the fry was counted after hatching. Fertilization rate is the number of fertilized embryos divided by the total embryos, and hatching rate is the

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number of hatched larvae divided by the fertilized embryos, in percentage.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from zebrafish testes or ovaries using the SV Total RNA Isolation System (Promega, Madison, WI). Double-stranded cDNA was synthesized using the GoScript Reverse Transcription System (Promega). The RT primers used for amplifying *mei4* were 5′-GCTACTGCTAACCAACCAACAACT-3′ and 5′-GAGGGAAAACTCATCCGCCA-3′, while primers for elongation factor 1α (*ef1α*) (endogenous control) were 5′-AGGCTGACTGTGCTGTGCTGA-3′ and 5′-CCAGGGTGAAAGCCAGGAGG-3′. Each experiment was performed in triplicate and data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Histological analysis and apoptosis assays

Adult gonads from WT and *mei4*$^{-/-}$ zebrafish at 4 months old were fixed in 4% paraformaldehyde for at least 24 hours at 4°C and embedded in paraffin wax. Hematoxylin and eosin (H&E) staining was performed on 4-μm-thick sections following the manufacturer's instructions. Histological analyses and staging of spermatogenesis and oogenesis were conducted as previously described (Figure 2) (Imai et al., 2021; Selman et al., 1993). For gonadal morphometric analysis, the number of all identifiable testicular or ovarian cell types was quantified. Testicular cells were counted across the entire cross-sectional area of seminiferous tubules within randomly selected, non-overlapping fields of view at 400× magnification under light microscopy. Ovarian follicles and interstitial cells were enumerated similarly at 200× magnification. To ensure statistical robustness, each experimental group included a minimum of three biological replicates ($n \geq 3$).

TUNEL staining was performed on 4-μm-thick sections to detect apoptotic cells using the In-Situ Cell Death Detection Kit, Fluorescein (Roche). The number of TUNEL-positive apoptotic cells was counted by cell type across the entire area of view of testis sections at 400× magnification.

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Testis and ovary chromosome spreads

For spermatocyte chromosome spreads, 4–6 intact testes from male zebrafishes (2-3 months old) were freshly dissected following anesthetization. Chromosome spreads were prepared using a modified version of the spread method described by (Blokhina et al., 2019).

For oocyte chromosome spreads, ovaries from approximately 4 female zebrafishes (2 months old) were freshly dissected after anesthetization. The gonads were placed in a 1.5 mL tube containing 60 μL PBS and minced with scissors. The cells were briefly centrifuged, and the supernatant was transferred to a clean 1.5 mL tube. A solution containing three volumes of 75 mM KCl and one-quarter volume of 4% paraformaldehyde was added. The cell suspension was then spread onto slides, air-dried completely, and either stored at -4°C or immediately used for immunostaining.

Immunostaining of spermatocyte/oocyte chromosome spreads

Chromosome spread slides were treated in boiled EDTA antigen retrieval buffer for 20 minutes, then permeabilized with 0.1% Tween 20 and 0.1% Triton X-100 in PBS for 10 minutes. To block nonspecific antibody binding, slides were incubated for 10 minutes at room temperature in 10% ADB (10% goat serum, 3% BSA, 0.05% Triton X-100 in PBS, stored at -20°C) diluted in PBS. The slides were incubated with primary antibodies overnight at 4°C in a humidified chamber. Secondary antibodies and DAPI (Sigma, United States) were applied for 1 hour at 37°C in the dark. All antibodies were diluted in ADB, as listed in Supplementary Table S1. After each incubation, slides were washed three times for 10 minutes each in PBS containing 0.04% Photo-Flo 200 and 0.03% Triton X-100. Finally, the slides were mounted with VECTASHIELD Antifade Mounting Medium (Vector Labs) and imaged using a Leica SP8 confocal microscope (Leica, Germany).

Image quantification

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The number of Sycp1 fragments in all mid-zygotene (-like) and pachytene (-like) spermatocytes was manually counted for each nucleus (Figure 3). Punctate Sycp1 signals were excluded from these counts. Lengths of Sycp3 in spermatocytes and oocytes were measured from traced photographs using ImageJ. To calculate the γH2AX signal-stained area and intensity from binary images, a consistent threshold was applied to all images within the same experiment to eliminate background signals in ImageJ. The total γH2AX fluorescence intensity was calculated as the product of the stained area and signal intensity across all nuclei stages (Supplementary Figure S2).

To quantify Rad51 and RPA2 foci in or near chromosome axes, chromosome axis-proximal regions were defined as within 1 μm of the Sycp3-stained axes, following the criteria used for human DMC1 analysis (Pratto et al., 2014). The total numbers of Rad51 and RPA2 foci per nucleus were counted using ImageJ (Figure 4; Supplementary Figure S3), with the same threshold applied across all images in each experiment to remove background signals. Additionally, Rad51 and RPA2 foci within chromosome axis-proximal regions were manually counted for all nuclei stages. All quantifications were performed within manually selected DAPI-positive areas.

Measurement of DNA content via flow cytometry

The embryo cells were collected from the chopped embryos in gastrula, and the red blood cells were collected from the clipped fin at adults, then mixed with 200 μL CyStain® DNA 1 Step solution (Sysmex Corporation, Japan) at 4°C. The DNA content of embryo and blood samples was measured using the Cytoflex S Flow Cytometer (Beckman, USA). Ploidy levels were determined by comparing the results to WT zebrafish, which served as the control.

Metaphase chromosome spreads

The preparation of metaphase chromosomes of kidney was carried out as described (Zhu and Gui, 2007). And metaphase chromosome spreads of blastocyst embryos were as follows. 4 hpf

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embryos were dechorionated and incubated in colchicine for 60 minutes to arrest cells in metaphase. Following hypotonic treatment with 0.05 nmol/L KCl, the embryos were fixed in a 3:1 methanol:acetic acid solution. Blastocyst cells were then suspended and spread onto glass slides. The slides were mounted and stained with Giemsa.

Statistics

Each experiment was performed at least three times. For biological replicates, a minimum of three zebrafish were analyzed for the same phenotypes. Comparisons of phenotypes were made between WT and mutant zebrafish from the same brood (siblings). The significance of Sycp1 signal counts, Rad51 and RPA2 foci, and γH2AX intensities was assessed using the Mann–Whitney two-tailed test in GraphPad Prism 6 (La Jolla, CA). Graphs were generated to present the results.

RESULTS

Molecular characterization and expression of zebrafish *mei4*

Zebrafish *mei4* (*zmei4*) (Gene ID: 568897) encodes a protein (RefSeq: XP_073789139) with 366 amino acids containing a conserved MEI4 domain (Supplementary Figure S1A). Despite sharing low full-sequence similarities with mammal Mei4 (28.50% and 26.32% identity with the human and mouse orthologs, respectively), zMei4 possesses six conserved short similarity motifs (SSMs) that are found in Mei4 proteins from yeast to mammals (Supplementary Figure S1B) (Kumar et al., 2010). *mei4* is highly expressed in gonads, showing sexual dimorphism with expression levels 11.4-fold higher in testis than in ovary (Figure 1A).

Spermatogenesis is blocked but oogenesis appears to be normal in zebrafish *mei4* mutants

To study the function of Mei4 in zebrafish, we used CRISPR/Cas9 to disrupt the Mei4 domain by targeting a site in the third exon of *mei4* (Figure 1B). Both the $mei4^{\Delta 2/\Delta 2}$ and $mei4^{+20\Delta 2/+20\Delta 2}$ mutations introduced a premature stop codon, resulting in truncated Mei4 proteins that retained

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only the N-terminal SSM1 and SSM2. Owing to the same phenotypes observed in both mutant lines, we chose *mei4*$^{\Delta 2/\Delta 2}$ for following experiments. Both male and female *mei4*$^{-/-}$ zebrafish showed normal body shape (Figure 1C), as well as typical secondary sexual characteristics and sex ratio (data not shown). While mutant testis appeared more transparent than WT testes, mutant ovaries displayed normal morphology.

Meanwhile, we analyzed the histology of testes and ovaries. WT testicular lobules exhibited complete spermatogenesis containing: spermatogonia (SPG, singly distributed basal cells with large ovoid nuclei), primary and secondary spermatocytes (SPC I and SPC II, clustered cells with second and third largest nuclei, respectively), spermatids (SPT, luminal clusters of cells with smallest nuclei) and numerous spermatozoa (SPZ, similar as SPT but devoid of discernible cytoplasm) (Figure 2A). In contrast, SPT and SPZ were completely absent in *mei4*$^{-/-}$ testes, and many SPC exhibited irregularly condensed nucleus (Figure 2A). The enlarged views shows that the nuclei of these cells exhibit a condensed and rod-shaped chromatin state, indicating that the cells are in the metaphase stage (MI: metaphase cell adjacent to SPC I. MII: metaphase cell adjacent to SPC II with samller nuclei than MI) (Figure 2B), consistent with reports in other studies (Feitsma et al., 2007; Ou et al., 2024; Selman et al., 1993). Unlike in the mutants, where all cells within each cyst are in metaphase, in WT, only a small number of cells in each cyst are in metaphase (Figure 2C, right). Unlike missing SPC II in mouse *mei4*$^{-/-}$ testes (Kumar et al., 2010), amounts of SPC II were found in *mei4*$^{-/-}$ testes. And the *mei4* mutants contained a significantly higher number of SPC II (858.0±116.9) compared to WT (338.7±55.8). Moreover, more germ cells in *mei4*$^{-/-}$ testes underwent apoptosis (Figure 2E, F), predominantly affecting spermatocytes (SPC I and SPC II) , as opposed to SPT and SPZ in WT testes.

Different from the testes, *mei4*$^{-/-}$ ovaries displayed similar structure and apoptosis levels as WT ovaries (Figure 2A, D, E). Both ovaries contained a large number of oocytes at all developmental stages, including primary-growth oocytes (PO), previtellogenic oocytes (PVO),

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vitellogenic oocytes (VO), and mature oocytes (MO). These findings suggest that while spermatogenesis is blocked, oogenesis seems relatively unaffected in *mei4* mutants.

Synapsis failure and differential responses in *zmei4*$^{-/-}$ females and males

To further analyze the gametogenesis in *mei4* mutants, we first monitored SC assembly by visualizing its lateral and transverse elements with Sycp3 and Sycp1 antibodies respectively (Hunter, 2015; Zickler and Kleckner, 2015). In WT spermatocytes and oocytes, Sycp3 formed short axis fragments at leptotene (L) (Figure 3A, B). At zygotene (Z), subsequent Sycp1 short fragments were colocalized with Sycp3 axial signals. Synapsed chromosome regions, characterized by colocalization of Sycp1 and Sycp3 signals, were gradually extended as meiosis progressed, ultimately forming 25 distinct, fully synapsed SCs at pachytene (P). However, WT pachytene oocytes had longer SCs compared to WT spermatocytes, consistent with previous studies (Kochakpour and Moens, 2008). The total length of SCs (presented by Sycp3 axis) in males ranged from 155 to 208 μm, while in females it varied between 325 and 738 μm (Figure 3C). The mean length of SCs in pachytene oocytes (19.44±5.11 μm) was 2.71 folds longer than that in pachytene spermatocytes (7.17±0.69 μm). There results indicate that female SCs are not only longer but also show greater variation compared to male SCs.

In *mei4* mutants, Sycp3 loading onto chromosome axis proceeded normally as in WT (Figure 3A, B). Based on the Sycp3 pattern, we staged prophase I of *mei4* mutants into leptotene/early zygotene-like (L/EZ-like), mid to late zygotene-like (MZ-LZ-like), and pre-pachytene-like to pachytene-like (Post-LZ-like), corresponding to those in WT. Sycp1-labeled stretches were largely absent in both *mei4*$^{-/-}$ spermatocytes and oocytes (Figure 3D). In contrast to oocytes, approximately 26% of spermatocytes still contained a few short Sycp1 fragments, either associated with a single Sycp3 axis, located between two Sycp3 axes, or as a single filament (Figure 3D, E, F). Additionally, average Sycp3 axes per cell in *mei4*$^{-/-}$ females are significantly longer than those in WT females, whereas they were almost unchanged in males (Figure 3G).

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This suggests that the longer chromosome axes in females, compared to males, are differently affected by synapsis failure caused by Mei4 deficiency.

Mei4 knockout impairs DSB formation in both females and males

To investigate the function of Mei4 on DSB formation in zebrafish, we examined the DSB formation marker γH2AX (Rogakou et al., 1999; Rogakou et al., 1998) and repair proteins Rad51 and RPA (Dernburg et al., 1998; Tesse et al., 2017). In WT cells, massive γH2AX signals were initially polarized to one side of the nucleus at L-EZ stage (Blokhina et al., 2019; Takemoto et al., 2020), spread throughout the nucleus at MZ-LZ stage, and gradually diminished by P stage (Supplementary Figure S2). Similarly, numerous Rad51 and RPA foci appeared close to or on Sycp3 axes from L-EZ stage, gradually diminishing by P stage as SC formation completed (Figure 4; Supplementary Figure S3).

Sexually dimorphic DSB patterns also exist in meiotic germ cells of zebrafish. In males, 51.16% of the Rad51 foci were concentrated within the first 20% of the chromosomal length near the ends, compared to only 23.91% in females (Figure 4E). Rad51 foci tended to localize in the distal regions of the SCs in males, whereas more evenly distributed along the SCs in females. Furthermore, an average of 1.54 Rad51 foci per SC was found in pachytene spermatocytes, lower than the 1.84 foci per SC observed in pachytene oocytes, and the proportion of female SCs with more than one Rad51 focus (52.00%) was higher than in males (39.29%) (Figure 4F). However, as the SC length in oocytes is longer, the number of Rad51 foci per unit length of SC in pachytene spermatocytes (0.10 foci/μm) was 2.86 times higher than that in pachytene oocytes (0.035 foci/μm).

In *mei4* mutants, Rad51 and RPA signals, as well as the γH2AX signals, were all drastically reduced in both spermatocytes and oocytes (Figure 4; Supplementary Figure S2, S3). These findings indicate that Mei4 depletion severely impairs DSB formation.

Crossover is absent in *mei4*$^{-/-}$ zebrafish

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Considering the defects of DSB formation in *mei4*$^{-/-}$ zebrafish, we next investigated the COs by monitoring Mlh1 (MutL protein homologue 1) (Baker et al., 1996; Hunter and Borts, 1997). In WT, Mlh1 signals appeared on the Sycp3 axis at Z-P stage, and one or occasionally two Mlh1 foci detected on most SCs (Figure 5A). The number of Mlh1 foci per nucleus varied from 6 to 27 in WT pachytene spermatocytes and from 9 to 26 in WT pachytene oocytes (Figure 5B). Consistent with the DSB patterns, females also showed a more equal distribution of COs along the SCs relative to males (Figure 5C). 50.52% of the Mlh1 foci were concentrated within the first 20% of the chromosomal length near the ends in spermatocytes, compared to only 9.52% in oocytes.

As expected, Mlh1 foci were completely absent in *mei4*$^{-/-}$ spermatocytes and oocytes (Figure 5A, B), indicating that no COs were formed without Mei4. No CO was the direct reason of chromosome mis-segregation during meiosis I.

mei4$^{-/-}$ females produce numerous aneuploid eggs and a few of unreduced eggs

Additionally, we examined the fertility of *mei4*$^{-/-}$ mutants. Consistent with the histological analyses (Figure 2A), *mei4*$^{-/-}$ males were able to induce spawning of WT females, but the fertilization failed (Figure 6A). In contrast to male sterility, *mei4*$^{-/-}$ females produced normal-size eggs that could be fertilized by WT sperm. However, these embryos showed high rates of malformation and mortality within the first few days of embryogenesis, with most gradually dying during development. Interestingly, approximately 7.2% of the surviving offspring from the mating between *mei4*$^{-/-}$ females and WT males were able to survive and developed into healthy adults (Figure 6A).

To investigate the reason, we analyzed the DNA content of cells from 30 WT ♀ × WT♂ and *mei4*$^{-/-}$♀ × WT ♂ embryos (Figure 6B). DNA content in embryos from WT ♀ × WT ♂ crosses reveals two distinct peaks, representing uniform ploidy cells in the normal or replicated (2C or 4C) state. In contrast, embryos from *mei4*$^{-/-}$ ♀ × WT♂ crosses show a broad and irregular

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distribution of DNA content, indicating a heterogeneous aneuploid phenomenon. And, we performed karyotype analysis on blastocyst cells derived from matings between WT males and either WT or *mei4*$^{-/-}$ females (Figure 6C). WT embryos predominantly contained the expected 50 chromosomes, whereas the embryos from *mei4*$^{-/-}$ females × WT males displayed a broader range of chromosome numbers from 28 to 75 (Figure 6D). These results indicate that Mei4 deficiency impairs proper chromosome segregation, and produce a lot of aneuploid eggs. Notably, among the analyzed 61 blastocysts, 59 exhibited a chromosome number ranging from 28 to 72, while only two contained exactly 75 chromosomes, indicating that *mei4*$^{-/-}$ females are able to generate unreduced eggs.

Viable offspring are triploids from *mei4*$^{-/-}$ females × WT males

Considering that *mei4*$^{-/-}$ females were able to produce unreduced eggs, we hypothesized that all the surviving offspring from the mating between *mei4*$^{-/-}$ females and WT males would be triploids. To test this, we analyzed the DNA content of blood cells and metaphase chromosomes of kidney cells from the survivors. As shown in Figure 7A, the DNA content of blood cells of all surviving offspring was 1.5 times that of WT. Consistently, all surviving offspring are triploids, in which there are 75 chromosomes (Figure 7B). The surviving offspring exhibited a normal sex ratio in adulthood (Figure 7C). These results demonstrate that *mei4*$^{-/-}$ females can produce viable triploid offspring when mated with WT males.

DISCUSSION

Meiosis, a highly conserved process that generates haploid gametes, requires proper DSB formation to ensure faithful segregation of homologous chromosomes (Subramanian and Hochwagen, 2014; Thacker et al., 2014; Zickler and Kleckner, 2015). The generation of unreduced gametes resulting from a rare meiotic error is widely considered a common pathway driving polyploidy. In this study, we focused on Mei4 to explore its functional significance in

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zebrafish meiosis. Our results not only demonstrate the conserved role of Mei4 in DSB formation across vertebrates but also provide critical insights into sex-specific meiotic behaviors in zebrafish. Notably, while the majority of oocytes in Mei4-deficient females were aneuploid, a small proportion of unreduced eggs were produced, enabling the successful generation of a significant number of triploid offspring through crosses with wild-type males.

In *mei4*$^{-/-}$ zebrafish, DSB formation, synapsis and recombination failed, leading to male sterility due to blocked spermatogenesis (Figure 2-5; Supplementary Figure S2, S3). These findings are consistent with observations in Mei4 mutants of yeast and mice (Kumar et al., 2010; Kumar et al., 2015; Menees et al., 1992). However, unlike the female sterility caused by ovarian arrest in MEI4 and SPO11 mutant mice (Baudat et al., 2000; Blokhina et al., 2019; Kumar et al., 2010), *mei4*$^{-/-}$ and *spo11*$^{-/-}$ females in zebrafish only showed impaired fertility. *mei4*$^{-/-}$ females produced mature oocytes but experienced chromosome mis-segregation, resulting in aneuploid and triploid progeny (Figure 6, 7). These findings suggest that male and female zebrafish respond differently to the same meiotic disturbances. Similarly, evidence from mice also indicates sexual dimorphism in the phenotypic outcomes of meiosis abnormalities, with spermatogenesis exhibiting more stringent quality control mechanisms that lead to developmental arrest or delay (Hunt and Hassold, 2002).

Nevertheless, the relaxed control in oogenesis comes at the cost of reduced gamete quality, as evidenced by an increase of aneuploid gametes (Figure 6). In mice, aneuploid gametes arise from chromosome mis-segregation, which may result from CO failure, premature cohesion loss, and impaired function of the spindle assembly checkpoint (SAC) (Lane and Kauppi, 2019; Santaguida and Amon, 2015). Multiple studies suggest that SAC is less stringent in oocytes than in spermatocytes (Lane and Kauppi, 2019). In yeast, SAC delays anaphase onset to allow correction when chromosome alignment errors occur during meiosis (Barisic et al., 2021). Interestingly, in *Caenorhabditis elegans*, oogenesis is governed by a stricter checkpoint than

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spermatogenesis, with apoptosis activated by meiotic checkpoints in oocytes but blocked in spermatocytes (Bhalla, 2010). These observations highlight sex-specific differences in checkpoint mechanisms or signaling, enabling certain gametocytes to persist despite meiotic disruptions. This phenomenon occurs independently of the conserved functions of genes involved in DSB formation and recombination.

A phenomenon observed in this study, and previously documented in MLH1 and spo11 mutants (Blokhina et al., 2019; Feitsma et al., 2007; Zhang et al., 2020), is that Mei4 deficiency leads to the production of aneuploid oocytes and, notably, a subset of unreduced (diploid) oocytes capable of generating triploid offspring. Without COs, homologous chromosomes lack the physical linkages (chiasmata) necessary for their proper alignment on the metaphase I plate and subsequent accurate segregation during anaphase I. This defect in chromosome pairing and segregation inevitably results in a high frequency of aneuploid gametes. The most extreme manifestation of this asymmetric division failure is the complete failure to extrude a polar body, leading directly to the formation of an unreduced (diploid) oocyte. The occurrence of these relatively rare unreduced oocytes could primarily be a stochastic outcome of the profound chromosome mis-segregation caused by the CO defect, although the involvement of specific regulatory mechanisms in driving this atypical asymmetric division cannot be entirely ruled out.

However, the Mei4-deficient females can produce viable triploid offspring with normal sex ratio, while the offspring from the mating between the *spo11*$^{-/-}$ females × WT males and triploid zebrafish prepared by temperature shock were basically males (Blokhina et al., 2019; Delomas and Dabrowski, 2018; Zhang et al., 2020). Sex determination in zebrafish is typically influenced by multiple factors, including the expression regulation of sex-related genes, insufficient primordial germ cell (PGC) numbers, or masculinization due to oogenesis arrest. Unlike WT triploid zebrafish where oogenesis is arrested, triploid offspring of *mei4*$^{-/-}$ females

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exhibit normal PGC numbers (data not shown) and ovarian development in females, consistent with their normal sex ratio. This suggests that in WT triploids, the presence of three functional *mei4* alleles may provide sufficient gene dosage to disrupt oogenesis via erroneous crossover recombination. Although Mei4 recruits Spo11 to regulate DSB formation, Spo11 may have additional functions during oogenesis. Such dysregulation could lead to aberrant gametogenesis, resulting in either masculinization or differential expression of sex-determining genes.

Similarly as seen in *mei4* mutants, defects in DSB formation, synapsis, recombination and crossover occur during oogenesis in unisexual organisms (Newton et al., 2016). In *C. gibelio*, significant variations are enriched in the genes related to meiosis, which may play a central role in the production of unreduced eggs by inhibiting meiosis I, thereby ensuring the reproductive success of this unisexual polyploid (Wang et al., 2022). This suggests that disruption of meiotic genes, particularly those involved in chromosome segregation based on DSB-mediated crossovers, is likely a critical initial process for neopolyploid formation (Lu et al., 2022; Lu et al., 2023a; Lu et al., 2023b). A majority of aneuploid and a few euploid progenies with diploidy and triploidy are also produced in *cntd1* mutant zebrafish, in which unreduced gametes may arise by chance (Ou et al., 2024). These findings provide direct evidence that defects in meiotic DSB-based crossovers play a critical function in polyploidy occurrence.

Furthermore, uncovering the underlying mechanisms could pave the way for developing methods to reliably produce unreduced oocytes, thereby advancing triploid synthesis techniques and unisexual reproduction breeding (Lu et al., 2023b). Studies in plants have identified some genes critical for polyploidy and led to the development of the MiMe system, in which meiosis is replaced by mitosis, thereby bypassing genetic segregation and producing unreduced gametes (D'erfurth et al., 2009; Khanday et al., 2019; Wang et al., 2024) These genes

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generally involve in key processes of gametogenesis, such as recombination, cell cycle regulation, spindle formation, and chromatid segregation (Chelysheva et al., 2005; D'erfurth et al., 2010; D'erfurth et al., 2008; De Storme and Geelen, 2011; Mercier et al., 2001; Mieulet et al., 2016). Considering that fish naturally exhibit a higher incidence of polyploidy, harnessing meiotic genes to manipulate gametogenesis to form unreduced gametes in fish represents a meaningful attempt for unisexual reproduction breeding in vertebrates (Lu et al., 2024).

In summary, this study uncovers the conserved function of Mei4 in zebrafish meiosis and highlights the sexual dimorphism in meiotic responses to DSB defects. Further investigation into the sexual dimorphism of gametogenesis is valuable for understanding the underlying mechanisms of meiosis and polyploidy.

CONCLUSIONS

This study provides new insights into Mei4's conserved role in vertebrate meiosis, highlighting its function in DNA double-strand break (DSB) formation and its instructive link to polyploidy. Here, we generated *mei4*$^{-/-}$ zebrafish mutants and observed severe meiotic defects, including impaired synapsis, defective DSB formation, and compromised homologous repair and crossover in both male and female germ cells. Notably, we identified a distinct sexual dimorphism in the meiotic response to Mei4 deficiency. While *mei4*$^{-/-}$ males were completely sterile, females produced a large number of aneuploid oocytes and a small proportion of unreduced eggs due to defective chromosome segregation. Strikingly, when *mei4*$^{-/-}$ females were crossed with wild-type males, we obtained a considerable number of viable triploid offspring. Our findings demonstrate the conserved function of Mei4 in DSB formation across vertebrates, provide new insights into sex-specific meiotic regulation, and highlight the critical

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role of meiotic DSB-based crossover defects in polyploidy occurrence.

SUPPLEMENTARY DATA

Supplementary Figure S1 Molecular characterization of Mei4 proteins in eukaryotes.

Supplementary Figure S2 γH2AX foci are impaired in *mei4*$^{-/-}$ zebrafish.

Supplementary Figure S3 RPA2 foci are impaired in *mei4*$^{-/-}$ zebrafish.

Supplementary Table S1 Antibodies used in this study.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

L.J.M. and Y.W. conceived the project. Y.C. and F.P. helped with experiments for immunostaining of spermatocyte/oocyte chromosome spreads. Z.L., X.J.Z. and Y.D.L. helped with the maintenance and generation of *mei4* mutant zebrafish. X.H. and L.Y. contributed to the editing of the figures. L.J.M. and Y.W. drafted the manuscript. L.J.M., Y.W., M.L., L.Z. and J.F.G. finalized the manuscript. All authors read and approved the final version of the manuscript.

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Contributor Information

Li-Jun Miao, Email: miaoli.jun@qq.com

Yang Wang, Email: wangyang@ihb.ac.cn

DATA AVAILABILITY

The data are available within the paper and its supplementary materials.

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Figure legends

Figure 1 Generation of zebrafish *mei4* mutants

A: Expression of zebrafish *mei4* in adult tissues. *ef1α* was used as control. B: Generation of *mei4* mutants using CRISPR/Cas9 strategy. The sequence of gRNA targeting site in the third exon of the zebrafish *mei4* locus is underlined, and the protospacer adjacent motif is marked on green. The conservative Mei4 domain and the frameshift amino acids are highlighted by red box and grey box, respectively. The six conserved SSMs are shown by small boxes. C: Body shape (left) and gonad morphological (right) comparison between adult WT and *mei4*$^{-/-}$. Scale bar: 1 mm.

Figure 2 Characterization of spermatogenesis and oogenesis in zebrafish *mei4* mutants.

A: Histological comparisons of adult gonads in WT and *mei4*$^{-/-}$ zebrafishes. SPG, spermatogonia; SPC I, primary spermatocytes; SPC II, secondary spermatocytes; SPT, spermatids; SPZ, spermatozoa; MI, meiotic metaphase I; MII, meiotic metaphase II. Lumens without spermatozoa and containing arrested spermatocytes with abnormal chromosome segregation are indicated by red asterisks and inside broken lines respectively. PO, primary-growth oocyte; PVO, previtellogenic oocyte; VO, vitellogenic oocyte; MO, mature oocyte. Scale bar: 50 μm. B: Histological comparisons of spermatocytes at MI and MII in WT and *mei4*$^{-/-}$ zebrafishes. Scale bar: 10 μm. C: Statistical analysis of testicular cells composition (left) and meiotic metaphase cells (right) in WT and *mei4*$^{-/-}$ male zebrafishes. D: The proportion of oocytes at every stage in WT and *mei4*$^{-/-}$ female zebrafishes. E: TUNEL staining in adult gonads of WT and *mei4*$^{-/-}$ zebrafishes. Apoptotic cells of SPC I (inside solid lines), SPC II (inside broken lines), SPT+SPZ (arrows) and MI or MII (arrowheads) are shown. Scale bar: 50 μm. F: Statistical analysis of apoptotic testicular cells in WT and *mei4*$^{-/-}$ zebrafishes. Data are present by mean ± SEM. Each group contains three samples for statistical purposes. Differences

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between two groups were determined by unpaired two-tailed Student's t-test. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$; ns, not significant.

Figure 3 SC formation of *mei4*$^{-/-}$ zebrafish

A: Immunofluorescence staining of WT and *mei4*$^{-/-}$ spermatocyte chromosome spreads using anti-Sycp3 (green) and anti-Sycp1 (magenta) antibodies. Spermatocytes are staged based on Sycp3 and Sycp1 staining: leptotene (L); early zygotene (EZ); mid zygotene (MZ); late zygotene (LZ); pachytene (P); leptotene to early zygotene-like (L/EZ-like); mid zygotene-like (MZ-like); pre-pachytene-like to pachytene-like (Post-LZ-like). Enlarged views of the box regions are shown. B: Immunofluorescence staining of WT and *mei4*$^{-/-}$ oocyte chromosome spreads using anti-Sycp3 (green) and anti-Sycp1 (magenta) antibodies. C: The total length of 25 SCs in pachytene spermatocyte and oocyte. Each group containing ten individuals for statistical purposes. D: Proportion of gametocytes with either no Sycp1 or visible Sycp1 stretches at prophase I in WT and *mei4*$^{-/-}$. E: Self-assembly of Sycp1 filaments in *mei4*$^{-/-}$ spermatocytes. Short Sycp1 stretches are shown by white arrows and enlarged views are shown in the bottom. F: Frequency distribution of Sycp1 stretch numbers from mid zygotene to pachytene (-like) spermatocytes with fully condensed axes in WT and *mei4*$^{-/-}$ spermatocytes. G: Average Sycp3 lengths in *mei4*$^{-/-}$ spermatocyte and oocyte during Post-LZ-like. Each group containing at least three cells. Data are present by mean ± SD. Differences between two groups were determined by unpaired two-tailed Student's t-test. **** $P < 0.0001$; ns, not significant.

Figure 4 Rad51 foci are impaired in *mei4*$^{-/-}$ zebrafish

A: Immunofluorescence staining of WT and *mei4*$^{-/-}$ spermatocyte chromosome spreads with anti-Sycp3 (green), anti-Rad51 (red), and anti-Sycp1 (blue) antibodies. B: Quantification of Rad51 fluorescent intensity per nucleus in WT and *mei4*$^{-/-}$ spermatocyte spreads. C: Immunofluorescence staining of WT and *mei4*$^{-/-}$ oocyte chromosome spreads with anti-Sycp3 (green), anti-Rad51 (red), and anti-Sycp1 (blue) antibodies. D: Quantification of Rad51

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fluorescent intensity per nucleus in WT and *mei4*$^{-/-}$ oocyte spreads. E: Comparison of the distribution pattern of DSBs between pachytene spermatocytes (left) and oocytes (right) in WT zebrafish. Each bar chart represents the number of Rad51 found in 5% lengths of SC measured from either end to the middle of the SC. On the x-axis, '0' represents either end of the SC. 43 Rad51 foci in 28 male SCs, 46 Rad51 foci in 25 female SCs were detected. F: A comparison of the frequency of Rad51 foci occurred in a SC between pachytene spermatocytes and oocytes in WT zebrafish. n shows the number of analyzed cells. Data are presented by mean ± SD. Differences between two groups were determined by unpaired two-tailed Student's t-test. $^{**}P < 0.01$; $^{****}P < 0.0001$; ns, not significant.

Figure 5 Crossover defects in *mei4*$^{-/-}$ zebrafish

A: Immunofluorescence staining of WT and *mei4*$^{-/-}$ spermatocyte and oocyte chromosome spreads with anti-Sycp3 (green) and anti-Mlh1 (red) antibodies. B: Quantification of Mlh1 focus numbers per nucleus in WT and *mei4*$^{-/-}$ P/Post-LZ-like spermatocyte and oocyte spreads. C: Comparison of the distribution pattern of crossovers between pachytene spermatocytes (left) and oocytes (right) in WT zebrafish. Each bar chart represents the number of Mlh1 found in 5% lengths of SC measured from either end to the middle of the SC. On the x-axis, '0' represents either end of the SC. 97 Mlh1 foci in 95 male SCs, 105 Mlh1 foci in 93 female SCs were detected. Data are presented by mean ± SD. n shows the number of analyzed spermatocytes and oocytes. Differences between two groups were determined by unpaired two-tailed Student's t-test. $^{****}P < 0.0001$.

Figure 6 Abnormal progenies of *mei4*$^{-/-}$ female zebrafishes

A: The fertilization rate and survival rate of progeny from different breeding combinations. Each group containing more than eight individuals for statistical purposes. B: Histograms of DNA content of embryo cells from WT ♀ × WT ♂ and *mei4*$^{-/-}$ ♀ × WT ♂. Each group containing 30 mixed embryos for statistical purposes. C: Chromosome spreads of blastocyst

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cells. Cells of WT embryos have the normal somatic chromosome number of 50, whereas embryos from mutant females have abnormal chromosome numbers. Scale bar: 20 μm. N shows the number of chromosomes per cell. D: Metaphase chromosome statistics of blastocyst cells. Each group containing more than 50 cells for statistical purposes. Data are presented by mean ± SEM. Differences between two groups were determined by unpaired two-tailed Student's t-test. **** $P < 0.0001$; ns, not significant.

Figure 7 Triploid progeny from *mei4*$^{-/-}$ females

A: Histograms of DNA content of blood cells from WT ♂, *mei4*$^{-/-}$♀ and the healthy offspring of *mei4*$^{-/-}$♀ ×WT ♂. B: Chromosome spreads of kidney cells. Cells of WT have the normal somatic chromosome number of 50, whereas cells from Triploid females have 75 chromosome numbers. Scale bar: 10 μm. n shows the number of chromosomes per chromosome set. C: Sex ratio of the healthy offspring from WT♀ ×WT ♂ and *mei4*$^{-/-}$♀ ×WT ♂. Data are presented by mean ± SD. Differences between two groups were determined by unpaired two-tailed Student's t-test. ns, not significant.

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