Mettl3 Mutation Disrupts Gamete Maturation and Reduces Fertility in Zebrafish

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ABSTRACT N⁶-methyladenosine (m⁶A), catalyzed by Mettl3 methyltransferase, is a highly conserved epigenetic modification in eukaryotic messenger RNA (mRNA). Previous studies have implicated m⁶A modification in multiple biological processes, but the *in vivo* function of m⁶A has been difficult to study, because *mettl3* mutants are embryonic lethal in both mammals and plants. In this study, we have used transcription activator-like effector nucleases and generated viable zygotic *mettl3* mutant, *Zmettl3^{mlm}*, in zebrafish. We find that the oocytes in *Zmettl3^{mlm}* adult females are stalled in early development and the ratio of full-grown stage (FG) follicles is significantly lower than that of wild type. Human chorionic gonadotropin-induced ovarian germinal vesicle breakdown *in vitro* and the numbers of eggs ovulated *in vivo* are both decreased as well, while the defects of oocyte maturation can be rescued by sex hormone *in vitro* and *in vivo*. In *Zmettl3^{mlm}* adult males, we find defects in sperm maturation and sperm motility is significantly reduced. Further study shows that 11-ketotestosterone (11-KT) and 17 β -estradiol (E2) levels are significantly decreased in *Zmettl3^{mlm}*, and defective gamete maturation is accompanied by decreased overall m⁶A modification levels and disrupted expression of genes critical for sex hormone synthesis and gonadotropin signaling in *Zmettl3^{mlm}*. Thus, our study provides the first *in vivo* evidence that loss of Mettl3 leads to failed gamete maturation and significantly reduced fertility in zebrafish. Mettl3 and m⁶A modifications are essential for optimal reproduction in vertebrates.

KEYWORDS *mettl3*; N⁶-methyladenosine; gamete maturation; fertility; zebrafish

TO date, >100 chemical modifications have been identified in eukaryotic RNA. Among these, N⁶-methyladenosine (m⁶A) is the most abundant RNA modification and the first one shown to be reversible in eukaryotic messenger RNA (mRNA) (Desrosiers *et al.* 1974; Fu *et al.* 2014).

A multicomponent methyltransferase complex, including methyltransferase-like 3 (Mettl3) (Bokar *et al.* 1997), methyltransferase-like 14 (Mettl14) (J. Liu *et al.* 2014), Wilms tumor 1-associated protein (Wtap) (Ping *et al.* 2014), and KIAA1429 (Schwartz *et al.* 2014), is responsible for adenosine methylation, while fat mass and obesity-associated

protein (Fto) and alkB homolog 5 (Alkbh5) have been identified as the demethylase for m⁶A (Jia *et al.* 2011; Zheng *et al.* 2013). Additionally, m⁶A can be recognized by multiple RNA-binding proteins, such as the YTH domain family proteins (Ythdf1–3, Ythdc1) and the heterogeneous nuclear ribonucleoprotein (HNRNP) protein families (HNRNPA2B1 and HNRNPC) (X. Wang *et al.* 2014, 2015; Alarcon *et al.* 2015a; Liu *et al.* 2015; Xiao *et al.* 2016). Together, these m⁶A methyltransferases, demethylases, and binding proteins function as “writers,” “erasers,” and “readers” of m⁶A and have implicated m⁶A in a variety of biological processes, such as mRNA metabolism (X. Wang *et al.* 2014, 2015), microRNAs maturation (Alarcon *et al.* 2015b), spermatogenesis (Hsu *et al.* 2017; Xu *et al.* 2017), stemness (Batista *et al.* 2014; Geula *et al.* 2015), circadian rhythm (Fustin *et al.* 2013), and disease (Cui *et al.* 2017).

First identified as a component of the methyltransferase complex, Mettl3 is highly conserved in eukaryotes from yeast to humans (Bokar *et al.* 1997; Yue *et al.* 2015). In *Saccharomyces cerevisiae*, Ime4 (inducer of meiosis 4; homolog of METTL3) has an important role in the initiation of meiosis

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doi: <https://doi.org/10.1534/genetics.117.300574>

Manuscript received June 17, 2017; accepted for publication November 29, 2017; published Early Online December 1, 2017.

Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.117.300574/-/DC1.

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and sporulation. Deletion of *ime4* leads to the loss of m⁶A defects in sporulation (Clancy *et al.* 2002; Schwartz *et al.* 2013). In *Arabidopsis thaliana*, MT-A (mRNA adenosine methylase; homolog of *METTL3*) is mainly expressed in dividing tissues, particularly the reproductive organs. Inactivation of MT-A in *A. thaliana* results in an embryonic lethal phenotype with developmental arrest at the globular stage (Zhong *et al.* 2008; Bodi *et al.* 2012). The *Drosophila melanogaster* homolog of *mettl3*, *Ime4*, is mainly expressed in ovaries and testes, and is required for Notch signaling during oogenesis. Loss of *Ime4* in fly leads to a lethal phenotype (Hongay and Orr-Weaver 2011), but this finding is challenged by recent studies finding that *Drosophila* *Ime4*-null mutants are viable and fertile, though flightless, and m⁶A is required for female-specific alternative splicing of *sxl* (Hausmann *et al.* 2016; Lence *et al.* 2016). In mammals, *Mettl3* affects cell division, differentiation, reprogramming, and spermatogenesis. Knockout of *mettl3* in mouse embryonic stem cells impairs differentiation and *mettl3*^{-/-} mice are embryonic lethal (Y. Wang *et al.* 2014; Chen *et al.* 2015; Geula *et al.* 2015). Recently, Yang's laboratory generated germ cell conditional *mettl3* knockout mice and found that *Mettl3* is essential for spermatogenesis by regulating spermatogonial differentiation and meiosis (Xu *et al.* 2017). Thus, *Mettl3* is clearly indispensable in different organisms, implicating an essential role for m⁶A in embryonic development.

Furthermore, He's laboratory reports that one-third of zebrafish maternal mRNAs are highly methylated at m⁶A, and clearance of m⁶A, mediated by Ythdf2, is critical for the maternal-to-zygotic transition (Zhao *et al.* 2017). Morpholino knockdown of *wtap* and *mettl3* in zebrafish embryos leads to tissue differentiation defects and the expression of somite marker *myod* (Ping *et al.* 2014). Recently, Liu's group demonstrated the critical function of m⁶A modification in the fate determination of hematopoietic stem/progenitor cells during zebrafish embryogenesis (Zhang *et al.* 2017). However, the *in vivo* functions of *Mettl3* and m⁶A function in zebrafish adults remains poorly understood. To elucidate the role of *Mettl3* and m⁶A in zebrafish reproduction, we employed transcription activator-like effector nucleases (TALENs)-mediated genomic editing technique and generated a null allele of *mettl3* (*Zmettl3*^{m/m}). We found that *Zmettl3*^{m/m} zebrafish are viable, in contrast to the lethal phenotype in mammals and plants. Loss of *Mettl3* leads to failed gamete maturation and impaired fertility that are accompanied by altered sex hormone synthesis and gonadotropin signaling. Our study provides the first line of evidence supporting that *Mettl3*-mediated modifications *in vivo* are essential for zebrafish reproduction.

Materials and Methods

Zebrafish maintenance

AB strain zebrafish used in this study were maintained and raised in recirculation systems at 28.5° under an alternating

14 hr:10 hr light/dark cycle. All animal experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals and were approved by the Institute of Hydrobiology, Chinese Academy of Sciences.

Establishment of *mettl3* mutant zebrafish lines

The TALEN target was the first exon of *mettl3*. The half-site length was 17 bp while the position "O" was T, and the spacer length was 15 bp. The pCS2-TALEN-ELD/KKR plasmids for TALENs were constructed as described (Y. Liu *et al.* 2014). The final TALEN plasmids were linearized by *NotI* and transcribed using the mMESAGE mMACHINE Sp6 Kit (Ambion, Austin, TX). TALEN mRNAs (300–500 pg) were microinjected into one-cell stage wild-type (WT) zebrafish embryos. Mutations were confirmed by competitive PCR and sequencing (Luo *et al.* 2015); the primers *mettl3*-F, *mettl3*-R1, and *mettl3*-R2 for PCR are listed in Supplemental Material, Table S1. The injected embryos were raised to adulthood and outcrossed with WT to obtain F1 offspring. The adults of F1 heterozygotes (*mettl3*^{m/+}) were then outcrossed with WT again. F2 heterozygotes with the same mutation were raised to adulthood and self-crossed to obtain F3 zebrafish, which would give rise to zygotic deficiency mutant lines (*Zmettl3*^{m/m}), then the *Zmettl3*^{m/m} self-crossed to obtain maternal and zygotic deficiency mutant lines (MZ*mettl3*^{m/m}). The flowchart for mutant line development is shown in Figure S1 in File S1.

Generation of *Mettl3* overexpressing transgenic zebrafish lines

The *mettl3* sequence was cloned into laboratory stocks of the pSK-GFP (Tol2-CMV-GFP-pA-CMV-MCS-pA-Tol2) vectors to get *Mettl3* overexpression construction (Tol2-CMV-GFP-pA-CMV-*mettl3*-pA-Tol2; Figure S2A in File S1). mRNA encoding Tol2 transposase (100 ng/ml) and Tol2-based *Mettl3* overexpression construction (50 ng/ml) were co-injected into one-cell stage WT zebrafish embryos to generate the *Mettl3* overexpression line (OE-*mettl3*). A flowchart of OE-*mettl3* lines is shown in Figure S2B in File S1. The positive embryos expressed fluorescence (Figure S2C in File S1). We crossed the *Mettl3* overexpression transgenic line (OE-*mettl3*) with the *mettl3* knockout line (*Zmettl3*^{m/m}) to obtain the *mettl3*-specific knockout and ectopic expression lines (OE-KO) to rescue the homozygotes.

RNA isolation and real-time quantitative PCR (qPCR)

Total RNA samples were isolated from the embryos, tissues, FG stage follicles, and pituitaries of WT and *Zmettl3*^{m/m} zebrafish using TRIzol Reagent (Invitrogen, Carlsbad, CA). The amount and purity of the RNA were determined by spectrophotometry and agarose gel electrophoresis. After a treatment with RNase-free DNase (Promega, Madison, WI), the total RNA was reverse-transcribed to complementary DNA (cDNA) using Rever Tra Ace M-MLV (TOYOBO, Osaka, Japan) with random primers. qPCR was carried out on an ABI 7900HT RT-PCR System using 2×SYBR Green mix

Figure 1 The expression pattern of *mettl3* and m⁶A level in zebrafish embryos and adult tissues. (A and B) qPCR analysis for the temporal and spatial expression profile of *mettl3* mRNA in WT embryos (A) and adult tissues (4 months postfertilization) (B). (C and D) Quantification of the m⁶A/A ratio of the total mRNA purified from WT embryos (C) and adult tissues (D) by LC-MS/MS. hpf, hours postfertilization.

(TOYOBO, Japan). The expression level of *mettl3* was normalized to that of β -actin, and the expression levels of target genes in ovaries and testes were normalized to that of the internal control *ef1a*. The gene names and primers used in this study are listed in Table S1.

Quantitative analysis of the m⁶A level using LC-MS/MS

Experiments followed the published procedures in Jia *et al.* (2011). The mRNAs were isolated from WT and *Zmettl3^{m/m}* total RNA using the PolyATtract Isolation Systems (Promega). Two hundred nanograms of mRNA was digested by nuclease P1 (2 U) at 50° for 1 hr, followed by the addition of NH₄HCO₃ (100 mM) and alkaline phosphatase (0.5 U). After incubation at 37° for 1 hr, the solution was diluted to 100 μ L, and 10 μ L of the solution was injected into LC-MS/MS. Nucleosides were separated by high-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry and quantified using the nucleoside to base ion mass transitions of 282.2–150 (m⁶A), and 268.2–136 (A). Quantification was performed in comparison with the standard curve obtained from pure nucleoside standards

running on the same batch of samples. The ratio of m⁶A to A was calculated based on the calculated concentrations.

In situ hybridization

To label the primordial germ cells (PGCs), we used *vasa* as the marker gene (Yoon *et al.* 1997). *In situ* hybridization was performed as described (Thisse and Thisse 2008). To verify the localization of *mettl3* in zebrafish gonads, fluorescent *in situ* hybridization on sections was performed with our published methods (Song *et al.* 2015). Fluorescence photomicrographs were collected using a laser scanning confocal microscope (Zeiss LSM710). Sense RNA probe was used as a negative control. Primers used in *in situ* hybridization can be found in Table S1.

Western blot

For Western blot analysis, tissues from WT and *Zmettl3^{m/m}* fish were lysed by the Total Protein Extraction Kit (Sangon Biotech, Shanghai, China). The lysates were first separated on a 12.5% SDS-PAGE gel and transferred onto PVDF membranes. The separated proteins were immunoblotted with

Figure 2 Localization of *mettl3* expression in the ovary and testis by fluorescent *in situ* hybridization. Cell nuclei from somatic cells are indicated by arrowheads, Leydig cells are indicated by asterisks, and Sertoli cells are indicated by arrows. NC, negative control using sense probe.

rabbit anti-Mettl3 (15073-1-AP; Proteintech) and rabbit anti- β -actin (bs-0061R; Bioss). After incubation overnight at 4°, the membrane was washed in phosphate buffered saline containing Tween-20 for 3×10 min and incubated with an HRP-conjugated anti-rabbit secondary antibody (#7074; Cell Signaling Technology). The signals were detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and visualized using ImageQuant LAS 4000 mini system (GE Healthcare).

Histological analysis and fertility assessment

After MS-222 anesthesia, we dissected the intact gonadal tissues from WT and *Zmettl3^{m/m}* adult zebrafish (4 months postfertilization) and calculated the gonadosomatic index (GSI; gonad weight/body weight $\times 100\%$). The gonad was fixed in 4% PFA (Sigma, St. Louis, MO) overnight at 4° and embedded in paraffin. Then the sections (7 μ m) were stained with hematoxylin and eosin (H&E) and examined under a microscope (Olympus BX53). The staging systems on oogenesis and spermatogenesis were identified as described previously (Selman *et al.* 1993; Shang *et al.* 2006; Leal *et al.* 2009; Q. Wang *et al.* 2015). As described in a previous report (Chu *et al.* 2014; Zhang *et al.* 2015), adult zebrafish (WT and *Zmettl3^{m/m}*) were transferred to a breeding aquaria at the ratio of one male to one female. Each pair of the following were used to assess the fertilization rate: WT male $\times$ WT female; WT male $\times$ *Zmettl3^{m/m}* female; *Zmettl3^{m/m}* male $\times$ WT female; *Zmettl3^{m/m}* male $\times$ *Zmettl3^{m/m}* female; OE-*mettl3* female $\times$ WT male; OE-*mettl3* male $\times$ WT female. The numbers of eggs ovulated

and ovulation rate (ovulation rate = number of spawned females/total number of females $\times 100\%$) were assessed by natural mating of WT, *Zmettl3^{m/m}*, and OE-*mettl3* females with WT males, respectively. If the females did not spawn after mating with males, the experiment was repeated 7 days later. Females were considered sterile after three such failed attempts.

Isolation and incubation of ovarian follicles

After being dissected from WT and *Zmettl3^{m/m}* females, the follicles of different stages were manually separated and divided into five groups based on their size and vitellogenic state: primary growth stage (PG, ~ 0.15 mm); previtellogenic stage (PV, ~ 0.25 mm); early vitellogenic stage (EV, ~ 0.35 mm); midvitellogenic stage (MV, ~ 0.45 mm); full grown stage (FG, ~ 0.65 mm). The incubation of FG stage follicles was carried out according to a previous report (Li *et al.* 2015). Briefly, the cells were incubated with human chorionic gonadotropin (hCG; 100 IU/ml) in medium 199 (Gibco) for 6 hr at 28°, and oocytes were examined microscopically for ooplasmic clearing, which occurs due to proteolytic cleavage of vitellogenin and is indicative of oocyte maturation (Selman *et al.* 1994; Li *et al.* 2015). No hormone was added in the control groups. Groups of four mutants and four controls were assayed in three replicate wells for a total of 24.

Intraperitoneal injection in adult zebrafish

The procedure was performed according to a previous report (Kinkel *et al.* 2010). After anesthetization, the *Zmettl3^{m/m}*

Figure 3 Establishment of *mettl3* knockout mutant lines. (A) Schematic representation of the genomic structures of zebrafish *mettl3* and the target sites of TALENs. Recognition sequences are boxed, and the spacer sequences are between the two black boxes. The start codon of translation ATG is underlined. Deletions and insertions are indicated by dotted line and red letters, respectively. (B) Western blot analysis of whole zebrafish and dissected testes from WT and *mettl3* mutant adults. (C) Quantification of m^6A /A ratio of the total mRNA purified from WT and *mettl3* mutant adult tissues by LC-MS/MS. (Student's *t*-test, *** $P < 0.001$; data are presented as mean $\pm$ SEM.)

females that did not spawn naturally and WT females were weighted, and then injected intraperitoneally with 0.65% isotonic saline (control) or the maturation-inducing hormone $17\alpha,20\beta$ -dihydroxyprogesterone ($17\alpha-20\beta$ -DHP, 5 μ g/g; Cayman Chemical, Ann Arbor, MI). After 4 hr, the ovaries were carefully dissected for quantification of oocyte maturation.

Sperm motility assessments

The sperm motility assessment was performed according to our previous report (Chen *et al.* 2016). Fresh semen samples were obtained by manual extrusion. Thereafter, 1 μ l of the diluted semen was dropped onto a 2X-CEL Chamber slide and analyzed with a CEROS sperm tracker (Hamilton Torne, Beverly, MA). The analysis parameter included: average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF).

Immunofluorescence of synaptonemal complexes

According to the method described previously (Chen *et al.* 2016), the synaptonemal complex was detected in spermatocyte bivalents from WT and *Zmettl3^{m/m}* adult males. The immunofluorescence was stained by anti-SYCP3 and anti-SYCP1 antibodies, which were prepared as described previously (Chen *et al.* 2016).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis

Gonadal tissues were dissected from 4 months postfertilization WT and *Zmettl3^{m/m}* adult fish and embedded in Optimal Cutting Temperature compound (O.C.T. SAKURA). The samples were sectioned at 7 μ m thickness. The TUNEL cell death assay was performed using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions. Images were obtained using a laser scanning confocal microscope (Zeiss LSM710).

Sex steroid measurements

Blood samples were collected from the caudal vein of the WT and *Zmettl3^{m/m}* adults as described by Pedrosa *et al.* (2012). Serum samples were extracted by centrifugation at $3000 \times g$ for 15 min at 4°. 11-keto-testosterone (11-KT) and estradiol- 17β (E2) were measured by competitive enzyme-linked immunosorbent assay kits (Cayman Chemical) following the manufacturer's instructions.

Prediction of m^6A modification sites

Most m^6A sites are found within the consensus sequence RRACH ([G/A/U][G > A] m^6A C[U > A > C]) (Dominissini *et al.* 2012; Meyer *et al.* 2012), so we wrote an in-house script to match RRACH motifs with the mRNA sequences of

Figure 4 Oocyte maturation defects in *Zmettl3^{m/m}* females. (A) Appearance of ovaries dissected from WT and *Zmettl3^{m/m}* females. (B) Gross anatomical appearance of ovaries from WT and *Zmettl3^{m/m}* females. Bar, 5 mm. (C) The GSI scatterplot of WT and *Zmettl3^{m/m}* females ($n = 12$). GSI, gonadosomatic index. (D and E) H&E staining of the ovaries from WT and *Zmettl3^{m/m}* females. PG follicles are indicated by arrowheads, and PV follicles are indicated by arrows. PG, primary growth stage; PV, previtellogenic stage; EV, early vitellogenic stage; MV, midvitellogenic stage; FG, full-grown stage. Bar, 200 μ m. (F) The relative distribution of different stage follicles in the WT and *Zmettl3^{m/m}* females ($n = 12$) (* $P < 0.05$; data are presented as mean $\pm$ SEM).

target genes from the NCBI database (<https://www.ncbi.nlm.nih.gov/>).

Statistical analyses

All data were expressed as mean $\pm$ SEM and analyzed by a Student's *t*-test or a one-way ANOVA using SPSS 18.0 (SPSS, Chicago, IL). Chi-square analysis was used to identify differences in sex ratio between WT, *Zmettl3^{m/m}*, and OE-KO groups. $P < 0.05$ was considered statistically significant. The figures were drawn using Prism 5 GraphPad Software. Results were confirmed in three independent experiments.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Strains used are available upon request. Supplemental Materials contain the Figures S1–S9 in File S1, Table S1, Table S2, and Table S3.

Results

Mettl3 expression and m⁶A levels are high in embryos and gonads

To study the functional role of *mettl3* and m⁶A in zebrafish, we first examined *mettl3* expression and m⁶A level in

embryos and tissues of WT zebrafish. The results show that *mettl3* is a maternally expressed gene. It is highly abundant in the early stages of embryonic development, but dramatically decreased at the 256-cell stage, and is further decreased to $\sim 28.4\%$ in the dome stage (Figure 1A). *Mettl3* mRNA was detected in adult tissues (brain, hypothalamus, eye, heart, liver, spleen, kidney, gill, and gonad), with especially high levels in ovaries and testes (Figure 1B).

Similarly, liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurement showed that high levels of m⁶A persist during the early stages of development with a temporary decrease in 12 and 24 hpf (Figure 1C). In addition, the m⁶A/A ratio was quantified at relatively low levels in liver and muscle, with the higher levels of expression being found in brain, ovaries, and testis (Figure 1D). *In situ* hybridization revealed that *mettl3* was expressed in both germ cells and somatic cells during gonad development (Figure 2). The high expression levels of *mettl3* and m⁶A in embryos and gonads led us to determine their roles in development and reproduction.

Generation of *mettl3* mutant using TALENs

To define the function of *Mettl3* and m⁶A *in vivo*, we targeted the first exon of *mettl3* using TALENs (Figure 3A and Figure S1 in File S1). Upon PCR and sequencing, two independent

Figure 5 Defects of oocyte maturation in mutants can be rescued by sex hormone. (A) Morphology of FG follicles dissected from WT and *Zmettl3^{m/m}* ovaries with incubation of hCG (100 IU/ml) after 6 hr. Follicles undergoing GVBD are marked by red arrows. Bar, 500 μ m. (B) Comparison of the %GVBD in WT and *Zmettl3^{m/m}* in control or hCG treatment. (C) Gross morphology of ovaries dissected from adult zebrafish 4 hr after injection of isotonic saline (control) or 17 α -20 β -DHP. Representative follicles undergoing GVBD are marked by red arrows. Bar, 500 μ m. (D) Quantitative assessment of oocyte maturation induction by injection of 17 α -20 β -DHP. ($n = 12$, one-way ANOVA; data are presented as mean $\pm$ SEM.)

mutant lines were obtained with a 4-bp deletion and 6-bp insertion (−4; +6) or a 10-bp deletion (−10) in the first exon of *mettl3* (Figure 3A and Figure S3A in File S1). The two mutations resulted in an open reading frame-shift and caused a truncated protein. Both truncated proteins lost the catalytic DPPW motifs and S-adenosylmethionine (SAM) binding domain (Figure S3B in File S1). Western blot analysis showed that Mettl3 protein was completely abolished in the mutants (Figure 3B). Using LC-MS/MS, we compared the m⁶A level of mRNA from WT zebrafish and *mettl3*-deficient zebrafish. We observed that the m⁶A/A ratio decreased significantly in brain ($P < 0.001$), liver ($P < 0.001$), muscle ($P < 0.01$), testis ($P < 0.001$), and ovaries ($P < 0.001$) from *mettl3*-deficient zebrafish (Figure 3C). Strikingly, MZ*mettl3^{m/m}* mutant embryos developed poorly at the dome stage (3–4 hpf) and all of them died by 24 hpf (Figure S4 in File S1 and Table S2). We therefore used the *Zmettl3^{m/m}* homozygote line

for subsequent experiments (marked in green, Figure S1 in File S1).

Oocyte maturation is disrupted in *Zmettl3^{m/m}* females

Through morphological and histological analyses, we found the GSI was significantly reduced in the *Zmettl3^{m/m}* (9.2%) compared to WT (16.2%) females (Figure 4, A–C, $P < 0.001$). Most oocytes were arrested in the early development stages of PG, PV, EV, and MV and showed a relatively loose arrangement in ovaries of *Zmettl3^{m/m}* females (Figure 4, D and E). The ratios at the PG, PV, EV, and MV stage had no significant difference with the corresponding stage of WT, but the ratio at FG follicles was significantly lower in *Zmettl3^{m/m}* (13.3%) than that of WT (25.6%) (Figure 4F, $P < 0.05$).

To determine if reproductive defects were related to the disruption of oocyte maturation in mutant females, FG stage

Figure 6 Sperm maturation is affected in *Zmettl3^{m/m}* male. (A) Appearance of ovaries dissected from WT and *Zmettl3^{m/m}* males. (B) Gross morphological appearance of ovaries from WT and *Zmettl3^{m/m}* males. Bar, 5 mm. (C) The GSI scatterplot of WT and *Zmettl3^{m/m}* males. (D and E) H&E staining of testes from WT and *Zmettl3^{m/m}*. SG, spermatogonia; SC, spermatocyte; ST, spermatid; SZ, spermatozoa. SG are marked by arrows. Bar, 100 μ m. (F) The ratio of different stage sperms in the WT and *Zmettl3^{m/m}* males. ($n = 15$, Student's t -test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; data are presented as mean $\pm$ SEM.)

follicles were isolated and treated with hCG *in vitro*. As expected, in the control group without hCG treatment, the percentage of germinal vesicle breakdown is 5.8% in WT follicles, but GVBD did not happen in *Zmettl3^{m/m}* follicles. After incubation with hCG, the percentage of GVBD was significantly increased to 14.5% ($P < 0.05$) in WT and 3.9% ($P < 0.05$) in mutants, respectively, though the % GVBD in mutant follicles is still lower than in WT follicles (Figure 5, A and B). The data indicate that the competency to respond to endogenous hormones was adversely affected in the follicles of *Zmettl3^{m/m}*. To further test the *in vivo* action of steroids on oocyte maturation of *Zmettl3^{m/m}*, we injected the 17α -20 β -DHP into adult zebrafish. In the control group, GVBD did not happen (Figure 5C). However, GVBD was evident in the ovaries of zebrafish injected with 17α -20 β -DHP (Figure 5C). Quantitation of oocyte maturation *in vivo* indicated significant induction of oocyte maturation in the injected *Zmettl3^{m/m}* (47.0%, $P < 0.05$) and WT (81.5%, $P < 0.001$) (Figure 5D). Taken together, these data suggest that oocyte maturation is impaired in *mettl3* mutant fish and the defects of oocyte maturation in mutants can be rescued by hormones *in vitro* and *in vivo*.

Sperm maturation is blocked in *Zmettl3^{m/m}* males

Similar to mutant females, the male GSI was also significantly decreased in *Zmettl3^{m/m}* (0.62%) compared to WT (1.02%) (Figure 6, A–C, $P < 0.001$). Histological examination revealed that the lobular cavities were filled with mature sperm, aligned in a tight and orderly manner in the testes of WT males. In contrast, the lobular cavities were smaller and contained very little or no mature sperm in the testes of the *Zmettl3^{m/m}* males (Figure 6, D and E). Early stages of spermatogenesis, particularly spermatogonia (SG) and spermatocytes (SC), were proportionally 24.4% ($P < 0.05$) and 56.1% ($P < 0.01$) in *Zmettl3^{m/m}* males, respectively. In contrast, the proportions of SG and SC were, respectively, only 7.5 and 26.7% in WT males, while the proportion of spermatozoa (SZ) was 50.1% in WT males and significantly reduced ($P < 0.001$) to 10.4% in *Zmettl3^{m/m}* (Figure 6F). Sperm motility parameters, such as average path velocity (VAP), curvilinear velocity (VCL), and straight-line velocity (VSL), were also significantly reduced ($P < 0.01$) in *Zmettl3^{m/m}* males, compared with WT sperm, but no significant difference was found in the amplitude of lateral head displacement (ALH) and BCF between *Zmettl3^{m/m}* and WT males (Table 1).

Table 1 Sperm activity is significantly decreased in *Zmettl3^{m/m}* males

	VAP (μm/sec)	VCL (μm/sec)	VSL (μm/sec)	ALH (μm)	BCF (Hz)
WT	51.04 ± 2.89	61.38 ± 4.21	37.08 ± 2.37	4.80 ± 0.67	15.04 ± 0.99
<i>Zmettl3^{m/m}</i>	33.1 ± 1.20**	38.18 ± 2.94**	27.75 ± 0.86**	3.07 ± 0.78	15.39 ± 1.96

VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency. *n* = 12, Student's *t*-test, ***P* < 0.01. Data are presented as mean ± SEM.

In addition, we examined the synaptonemal complex in *Zmettl3^{m/m}* and WT spermatocytes using immunofluorescence. Synaptonemal complex protein Sycp1 is a component of the transverse filaments of synaptonemal complexes, which marks the synapsed chromosome regions (Handel and Schimenti 2010), and Sycp3 is a component of the axial/lateral synaptonemal complex elements. We found that both Sycp1 and Sycp3 protein assembled to the synaptonemal complex, similar to WT spermatocytes (Figure 7). In addition, expression levels of several genes involved in meiosis during gametogenesis were not different between *Zmettl3^{m/m}* and WT (Figure S5, A and B in File S1). These data suggest that synapsis is normal in *Zmettl3^{m/m}* spermatocytes, and *mettl3* mutation primarily impairs sperm maturation.

Fertility and sex differentiation are affected in *Zmettl3^{m/m}* adults

Since the maturation of gametes was severely altered, we next assessed natural fertilization rates. We found that

fertilization rate in WT fish was ~91.4%. In contrast, *Zmettl3^{m/m}* females or males outcrossed with the WT adults had the fertilization rate of 42.6% (Figure 8A, *P* < 0.001) and 48.4% (Figure 8A, *P* < 0.001), respectively, and the fertilization rate in self-cross homozygotes was only 33.3% (Figure 8A, *P* < 0.001). The fertilization rate of OE-KO females and males outcrossed with the WT adults was increased to 87.2 and 90.7%, respectively (Figure 8A).

Compared with the 96.7% ovulation rate of WT females, only 51.0% of the *Zmettl3^{m/m}* females were able to spawn naturally (Figure 8B, *P* < 0.001), but 83.3% of OE-KO females ovulated (Figure 8B). Those *Zmettl3^{m/m}* females that spawned naturally only ovulated 158 eggs, whereas WT females ovulated 382 eggs (Figure 8C, *P* < 0.001). Ectopic expression of *Mettl3* in *Zmettl3^{m/m}* increased the number of eggs ovulated to 240 in OE-KO females, indicating a degree of rescue (Figure 8C). To test the fertility of mutant males, we crossed the WT females with the WT, *Zmettl3^{m/m}*, and OE-KO

Figure 7 Synapsis proceeds normally in the *Zmettl3^{m/m}* spermatocytes. Bar, 10 μm (*n* = 12).

Figure 8 Fertility and sex ratios are altered in *Zmettl3^{m/m}* adults. (A) The fertilization rate (fertilization rate = fertilized eggs/total eggs × 100%) of WT, *Zmettl3^{m/m}*, and OE-KO adults. ♀, female, ♂, male ($n = 30$, one-way ANOVA). (B) The ovulation rate (ovulation rate = number of spawned females/total number of females × 100%) of WT, *Zmettl3^{m/m}*, and OE-KO females crossed with WT males ($n = 30$, Student's *t*-test). (C) The numbers of eggs ovulated by WT, *Zmettl3^{m/m}*, and OE-KO females crossed with WT males ($n = 30$, Student's *t*-test). (D) The ovulation rate of WT females crossed with WT, *Zmettl3^{m/m}*, and OE-KO males ($n = 30$, Student's *t*-test). (E) Sex ratio of WT, *Zmettl3^{m/m}*, and OE-KO adults ($n = 120$, one-way ANOVA; data are presented as mean ± SEM).

males. We found 94.4% of WT male × WT female crosses spawned naturally, but only 8.1% of *Zmettl3^{m/m}* male × WT female pairs spawned successfully (Figure 8D, $P < 0.001$). Ovulation rate of WT females increased to 87.5% when crossed with OE-KO males (Figure 8D). The TUNEL assays did not reveal any major apoptotic signals in *Zmettl3^{m/m}* ovaries (Figure S6 in File S1) and testes (Figure S7 in File S1). These data indicate that the fertility of *Zmettl3^{m/m}* females and males is significantly lower than that in WT adults.

Compared with the 1:1 sex ratio of WT adults, the sex ratio was significantly altered in *Zmettl3^{m/m}* adults with ~85.2% being males (Figure 8E, $P < 0.001$). The sex ratio of OE-KO adults approached normal expectations with 44.6% of the OE-KO being males (Figure 8E). The number of *vasa*-positive PGCs had no significant difference between *Zmettl3^{m/m}* and WT embryos (Figure S8A in File S1). The PGC numbers,

quantified at the bud stage by whole-mount *in situ* hybridization for *vasa* expression, were 24.8 for WT and 23.7 for *mettl3* mutants (Figure S8B in File S1), indicating that deficits in PGCs do not account for the male bias. These data suggest that both *mettl3* null females and males show significantly reduced reproduction and loss of *Mettl3* affects sex ratio in the mutants.

***Mettl3* deletion alters expression pattern of sex hormone synthesis and gonadotropin-related genes**

To further analyze the molecular mechanism underlining the fertility defects observed in *mettl3* mutants, the expression levels of a panel of genes (Table S1) involved in gamete maturation and steroidogenesis were assessed in pituitaries, FG stage follicles, and testis from the *Zmettl3^{m/m}* and WT lines by qPCR. Compared with WT adults, *lhβ* mRNA was

Figure 9 Gene expression profiles in WT and *Zmettl3^{m/m}* adults. Expression of genes in pituitaries of females (A) and males (C), FG stage follicles (B), and testes (D) by qPCR. (Student's *t*-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; data are presented as mean $\pm$ SEM.)

significantly lower in the pituitaries of *Zmettl3^{m/m}* females ($P < 0.05$), but there was no difference for *fshβ*, *gh*, *prl* expression level (Figure 9A). For key genes involved in steroidogenesis, oocyte maturation, and ovulation, the expression of *npr*, *igf3*, *star*, *3βhsd*, and *cyp19a1a* was significantly reduced while *fsta* expression was significantly increased in mutant FG stage follicles. In contrast, *acvr2aa*, *acvr2b*, *cyp17a2*, *lhr*, *cyp11a1*, *17βhsd*, *hsd17b3*, *amh*, *cyp26b1*, *esr2a*, *esr2b*, *ptgs2a*, *ptgs2b*, *piwil*, *wt1a*, and *fshr* mRNA levels were normal in *Zmettl3^{m/m}* FG follicles (Figure 9C). Unlike the females, there was a significant decrease in *fshβ* mRNA level in the pituitaries of *Zmettl3^{m/m}* males (Figure 9B,

$P < 0.05$); *Lhβ* expression was also decreased, but this was not statistically significant. There was no difference in the levels of expression of *gh* and *prl* (Figure 9B). Additionally, the expression of *fshr*, *star*, *cyp11a1*, *cyp17a1*, *cyp17a2*, *3βhsd*, and *hsd17b3* was significantly reduced, while *cyp26b1*, *yp19b*, *cyp19a1a*, *17βhsd*, *esr1*, *esr2a*, *esr2b*, and *lhr* mRNA levels were normal in the *Zmettl3^{m/m}* testes (Figure 9D).

Since the expression of genes involved in sex hormone synthesis was changed, we assessed the serum concentrations of sex hormones in adult fish. The results indicate that 11-KT and E2 levels are significantly decreased in *Zmettl3^{m/m}*

Figure 10 Serum 11-KT and E2 level are significantly decreased in *Zmettl3^{m/m}* adults. (A) Serum concentration of 11-KT and E2 in WT and *Zmettl3^{m/m}* females. (B) Serum concentration of 11-KT and E2 in WT and *Zmettl3^{m/m}* males. ($n = 12$, Student's t -test, * $P < 0.05$, *** $P < 0.001$; data are presented as mean $\pm$ SEM.)

females (Figure 10A, $P < 0.05$). Similarly, 11-KT and E2 levels were significantly decreased in *Zmettl3^{m/m}* males (Figure 10B, $P < 0.001$).

To provide supportive evidence for the relationship between m⁶A modification and these differentially expressed genes including *3bhsd*, *cyp11a1*, *cyp17a1*, *cyp17a2*, *cyp19a1a*, *fshb*, *fshr*, *fsta*, *hsd17b3*, *igf3*, *lhb*, *npr*, and *star*, we predicted the probable m⁶A modification site within the consensus sequence RRACH. We found the mRNA sequences of these genes had many of the consensus RRACH ([G/A/U] [G > A] m⁶AC [U > A > C]) (Table S3) and the motifs were mainly located in CDS and 3'UTR Region (Figure S9 in File S1), so these genes are potentially targets for the m⁶A modification.

Discussion

Unlike the lethal effect of *mettl3* knockout in mammals (Geula *et al.* 2015) and plants (Zhong *et al.* 2008; Bodi *et al.* 2012), we obtained viable *Zmettl3^{m/m}*-deficient homozygote zebrafish. In the *mettl3* mutant, we reported that m⁶A methylation levels were significantly lower than in the WT line. The *mettl3* deletion was detrimental to gamete maturation and fertility in zebrafish. This study provides the first *in vivo* evidence that Mettl3, the “writer” of m⁶A modification, plays an important role in regulating reproduction in zebrafish.

The process of oogenesis is divided into three different phases: growth, maturation, and ovulation (Kagawa 2013). Germinal vesicle breakdown is usually regarded as a hallmark of the progress of oocyte maturation. In response to hormonal stimulation, the germinal vesicle (GV) migrates to the animal pole. As maturation proceeds the GV becomes visible under a dissecting microscope and then disappears (Suwa and Yamashita 2007; Nagahama and Yamashita 2008). Compared with WT adults, there was no significant difference in the proportion of the early developmental stages, including PG, PV, EV, and MV stages. However, the number of FG stage follicles was significantly lower in the *Zmettl3^{m/m}* line than in WT female fish, and the % GVBD

and numbers of eggs ovulated were also significantly decreased. The disruption of gamete maturation was successfully rescued by overexpression of *mettl3* in *Zmettl3^{m/m}*, indicating that the deletion of *mettl3* is the cause of the disruption of gamete maturation. These results indicate that the *mettl3* mutants have defects in oocyte maturation.

In female zebrafish, Lh signaling is mainly responsible for stimulating oocyte maturation and ovulation, and Fsh signaling is mainly responsible for promoting follicular growth (Zhang *et al.* 2014; Chu *et al.* 2015). Compared to WT, the pituitaries of *Zmettl3^{m/m}* females expressed lower levels of *lhb* but there was no difference for the *fshb*. Furthermore, the expression of *fsta* was significantly increased, while the *npr* and *igf3* expression were significantly reduced in mutant FG stage follicles. *Fsta* inhibits hCG-induced oocyte maturation (Wu *et al.* 2000; Ge 2005), while the *npr* and *igf3* activate oocyte maturation and ovulation (Li *et al.* 2015; Tang *et al.* 2016). Hence, these results show oocyte maturation and ovulation are blocked in *Zmettl3^{m/m}* adults at multiple steps.

In most teleost fish, three major phases compose spermatogenesis: mitotic proliferation of spermatogonia, meiosis of spermatocytes, and spermiogenesis, which is characterized by the restructuring of spermatids into flagellated spermatozoa (Schulz and Miura 2002; Nobrega *et al.* 2009; Schulz *et al.* 2010). In *S. cerevisiae*, the initiation of meiosis and sporulation are dependent upon m⁶A methylation mediated by *Ime4* (Clancy *et al.* 2002). In mice, the ablation of Mettl3 in germ cells severely inhibited spermatogonial differentiation and blocked the initiation of meiosis (Xu *et al.* 2017), while in *Zmettl3^{m/m}* spermatocytes, the synaptonemal complex can be assembled and synapsis was normal. However, the proportion of SG and SC stages was significantly higher, yet the proportion of SZ stage decreased. In addition, sperm motility was decreased in *Zmettl3^{m/m}* males. Therefore, in the *Zmettl3^{m/m}* males, the decreased levels of m⁶A mediated by Mettl3 do not affect spermatogonial proliferation and the first meiotic division of spermatocytes, but hinder sperm maturation. This is entirely different from the situation in mice (Hsu *et al.* 2017; Xu *et al.* 2017), suggesting Mettl3 and m⁶A

regulate gametogenesis differently in fishes and mammals. In the pituitaries of *Zmttl3^{m/m}* males, there was a significant decrease in the *fsh β* mRNA level. The expression of *fshr* was also significantly reduced in mutant testes. In male zebrafish, *fsh β* plays an important role in sperm maturation, while *lh β* and *Lhr* seem to be less important (Zhang *et al.* 2014; Chu *et al.* 2015). Therefore, the defect in sperm maturation in *Zmttl3^{m/m}* males is correlated to changes in Fsh signaling.

In zebrafish, the sex steroids 11-KT and E2 have significant regulatory effects on germ cells to promote gametogenesis and gamete maturation (Lubzens *et al.* 2010; Schulz *et al.* 2010). Levels of mRNA for the cholesterol transporter *star* and key enzymes in the steroidogenesis pathway such as *3 β hsd* and *Cyp19a1a* were significantly reduced in *Zmttl3^{m/m}* mutant females. Moreover, *star*, *cyp11a1*, *cyp17a1*, *cyp17a2*, *3 β hsd*, and *hsd17 β 3* were all significantly reduced in *Zmttl3^{m/m}* mutant males. Serum concentrations of 11-KT and E2 were also significantly decreased in *Zmttl3^{m/m}* females and males. The disruption of gamete maturation can be successfully rescued by injecting the maturation-inducing hormone 17 α -20 β -DHP in *Zmttl3^{m/m}*. These data show decreased sex hormone levels also contribute to defects in gamete maturation. We also report that the sex ratio is significantly altered with 85.2% of adult *Zmttl3^{m/m}* being male. This was not associated with any changes in PGC numbers in embryos. It is reported that m⁶A can also modulate sex determination by potentiating sex lethal (*Sxl*) alternative pre-mRNA splicing in *Drosophila* (Hausmann *et al.* 2016; Lence *et al.* 2016; Kan *et al.* 2017). In zebrafish, *Mettl3* and m⁶A may have an important role in sexual development by regulating the expression of genes important for sex steroid synthesis.

It is reported that reversible m⁶A methylation can dynamically tune the stability and translation of the target mRNAs by the “erasers” of m⁶A. In the cytosol, Ythdf1 and Ythdf3 act in concert to affect the translation of their targets by facilitating ribosome loading in HeLa cells (X. Wang *et al.* 2015; Li *et al.* 2017), whereas Ythdf2 decreases mRNA stability by recruiting the CCR4-NOT deadenylase complex (X. Wang *et al.* 2014; Du *et al.* 2016). In the nucleus, Ythdc1 influences mRNA splicing (Xiao *et al.* 2016). The differentially expressed genes identified here had many m⁶A consensus motifs that were mainly located in CDS and 3'UTR regions, which is consistent with m⁶A-seq results in human and mouse (Dominissini *et al.* 2012; Meyer *et al.* 2012). We infer that the decreased m⁶A level may lead to differential expression of these reproduction-related genes in *mettl3* mutant adults. Moreover, the m⁶A-binding protein Ythdf2 targets genes with the m⁶A that are involved in phosphorus metabolism, cell cycle processes, and reproduction (Zhao *et al.* 2017), supporting the relationship between m⁶A modifications and control of reproduction in zebrafish.

In summary, our results demonstrate that loss of *Mettl3*, the “writer” for m⁶A modifications, leads to failed gamete maturation and impaired fertility, likely as the result of decreased m⁶A levels and disrupted expression of genes important for

sex hormone synthesis and gonadotropin signaling. We provided further evidence that these genes are likely the targets of m⁶A modifications. In contrast to previous studies in mammals and plants, we provide functional *in vivo* support for the theory that m⁶A is essential for zebrafish reproduction, highlighting a key role for m⁶A modification in gamete maturation.

Acknowledgments

We are grateful to Jie Mei (Huazhong Agricultural University) for providing help to test sperm quality parameters and Zhaolan Zhou (University of Pennsylvania School of Medicine) for his valuable discussion and critical reading of the manuscript. This work was supported financially by the National Natural Science Foundation (grant nos. 31325026, 31721005, and 31461163006) and the Chinese Academy of Sciences (grant nos. XDA08010106 and 2016FBZ03). Funding from the University of Ottawa International Research Acceleration Program (to V.L.T. and W.H.) is acknowledged with appreciation.

Author contributions: WH, HX, and CZ designed the research; HX, CZ, XW, JC, and BT developed methods and performed research; XX and MS contributed bioinformatics analysis; WH and ZZ provided reagents; WH, HX, CZ, ZZ, and VLT analyzed data; WH, HX, and VLT wrote the manuscript.

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Communicating editor: M. Halpern