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Author(s)	Otsuka-Kanazawa, Saori; Ichii, Osamu; Kon, Yasuhiro
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Testicular oocytes in MRL/MpJ mice possess similar morphological, genetic, and functional characteristics to ovarian oocytes

Saori Otsuka-Kanazawa, Osamu Ichii, Yasuhiro Kon

5 *Laboratory of Anatomy, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan*

Corresponding author:

Dr. Saori Otsuka-Kanazawa

10 Laboratory of Anatomy, Graduate School of Veterinary Medicine, Hokkaido University

Kita 18 Nishi 9, Kita-ku, Sapporo 060-0818, Japan

Tel: +81-11-706-5188, Fax: +81-11-706-5189,

E-mail: saori-o@vetmed.hokudai.ac.jp

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## ABSTRACT

In general, mammalian males produce only spermatozoa in their testes and females produce only oocytes in their ovaries. However, newborn MRL/MpJ male mice produce oocytes within their testes. In this study, we examined the initiation and progression of oogenesis in fetal and neonatal MRL/MpJ mouse testes and evaluated the characteristics of testicular oocytes. Germ cells with positive reactions to oogenesis markers such as NOBOX oogenesis homeobox and synaptonemal complex protein 3 were observed in the MRL/MpJ fetal testes on embryonic day 18.5. These fetal testicular oocytes possessed maternal-specific methylation patterns of histone and DNA. The level of DNA methylation was still low in postnatal testicular oocytes at day 14 after birth. Additionally, the postnatal testicular oocytes contained both X and Y chromosomes and had the ability to fuse with sperm. These results suggest that some XY germ cells in fetal testes of MRL/MpJ mice enter meiosis prematurely, undergo oogenesis, and differentiate into oocytes. In addition, MRL/MpJ testicular oocytes have the ability to carry on oogenesis before and shortly after birth until they obtain some of the morphological, epigenetic, and functional characteristics of oocytes.

**KEYWORDS:** DNA methylation, H3K9me2, meiosis, MRL/MpJ mouse, oogenesis, testicular oocytes

## 1. INTRODUCTION

45 Germ cells are highly specialized cells that transmit genomic information to the next generation. They have unique developmental processes because of their ability to differentiate into either oocyte or prospermatogonia. The key factor for sex determination of primordial germ cells is the initiation of meiosis at different time points between males and females (McLaren, 1995). In mice, this phenomenon occurs at embryonic day (E) 13.5, when  
50 ovarian germ cells enter meiosis and testicular germ cells are arrested at the G0/G1 phase of mitosis (McLaren, 1984). After sex determination, ovarian germ cells become oocytes and undergo oogenesis.

Oogenesis consists of many complicated processes, such as meiosis, oocyte maturation, and epigenetic modification (histone modifications and DNA methylation) (summarized in  
55 Supplementary Fig. 1). The oocytes carry out meiotic prophase I until the diplotene stage at birth. During meiotic prophase I, methylation of lysine 9 on histone H3 (H3K9), which is necessary for the proper progression of the meiotic prophase, is completed (Tachibana et al., 2007; Sasaki and Matsui, 2008). After birth, oocytes develop in the collaboration with surrounding follicular epithelial cells (Li and Albertini, 2013). They form the zona pellucida  
60 which works as a sperm-receptor and blocks against polyspermy at the time of fertilization (Wassarman et al., 2004). In addition, oocytes initiate and carry on *de novo* DNA methylation of female-specific imprints which is essential for fetal development of the next generation (Reik and Walter, 2001; Trasler, 2006). The oocytes resume and complete meiosis I at the antral stage just before ovulation in mice. After fertilization with sperm, murine  
65 oocytes continue meiosis II and finally become ovum with 20 single-sets of chromosomes. Thus, the abnormalities in these processes cause failure in the production of functional ovum, fertilization, and embryogenesis.

It is generally believed that mammalian males produce only spermatozoa in their testes

and females produce only oocytes in their ovaries. However, MRL/MpJ mouse strains,  
70 MRL/MpJ-+/+ (MRL) and MRL/MpJ-*lpr/lpr* produce oocytes within their testes  
(Supplementary Fig. 2) (Otsuka et al., 2008a, 2008b, 2012). These testicular oocytes coexist  
in seminiferous tubules with normal spermatogenic cells and possess many characteristics of  
ovarian oocytes, including expression of oocyte-specific genes and morphological features,  
such as cell size, zona pellucida glycoprotein 3 (Zp3)-positive zona pellucidae, and follicular  
75 epithelial-like cells.

However, it is still unknown whether these MRL testicular oocytes are produced via  
oogenesis and meet the requirements for fertilization and further embryonic development.  
Therefore, in the present study, we focus on the initiation and progression of meiosis and  
oogenesis, epigenetic status, and the ability to fuse with the sperm of testicular oocytes in  
80 order to evaluate the potential of MRL testicular oocytes to differentiate into fertile gametes.

## 2. MATERIALS AND METHODS

### 2.1 Mice

Eight-week-old male and female MRL and C57BL/6 (B6) mice purchased from Japan  
85 SLC were maintained with free access to food and water in our facility approved by the  
Association for Assessment and Accreditation of Laboratory Animal Care. In this study,  
newborn mice at days 8-14 after birth (D8-14) and adult F1 mice generated between female  
B6 and male MRL mice (B6MRLF1) obtained by free-breeding were sacrificed by  
detruncation or CO<sub>2</sub> inhalation euthanasia. For embryo collection, timed mating was  
90 established by housing females with males overnight. At noon on the following day, females  
were checked for the presence of vaginal plugs, which denoted pregnancy, and the embryos  
were recorded as being in E0.5 of development.

For the handling of experimental animals, the investigators adhered to the “Guide for

the Care and Use of Laboratory Animals, Hokkaido University, Graduate School of  
95 Veterinary Medicine,” and this project was approved by the Animal Experiment Committee  
of the Graduate School of Veterinary Medicine, Hokkaido University (approval no. 13009)  
and the Animal Experiment Committee of the Hokkaido University (approval no. 13-0032).

### 2.2 Testicular oocyte detection and isolation in postnatal mice

100 To detect for testicular oocytes, fresh whole-mount preparations were used. The testes  
of MRL mice were removed, and immediately mounted on glass slides with cover slips.  
They were then crushed to make whole-mounted single tubular sheets, and were observed  
under a differential interference contrast microscope (BX50F4, Olympus). After observation  
and counting, oocytes or seminiferous tubules containing oocytes in MRL mice were isolated  
105 under a dissection microscope (SZX7, Olympus) with 27 gauge needles for  
immunohistochemical analysis, fluorescence *in situ* hybridization (FISH), and sperm-egg  
fusion assay.

### 2.3 Immunohistochemical analysis

110 Immunohistochemical and immunofluorescence analyses were performed using fetal  
gonads of MRL and B6 mice, and the primary and secondary antibodies listed in  
Supplementary Table 1. Briefly, the gonads of MRL and B6 mice were removed,  
immediately fixed with 4% paraformaldehyde/0.1 M phosphate buffer (PFA) overnight at  
4°C, and cut into 5- $\mu$ m-thick complete serial paraffin sections. The deparaffinized sections  
115 were autoclaved with 10 mM citrate buffer (pH 6.0) for 20 min at 105°C. Some samples  
were then incubated in 3% hydrogen peroxide/methanol solution for 20 min. Sections  
blocked in either normal goat serum, donkey serum, and/or 0.25% casein/PBS were then  
incubated with a primary antibody or mixture of primary antibodies at 4°C overnight. For

immunohistochemistry, the sections were incubated with goat anti-rabbit IgG conjugated  
120 with biotin (SABPO Kit, Nichirei Bioscience) and subsequently with streptavidin-peroxidase  
complex (SABPO Kit) each for 30 min at room temperature. The labeled sections were  
developed using 3,3'-diaminobenzidine-H<sub>2</sub>O<sub>2</sub> solution and counterstained with hematoxylin.  
For immunofluorescence, the sections were treated with the corresponding secondary  
antibodies conjugated with a fluorescent dye. The sections were counterstained with Hoechst  
125 33342 (Dojindo) before being observed under a confocal laser scanning microscope  
(LSM700, Carl Zeiss). After the observation, all serial sections including those used for  
immunofluorescence were stained with hematoxylin and eosin (HE) to measure the  
diameters of some oocytes and nuclei of germ cells.

130

#### *2.4 Histometric analysis*

The diameters of postnatal oocytes and nuclei of fetal germ cells were measured using  
Zeiss LSM Image Browser Version 4.2.0.121 (Carl Zeiss). To measure the diameters of  
postnatal oocytes, all serial sections, including those used for immunofluorescence, were  
135 stained with hematoxylin and eosin. The sections with the maximum cross-section of  
oocytes were then selected, and the diameters were measured.

In order to investigate the number of testicular oocytes per one fetal testis (oocyte  
score) and the percentage of fetal MRL mice with testicular oocytes at E18.5, one quarter of  
5- $\mu$ m-thick complete serial sections (an interval of sections was 20  $\mu$ m) of MRL fetal testis  
140 were immunostained for NOBOX oogenesis homeobox (Nobox), and the testes and mice  
with immunopositive cells were counted. Since Nobox was immunolocalized in nuclei with  
diameters of approximately 10  $\mu$ m, the oocyte scores of fetal mice were calculated with the  
following equation: number of oocytes detected/number of testes observed  $\times$  diameter of

nuclei of germ cells/thickness of paraffin section. For example, if 100 testicular oocytes were  
145 observed in 25 testes, oocyte scores would be 8.0 (100/25×10/5). The postnatal testicular  
oocytes were detected using whole-mount specimens of testes at D14.

## 2.5 RT-PCR and quantitative real-time PCR

To examine the expression of *Nobox*, total RNA was obtained from the gonads of MRL  
150 and B6 mice at 18.5 using TRIzol reagent (Life Technologies) according to the  
manufacturer's instructions and treated with DNase for DNA digestion (Nippon Gene).  
Complementary DNA was then synthesized with ReverTra Ace (Toyobo) and Oligo-dT  
primers (Life Technologies). PCR was carried out with ExTaq (Takara) under the following  
conditions: 5 min at 95°C, 35 cycles of 40 sec at 95°C, 30 sec at 60°C, and 1 min at 72°C,  
155 followed by 5 min at 72°C. The PCR primer sequences of *Nobox* were 5'-  
CATGAAGGGGACCTGAAGAA-3' (forward) and 5'- GGAAATCTCATGGCGTTTGT-3'  
(reverse) and amplified product was 153bp in size. The quantitative real-time PCR analysis  
was performed using the Brilliant III SYBR Green QPCR Master Mix and a real-time  
thermal cycler (MX 3000P; Agilent Technologies, Santa Clara, CA, USA), according to the  
160 manufacturer's instructions. The mRNA expression level of *Nobox* was normalized against  
that of a germ cell marker, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*Ddx4*). The PCR  
primer sequences of *Ddx4* were 5'- TGGGTTTTGGACCAGAGATG -3' (forward) and 5'-  
CCCAACAGCGACAAACAAG -3' (reverse).

## 165 2.6 FISH for sex chromosomes

To determine the constitution of sex chromosomes of testicular oocytes, Cy5 labeled X  
chromosome and Cy3 labeled Y chromosome probes were purchased from Chromosome  
Science Labo Inc. The ovaries and seminiferous tubules containing oocytes obtained from

MRL mice at D8 were removed and immediately fixed with 4% PFA overnight at 4°C, cut  
170 into 5-µm-thick complete serial paraffin sections. The deparaffinized sections were  
incubated with 2× sodium chloride/sodium citrate (SSC) for 5 min and heated in a  
microwave for 10 min. Then the slides were treated with 0.02% pepsin/0.1N HCl for 1 min  
at 37°C, dehydrated with ethanol, and incubated with a mixture of probes for 10 min at 90°C  
followed by 37°C overnight. The next morning, the slides were washed in 50%  
175 formamide/2×SSC for 20 min at 37°C and 1×SSC for 15 min, counterstained with DAPI for  
5 min, and finally observed under a laser microscope (LEICA DC350FX, Leica) with CW  
40000 FISH application program (Leica).

### *2.7 Sperm-oocyte fusion assay*

180 The sperm-egg fusion assay was performed as described previously (Conover and  
Gwatkin, 1998). Briefly, fresh sperm from the cauda epididymis of an adult B6MRLF1 male  
mouse were dispersed in 0.2 mL drops of TYH medium (Mitsubishi Chemical Medience  
Co.) and incubated for 1 h to induce capacitation. The isolated testicular oocytes and ovarian  
oocytes from MRL mice at D12 were treated with acidic Tyrode's solution (Sigma-Aldrich)  
185 for 30 sec to remove the zona pellucida, washed three times with M2 medium (Life  
Technologies), and incubated with M2 medium for 2 h to allow the surface proteins to  
recover. Then the oocytes were incubated with 1 µg/mL Hoechst 33342 for 30 min at 37°C  
in 95% air/5% CO<sub>2</sub> and washed three times with TYH medium. The dye-loaded oocytes  
were inseminated with the sperm and fixed with 2% PFA. The transfer of Hoechst 33342  
190 from dye-preloaded oocytes to sperm was observed under UV excitation light with the  
U-MWU filter set on a confocal laser scanning microscope (Fluoview FV500, Olympus).

### *2.8 Statistical analysis*

The experimental values for quantitative real-time PCR and The oocyte scores were  
195 statistically compared using one-sided Student's *t*-tests using PASW version 18 (IBM). The  
oocyte scores were statistically compared using the non-parametric Mann-Whitney *U*-test  
using PASW version 18 (IBM). Statistical significance was set at  $P < 0.05$ .

### 3. RESULTS

#### 200 3.1 Initiation of meiosis and oogenesis within fetal MRL testes

In our previous study, we found meiotic germ cells in MRL fetal testis at E13.5-18.5  
(Otsuka et al., 2008a); however, we did not have direct evidence that MRL fetal testes  
actually carry out oogenesis. In order to examine whether these meiotic germ cells were  
future testicular oocytes, an oocyte marker, *Nobox*, was detected using RT-PCR. mRNA  
205 expression of *Nobox* was detected in MRL fetal ovaries and testes at E18.5, but weakly in  
the testes of age-matched B6 mice (Fig. 1A). Quantitative real-time PCR revealed that the  
*Nobox* gene expression level was approximately 2-fold higher in MRL mouse testes than in  
B6 mouse testes at E18.5 (Fig. 1B). Additionally, immunohistochemistry for a meiotic  
marker, synaptonemal complex protein 3 (Sycp3), and *Nobox* revealed the existence of germ  
210 cells with positive reactions to both proteins, fetal testicular oocytes, in MRL testes at E18.5  
(Fig. 1C, lower panels and D, middle panels). On the other hand, there were no Sycp3 or  
*Nobox* positive cells observed in B6 testes at E18.5 (Fig. 1D, lowest panels).

We then calculated the oocyte score and the percentage of fetal MRL mice with  
testicular oocytes at E18.5 and compared these values to those of postnatal MRL mice at  
215 D14. As a result, the oocyte score of fetal MRL mice was 14.85 at E18.5, which was  
significantly higher than that of postnatal MRL mice at D14, 1.28 (Table 1). In addition,  
testicular oocytes were observed in all of the fetal MRL mice investigated, and in 61% of  
MRL mice at D14 (94 out of 154 mice) (Table 1). Thus, some primordial germ cells in MRL

fetal testes, which entered meiosis prematurely, differentiated into oocytes; however, their  
220 number drastically decreased during the perinatal period.

### 3.2 H3K9 dimethylation of fetal testicular oocytes in MRL mice

The epigenetics of fetal testicular oocytes was examined by immunofluorescence. Recent studies reported the importance of histone methylation, especially mono-, di-, and  
225 tri-methylation of H3K9, during early meiotic progression (Peters et al., 2001; Tachibana et al., 2007) (Supplementary Fig. 1). In the present study, the level of histone methylation was evaluated by the detection of dimethylation of H3K9 (H3K9me2) in MRL testicular oocytes at E16.5 due to not only its importance in early meiosis, but also its sex-dependent manner of methylation at this stage (Abe et al., 2011). The previous studies reported that the level of  
230 H3K9me2 was low in spermatogenic cells compared to the oocytes during fetal life after sex determination (Suzuki and Saga, 2008; Deguchi et al., 2013). Further, TRA98 and DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (Dmc1) were used for markers of germ cells and meiotic cells, respectively. Therefore, TRA98<sup>+</sup>/Dmc1<sup>+</sup> cells are considered as meiotic oocytes in fetal gonads. A positive reaction  
235 of TRA98, Dmc1, or H3K9me2 was observed in the nuclei of germ cells or somatic cells (Fig. 2). In MRL ovaries, TRA98<sup>+</sup>/Dmc1<sup>+</sup> ovarian oocytes showed a positive reaction to H3K9me2 (Fig. 2, upper panels). In MRL testes, TRA98-positive reactions were observed in the nuclei of germ cells (Fig. 2, middle panels). TRA98<sup>-</sup>/Dmc1<sup>-</sup> somatic cells were positive for H3K9me2 (Fig. 2, middle panels). In addition, we found TRA98<sup>+</sup>/Dmc1<sup>+</sup> cells, i.e.  
240 testicular oocytes, with a positive reaction to H3K9me2, showed signal intensity comparable to that of ovarian oocytes (Fig. 2, middle panels, indicated by an arrow). Importantly, TRA98<sup>+</sup>/Dmc1<sup>-</sup> spermatogenic cells were negative to H3K9me2 (Fig. 2, middle panels). In B6 testes at E16.5, no cells showed positive reaction to Dmc1. Additionally, in B6 testes, the

TRA98<sup>-</sup>/Dmc1<sup>-</sup> somatic cells were positive for H3K9me2, whereas the TRA98<sup>+</sup>/Dmc1<sup>-</sup> spermatogenic cells showed a negative reaction to H3K9me2 similar to those in MRL testes (Fig. 2, lowest panels).

### 3.3 DNA methylation status of fetal and postnatal testicular oocytes in MRL mice

First, we examined the DNA methylation status of fetal testicular oocytes. DNA methylation in murine primordial germ cells is erased by E12.5, and sex-specific genomic imprinting is reestablished according to the sex of germ cells under the regulation of DNA methyltransferases (Dnmt), Dnmt3a, Dnmt3b, and Dnmt3l (Hajkova et al., 2002; Kaneda et al. 2004; Sasaki and Matusi, 2008). The oocytes keep their DNA methylation level low after the onset of meiosis, while spermatogenic cells initiate *de novo* DNA methylation of male-specific imprints at E15.5 and complete around birth during mitotic arrest (Li et al., 2004; Sasaki and Matusi, 2008) (Supplementary Fig. 1). To examine the DNA methylation status of fetal testicular oocytes, double immunofluorescence was performed using an oocyte marker, Nobox, and DNA methylation indicators, Dnmt3a or 5-methylcytosine (5-Mec). In MRL ovaries at E18.5, ovarian oocytes identified by Nobox showed a negative reaction to both Dnmt3a and 5-Mec, while surrounding somatic cells showed positive reactions to both DNA methylation markers (Fig. 3A and B, upper panels). In MRL testis at E18.5, testicular oocytes identified by Nobox showed negative reactions to both Dnmt3a and 5-Mec, whereas Nobox<sup>-</sup> spermatogenic cells were immunopositive to both DNA methylation markers (Fig. 3A and B, middle panels). There were no Nobox<sup>+</sup> cells in B6 testes at E18.5 (Fig. 3A and B, lowest panels).

Next, the DNA methylation status of postnatal testicular oocytes at D14 was examined in MRL mice. The oocytes reestablish female-specific imprints after birth, during the oocyte growth phase (Lucifero et al., 2004; Hiura et al., 2006; Sasaki and Matsui, 2008)

(Supplementary Fig. 1). In MRL ovaries at D14, a few oocytes and surrounding somatic cells  
270 showed Dnmt3a-positive reactions in their nuclei (Fig. 4A, upper panels). Interestingly,  
Dnmt3a<sup>+</sup> oocytes had diameters of more than 50  $\mu\text{m}$  in the secondary or tertiary follicles,  
while Dnmt3a<sup>-</sup> oocytes had diameters of less than 50  $\mu\text{m}$  in the primordial, primary, and  
secondary follicles (Fig. 4A, upper panels). The ovarian somatic cells showed 5-Mec<sup>+</sup> nuclei;  
however, 5-Mec<sup>+</sup> positive ovarian oocytes were seldom observed (Fig. 4A, lower panels).  
275 The Nobox<sup>+</sup> postnatal testicular oocytes were negative for both of the DNA methylation  
markers (Fig. 4B). Importantly, the diameters of testicular oocytes were less than 50  $\mu\text{m}$  at  
D14.

#### *3.4 Sex chromosome constitution of testicular oocyte in MRL mice*

280 Based on the initiation of meiosis and oogenesis in MRL fetal testes, the testicular  
oocytes were speculated to derive from primordial germ cells containing X and Y  
chromosomes. Additionally, sex chromosomes in primary spermatocytes are known to settle  
in different secondary spermatocytes, therefore the constitution of sex chromosomes can  
indicate the meiotic progression of testicular oocytes. To confirm these hypotheses, the  
285 constitution of sex chromosomes in postnatal testicular oocytes was examined by FISH in  
MRL testes at D8. FISH analysis revealed that ovarian oocytes possessed only X  
chromosomes (Fig. 5, left panel), while testicular oocytes contained both X and Y  
chromosomes (red signal: X; yellow signal: Y) similar to other surrounding somatic cells  
(Fig. 5, right panel).

290

#### *3.5 Ability of testicular oocytes to fuse with sperm in MRL mice*

In the sperm-egg fusion assay, sperm invaded oocytes during pre-loading  
incorporating the fluorochrome, Hoechst 33342, and fluoresced upon fusion with the oocyte

plasma membrane (Conover and Gwatkin, 1998). Because of this assay, fluorescent sperm  
295 were observed in both MRL ovarian and testicular oocytes at D12 (Fig. 5B, left panels,  
arrows). Under a differential interference contrast microscope, there were numerous sperm  
surrounding MRL ovarian and testicular oocytes (Fig. 5B, right panels).

#### 4. DISCUSSION

##### 300 *4.1 Testicular oocytes are derived from XY primordial germ cells via meiosis and oogenesis in MRL mice*

One of the first steps of the female pathway in mammalian germ cells is to initiate  
meiosis during the embryonic period. Based on our immunohistochemistry results, we  
confirmed the existence of Sycp3<sup>+</sup> meiotic germ cells within the testes of MRL mice. It has  
305 been reported that the initiation of meiosis is regulated by retinoic acid produced in the  
mesonephros, while the prevention of meiosis is controlled by both somatic and germ cell  
factors in the fetal testis (Bowles et al., 2006; Koubova et al., 2006). One factor is sex  
differentiation of somatic cells. When partially feminized XY embryonic gonads are  
observed, meiotic germ cells are localized in the ovarian region with feminized somatic cells  
310 in ovotestes (Menke et al., 2003). The production of cytochrome P450, family 26, subfamily  
b, polypeptide 1 (Cyp26b1), an enzyme involved in retinoid metabolism, is another somatic  
cell factor, since germ cells in *Cyp26b1* knockout mouse testes enter meiosis during the fetal  
period (Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007). Nanos homolog 2  
(*Drosophila*) (*Nanos2*) is a germ cell factor that suppresses meiosis, and germ cells in  
315 *Nanos2* knockout mouse testes also enter meiosis during the fetal period (Suzuki and Saga,  
2008). Our previous report revealed that meiotic germ cells are concentrated in the cranial  
region in MRL fetal testes. The cranial part of the testis is the region in which  
masculinization of somatic cells occurs last, and the meiotic wave of germ cells is first

initiated (Menke et al., 2003). However, the meiotic germ cells in *Nanos2* knockout mice  
320 spread widely within the testes (Suzuki and Saga, 2008). Therefore, although further  
research is necessary to reveal the mechanism of testicular oocyte production, we assume  
that some type of disturbance in somatic cell factors is involved in this phenotype rather than  
a disturbance in *Nanos2* expression. Interestingly, the meiotic germ cells in the fetal testes of  
both *Cyp26b1* knockout mice and *Nanos2* knockout mice disappear via apoptosis during the  
325 fetal period (MacLean et al., 2007; Suzuki and Saga, 2008). However, in the MRL fetal testis,  
the meiotic germ cells survived and subsequently expressed an oocyte marker, Nobox, by  
E18.5, which means they successfully differentiated into fetal oocytes. These findings  
indicated that the MRL fetal testis is a unique environment which permit female pathway of  
germ cell differentiation.

330 Although only 61% of MRL mice at D14 had testicular oocytes within their testes, these  
fetal testicular oocytes were observed in all examined MRL mice. We have previously  
reported a peak in the appearance of testicular oocytes at D14 (Otsuka et al., 2008a);  
however, continuous observations have revealed that the oocyte score in postnatal MRL  
mice is stable at around 1 between D0 and D16. In the present study, the oocyte score in fetal  
335 MRL mice at E18.5 was approximately 15. Furthermore, we demonstrated that the testicular  
oocytes at D8 contained both X and Y chromosomes by FISH analysis. These findings  
indicate that the testicular oocytes detected in newborn MRL mice originated from XY  
primordial germ cells and differentiated via the female pathway during fetal life. However,  
many of the testicular oocytes are thought to be eliminated during the transition from  
340 prenatal to postnatal life.

The existence of both X and Y chromosomes in the testicular oocytes in MRL mice at  
D8 also indicates that they pursued the female process through the embryonic period after  
entering meiosis and stayed within the diplotene stage similar to ovarian oocytes.

Additionally, it revealed that they had not completed the first meiotic division yet by D8.  
345 Since normal spermatogenic cells, which undergo spermatogenesis and mature as sperm with  
fertility coexist with testicular oocytes, MRL mice have the ability to carry out both  
spermatogenesis and oogenesis at the same time. However, some of the testicular oocytes in  
the later period, after D16, were seen to have two nuclei because of the resumption of  
meiosis, a nuclear division in an oocyte without subsequent cytokinesis (Otsuka et al.,  
350 2008a). Therefore, it is suggested that although testicular oocytes undergo the female  
pathway from fetal to neonatal life, they can no longer carry out normal meiosis within the  
postnatal testicular environment.

#### 4.2 Testicular oocytes possess maternal patterns of H3K9me2 and DNA methylation in MRL 355 mice

The H3K9 dimethylation is sex-differentially regulated and plays important roles during  
the meiotic prophase (Tachibana et al., 2007; Suzuki and Saga, 2008; Deguchi et al., 2013).  
In oocytes, dimethylation of H3K9 is completed by the early leptotene stage (Tachibana et  
al., 2007). In the present study, the testicular oocytes in MRL mice at E16.5 successfully  
360 obtain the oocyte-pattern of H3K9 dimethylation, but not the spermatogenic cell-pattern  
during meiotic prophase I. This result suggests that H3K9 dimethylation in germ cells is  
independent of its sex chromosome constitution or surrounding environment.

Germ cells reestablish DNA methylation on imprinted genes under a sex-specific  
manner during gametogenesis. After the erasure of parental imprints by E12.5, DNA  
365 methylation in oocytes is kept at a low level until after birth (Hajkova et al., 2002; Sasaki  
and Matsui, 2008). In the present study, fetal testicular oocytes at E18.5 showed low levels  
of methylated DNA that were similar to those of ovarian oocytes at the same age. The  
reestablishment of DNA methylation in oocytes gradually progresses during follicular

development, and its progression is correlated with the size of oocytes (Hiura et al., 2006). In  
370 the present study, the testicular oocytes at D14 had diameters of less than 50  $\mu\text{m}$  and were  
surrounded by 1 or 2 layers of granulosa cells. Morphologically, these testicular oocytes  
resembled the ovarian oocytes within primary or early secondary follicles. In addition, low  
DNA methylation was observed in testicular oocytes and ovarian oocytes with diameters of  
less than 50  $\mu\text{m}$  within the secondary, primary, and primordial follicles. Thus, not only  
375 morphology but also DNA methylation status of testicular oocytes at D14 is similar to  
ovarian oocytes within primary to early secondary follicles at the same age.

Durcova-Hills et al. and Isotani et al. reported that XY ovarian oocytes and XX  
testicular oocytes carry out maternal imprinting, respectively (Durcova-Hills et al., 2006;  
Isotani et al., 2005). The DNA methylation status of both fetal and postnatal XY testicular  
380 oocytes in MRL mice revealed that oocytes still have the ability to reestablish maternal  
imprinting even though they carry XY sex chromosomes and settle in a testicular  
environment. However, due to the low appearance rate of testicular oocytes in MRL mice,  
we were unable to analyze the methylation status of each imprinting gene. In addition, since  
the testicular oocytes start to degenerate and disappear after a few weeks of birth, we cannot  
385 deny the possible effect of degeneration on DNA methylation. Therefore, further research is  
needed to confirm whether testicular oocytes have the ability to accomplish maternal  
patterns of DNA methylation.

#### *4.3 Testicular oocytes have the ability to fuse with sperm in MRL mice*

390 The sperm-egg fusion assay revealed the ability of testicular oocytes to fuse with sperm.  
Although, during normal fertilization, the oocyte will not meet the sperm until ovulation of  
an 80- $\mu\text{m}$ -diameter oocyte that is arrested in metaphase II, oocytes with diameters of  
approximately 20  $\mu\text{m}$ , which are still arrested in the prophase of the first meiotic division,

can fuse with sperm (Zuccotti et al., 1994). The molecular mechanisms of the interactions  
395 between the sperm and oocyte plasma membranes are not well understood yet; however,  
some essential proteins on the oocyte membrane have been identified, such as Zp3, CD9  
antigen, folate receptor 4, and phosphatidylinositol glycan anchor biosynthesis, class A  
(Chen et al., 1999; Miyado et al., 2000; Alfieri et al., 2003; Bianchi et al., 2014). The success  
of the insemination showed that testicular oocytes are recognized by sperm as an oocyte and  
400 contained all factors essential for membrane fusion.

In conclusion, XY germ cells, which entered meiosis prematurely in MRL fetal testes,  
can carry out oogenesis until they obtain some of the morphological, epigenetic, and  
functional characteristics as oocytes, such as maternal patterns of H3K9me2, DNA  
methylation, and ability to fuse with sperm. The pairing and recombination of homologous  
405 chromosomes and their segregation at two consecutive meiotic divisions, and ultimately the  
development of embryos after fertilization must be examined. If the testicular oocytes  
undergo fertilization, an embryo of paternally derived mice may be obtained, i.e., an embryo  
of mice carrying genetic information derived from the male parent alone. In addition, the  
unique characteristics in which spermatogenesis and oogenesis take place at the same time in  
410 the same organ found in MRL mice will provide us with a useful model to investigate sexual  
determination and differentiation of gonads and germ cells.

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505

## FIGURE LEGENDS

### **Fig. 1. Expression of oogenesis markers in fetal gonads of MRL and B6 mice.**

(A) Expression of *Nobox* in MRL and B6 gonads at E18.5 by RT-PCR. The left numeral  
510 denotes the size of amplicons (bps). M, size marker.

(B) Quantitative real-time PCR of *Nobox* in MRL and B6 testes at E18.5. Each bar  
represents the mean  $\pm$  SE ( $n \geq 3$ ). \*: significant difference (one-sided Student's *t*-test,  $P <$   
0.05).

(C) Double immunofluorescence for Sycp3 and Nobox using MRL ovary (upper panels) and  
515 testis (lower panels) at E18.5. Green, Sycp3; red, Nobox. Scale bars: 50  $\mu$ m.

(D) Immunohistochemistry for Sycp3 (left) and Nobox (right) using MRL ovary (upper  
panels), MRL testis (middle panels), and B6 testis (lowest panels) at E18.5. Scale bars: 50  
 $\mu$ m.

### **520 Fig. 2. Dimethylation status of H3K9 of germ cells in MRL and B6 gonads at E16.5.**

Triple immunofluorescence for TRA98, Dmc1, and H3K9me2 using MRL ovaries (upper  
panels), MRL testes (middle panels), and B6 testes (lowest panels) at E16.5. Green, TRA98;  
blue, Dmc1; red, H3K9me2; arrows, testicular oocytes. Scale bars: 50  $\mu$ m.

### **525 Fig. 3. DNA Methylation status of germ cells in MRL and B6 gonads at E18.5.**

(A) Double immunofluorescence for Dnmt3a and Nobox using MRL ovaries (upper panels),  
MRL testes (middle panels), and B6 testes (lowest panels) at E18.5. Green, Dnmt3a; red,  
Nobox; arrowheads, spermatogenic cells; arrows, testicular oocytes. Scale bars: 50  $\mu$ m.

(B) Double immunofluorescence for 5-Mec and Nobox using MRL ovaries (upper panels),  
530 MRL testes (middle panels), and B6 testes (lowest panels) at E18.5. Green, 5-Mec; red,  
Nobox; arrowheads, spermatogenic cells; arrows, testicular oocytes. Scale bars: 50  $\mu$ m.

**Fig. 4. DNA Methylation status of testicular oocytes at D14.**

(A) Double immunofluorescence for Dnmt3a and Nobox (upper panels) and for 5-Mec and  
535 Nobox (lower panels) using serial sections of MRL ovaries at D14. Green, Dnmt3a; red,  
Nobox; arrowheads, ovarian oocytes with diameters of less than 50  $\mu\text{m}$ ; an arrow, ovarian  
oocyte with diameter of more than 50  $\mu\text{m}$ . Scale bars: 50  $\mu\text{m}$ .

(B) Double immunofluorescence for Dnmt3a and Nobox (upper panels) and for 5-Mec and  
Nobox (lower panels) using serial sections of MRL testis at D14. Green, 5-Mec; red, Nobox.  
540 Scale bars: 50  $\mu\text{m}$ .

**Fig. 5. Constitution of sex chromosomes and ability to fuse with sperm of postnatal testicular oocyte in MRL mice.**

(A) Detection of sex chromosomes in ovarian (left) and testicular (right) oocytes at D8 by  
545 the FISH method. Red dots, X chromosomes; yellow dots, Y chromosomes. Scale bar in left  
figure: 100  $\mu\text{m}$  (scale bar in inset: 12.5  $\mu\text{m}$ ). Scale bar in right figure: 50  $\mu\text{m}$  (scale bar in  
inset: 12.5  $\mu\text{m}$ ).

(B) Sperm-egg fusion assay using testicular and ovarian oocytes at D12. The ovarian oocytes  
(upper panels) and testicular oocytes (lower panels) under a confocal laser scanning  
550 microscope (left panels) and a differential interference contrast (DIC) microscope (right  
panels). Arrows, heads of invading sperms. Scale bars: 50  $\mu\text{m}$ .

**Supplementary Fig. 1. Oogenesis in mice.**

Primordial germ cells settled in the ovary enter meiosis prophase I around E13.5 and become  
555 primary oocytes. H3K9 of primary oocytes becomes highly methylated by the meiotic arrest  
at the diplotene stage. After birth, primary oocytes are surrounded by follicular epithelial

cells and form zona pellucida. In addition, they carry out *de novo* DNA methylation of female-specific imprints. At the antral stage, they resume and complete meiosis I and release first polar body to become secondary oocytes prior to ovulation. After fertilization with sperm, secondary oocytes continue meiosis II and finally become fertilized ovum.

**Supplementary Fig. 2. Appearance of postnatal testicular oocytes in newborn MRL mice.**

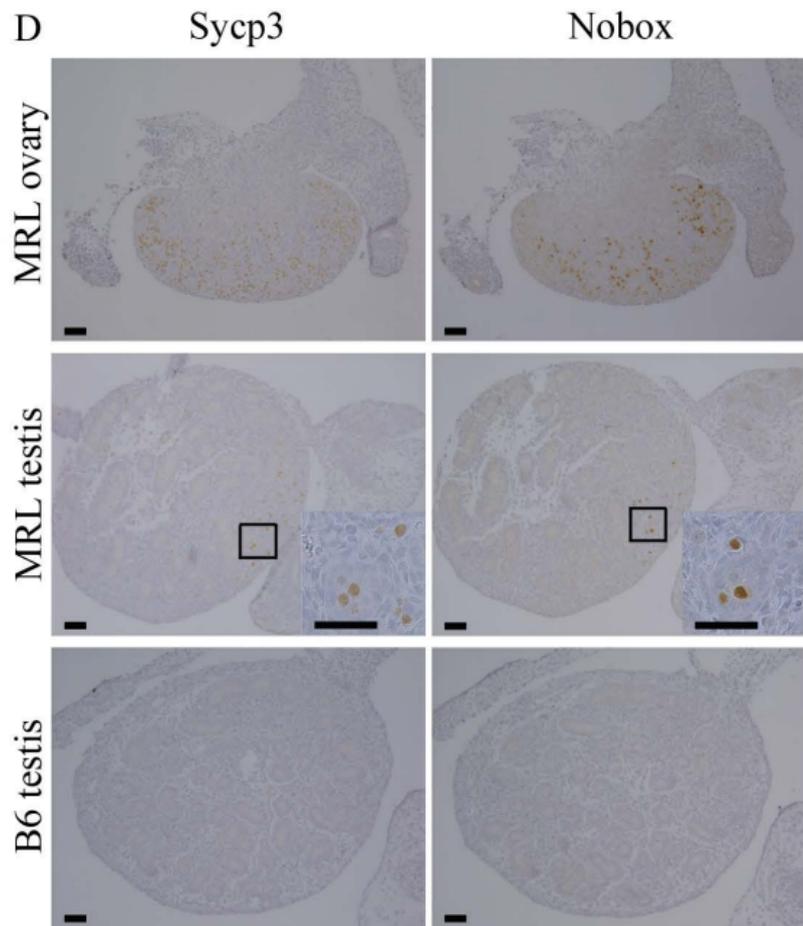
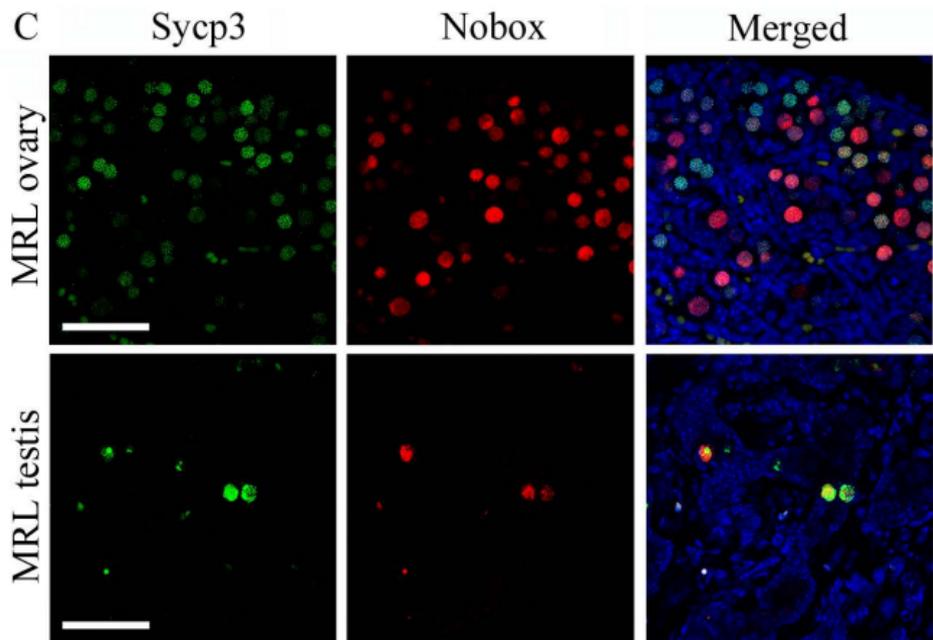
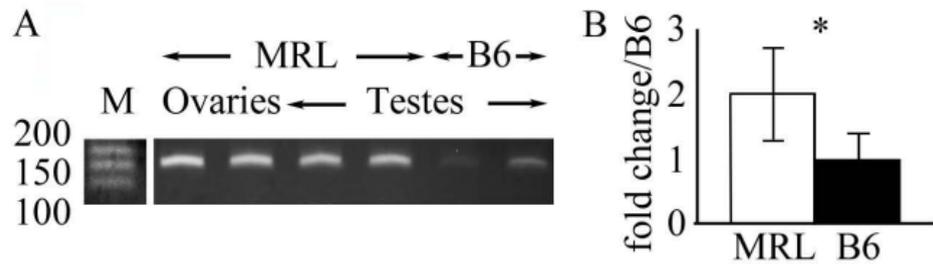
Whole-mount specimen of testicular oocytes at D14 under a differential interference contrast (DIC) microscope (left) and a hematoxylin and eosin (HE)-stained testicular oocyte under a light microscope (right). FE, follicular epithelial like-cells; O, testicular oocytes; ZP, zona pellucida. Scale bars: 50  $\mu$ m.

570

Table 1. Appearance of testicular oocytes in MRL mice

Age	No. of testes	No. of oocytes detected	Oocyte score	Percentage of mice with testicular oocytes
E18.5	26	193	14.85 <sup>*†</sup>	100
D14	308	394	1.28	61

Oocyte Score, no. of oocytes detected/no. of testes; \*, number of oocytes detected/number of testes observed  $\times 2$ ;  
<sup>†</sup>, significant difference (Mann-Whitney *U*-test,  $P < 0.05$ ).



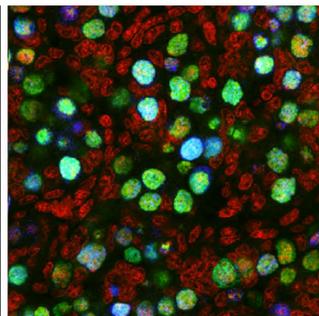
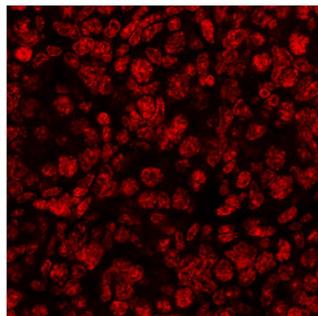
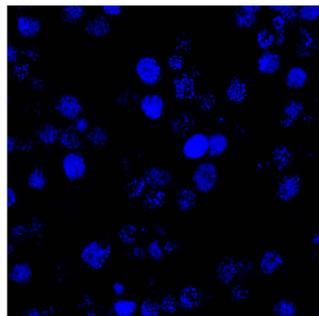
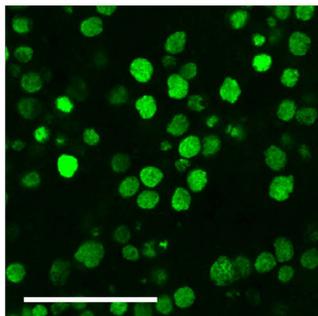
TRA98

Dmc1

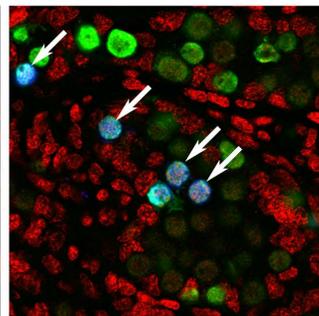
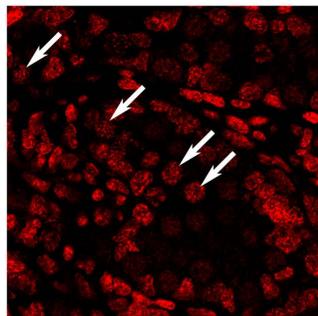
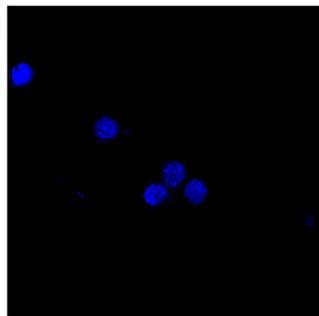
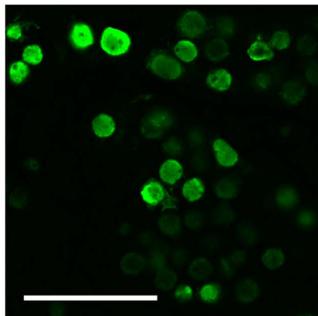
H3K9me2

Merged

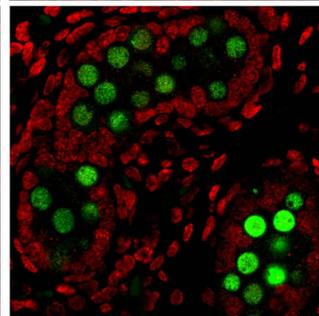
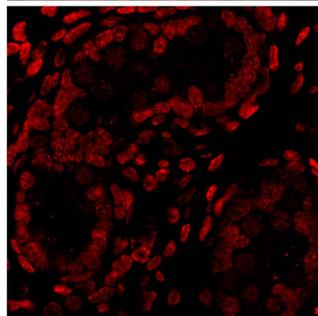
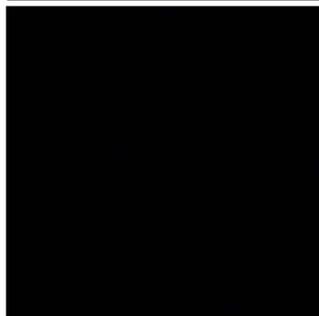
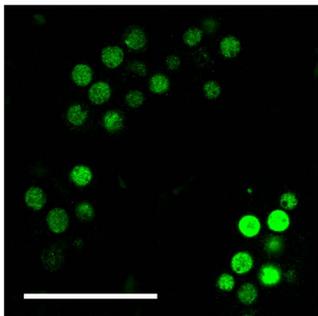
MRL ovary

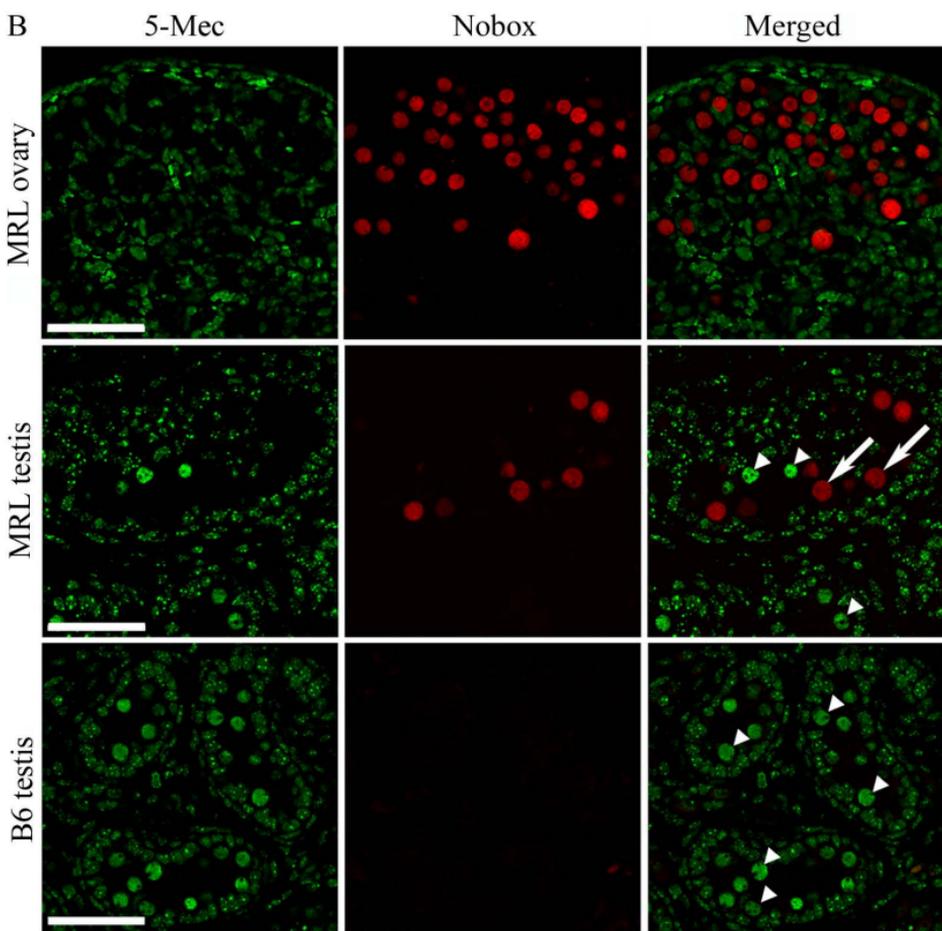
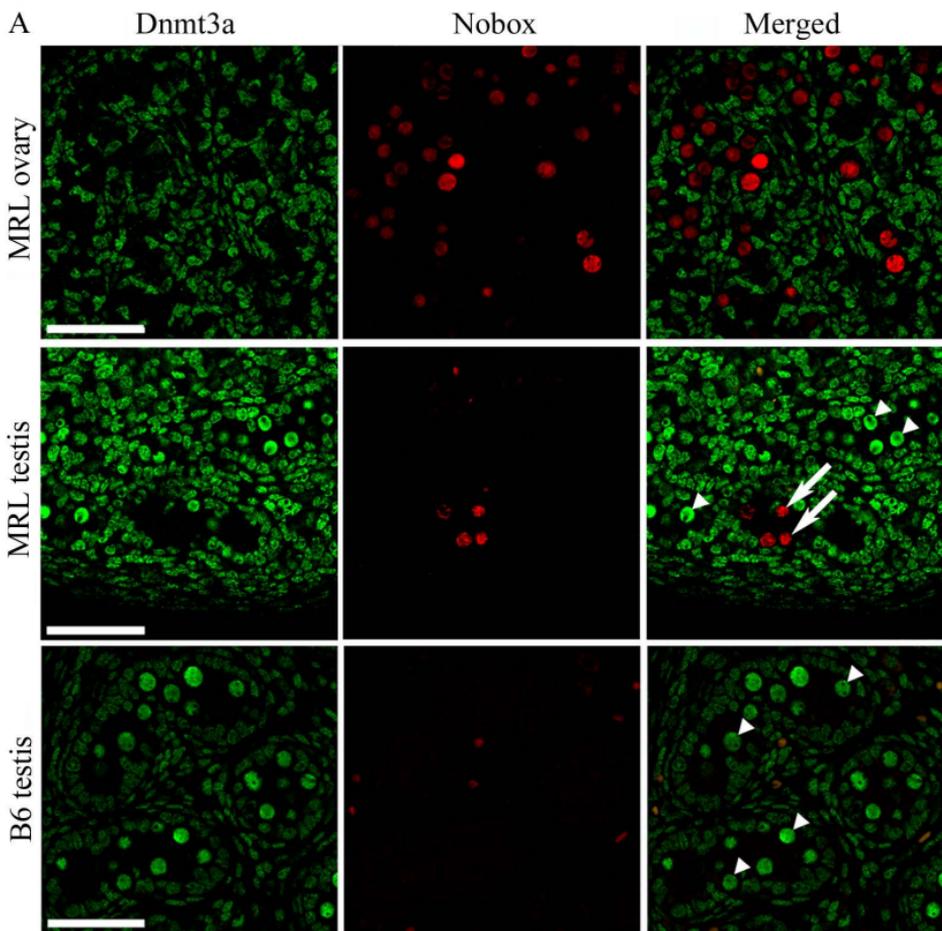


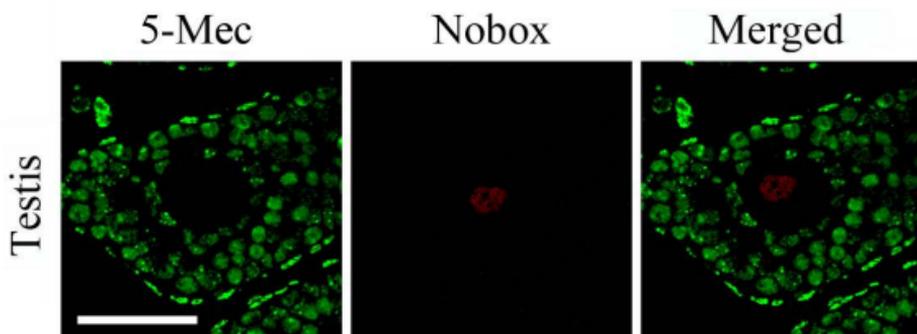
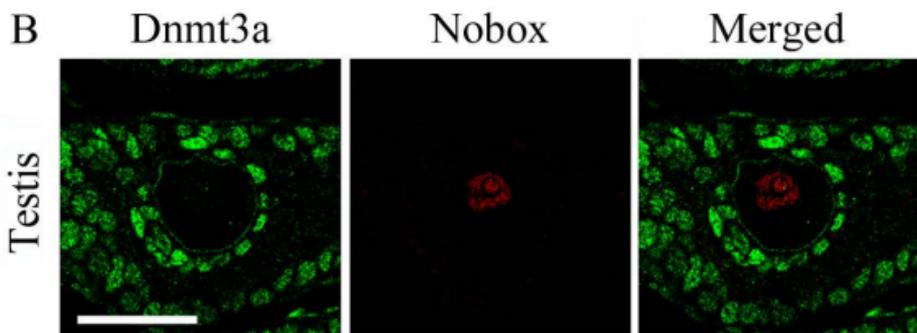
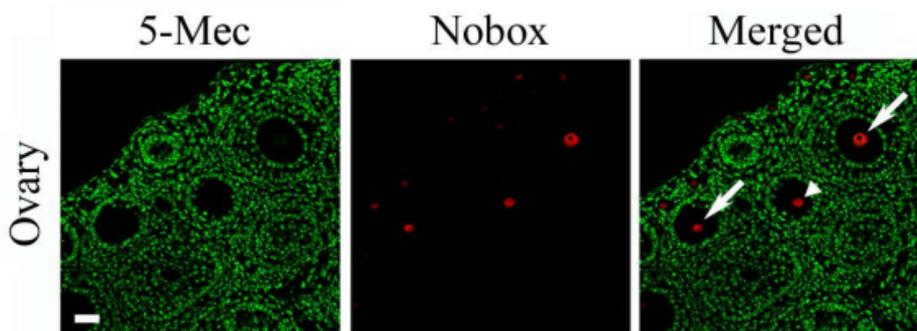
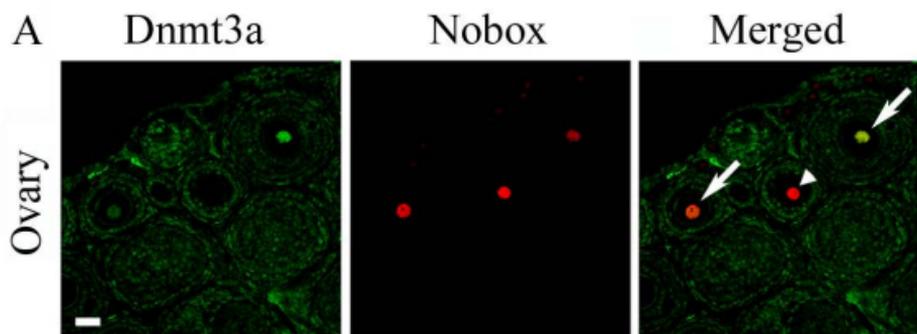
MRL testis



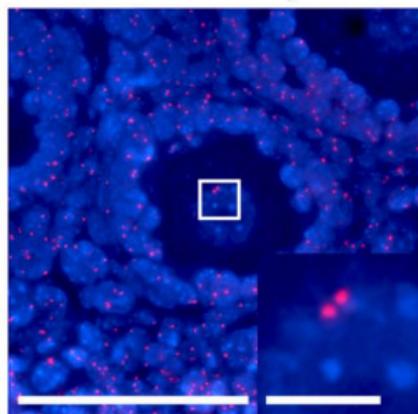
B6 testis



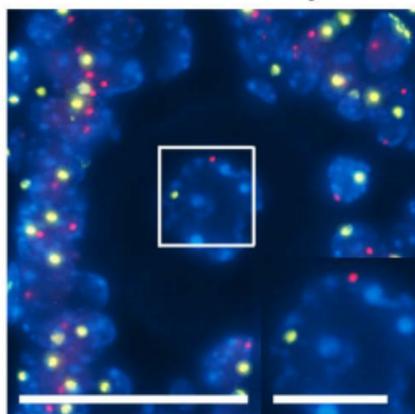




**A** Ovarian oocyte

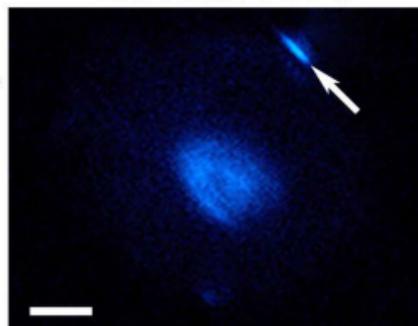


Testicular oocyte

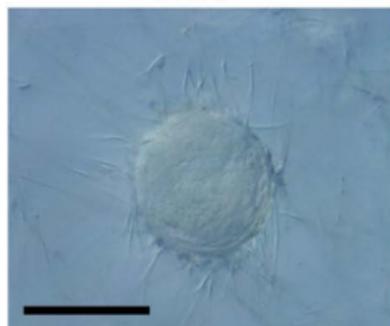


**B** Hoechst 33342

Ovarian oocyte



DIC



Testicular oocyte

