Spermatogonial deubiquitinase USP9X is essential for proper spermatogenesis in mice

Kasane Kishi¹, Aya Uchida¹, Hinako M Takase², Hitomi Suzuki², Masamichi Kurohmaru¹, Naoki Tsunekawa¹, Masami Kanai-Azuma², Stephen A Wood³ and Yoshiakira Kanai¹

¹Department of Veterinary Anatomy, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, ²Department of Experimental Animal Model for Human Disease, Centre for Experimental Animals, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan, and ³Griffith Institute for Drug Discovery, Griffith University, Brisbane, Queensland, Australia

Correspondence should be addressed to Y Kanai; Email: aykanai@mail.ecc.u-tokyo.ac.jp

Abstract

USP9X (ubiquitin-specific peptidase 9, X chromosome) is the mammalian orthologue of *Drosophila* deubiquitinase fat facets that was previously shown to regulate the maintenance of the germ cell lineage partially through stabilizing Vasa, one of the widely conserved factors crucial for gametogenesis. Here, we demonstrate that USP9X is expressed in the gonocytes and spermatogonia in mouse testes from newborn to adult stages. By using Vasa-Cre mice, germ cell-specific conditional deletion of *Usp9x* from the embryonic stage showed no abnormality in the developing testes by 1 week and no appreciable defects in the undifferentiated and differentiating spermatogonia at postnatal and adult stages. Interestingly, after 2 weeks, *Usp9x*-null spermatogenic cells underwent apoptotic cell death at the early spermatocyte stage, and then, caused subsequent aberrant spermiogenesis, which resulted in a complete infertility of *Usp9x* conditional knockout male mice. These data provide the first evidence of the crucial role of the spermatogonial USP9X during transition from the mitotic to meiotic phases and/or maintenance of early meiotic phase in *Usp9x* conditional knockout testes.

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Introduction

In mammalian testes, spermatozoa are constantly produced by well-balanced process between the maintenance of undifferentiated spermatogonia including spermatogenic stem/progenitor cells and their differentiation into differentiating spermatogonia in the basal compartment of the seminiferous epithelia. Subsequently, the differentiating spermatogonia undergo meiotic initiation, leading to the cyclical differentiation into spermatocytes, round and elongated spermatids and spermatozoa in the adluminal compartment of the seminiferous tubules (Russell *et al.* 1990). During the postnatal development of mouse testes, a certain population of the gonocytes, the precursors of spermatogonia, directly undergoes the 1st wave of spermatogenesis, followed by the initial onset of meiosis by 2 weeks at the adluminal compartment. The remaining population of the gonocytes is differentiated into the spermatogenic stem/progenitor cells within the basal compartment, which subsequently contribute to the 2nd and later cycles of the spermatogenesis throughout the reproductive life (~1.5-year-old mice) (Yoshida *et al.* 2006).

The ubiquitin-protease degradation system is well known to be involved in various steps of spermatogenesis

including the maintenance/proliferation of the spermatogonia, meiotic entry, chromosomal recombination, acrosome biogenesis and spermiogenesis (Hou & Yang 2013). For example, the deletion of *Ubb*, the polyubiquitin gene with predominant expression in male germ cells, resulted in the complete failure of meiotic progression of spermatocytes beyond early pachytene stage as well as more than 50% reduction of ubiquitin concentration in mouse testes (Ryu *et al.* 2008). It is well known that ubiquitin proteasome-related genes including ubiquitin-ligase and deubiquitinase are stage-specifically expressed in the spermatogenic cells and they appear to be involved in multiple steps of spermatogenesis (review by Bose *et al.* 2014). The murine deubiquitinase genes such as *Usp2*, *Usp14*, *Uchl1* and *Cyld* in male germ cells were also shown to be crucial for the proper spermatogenesis by a number of forward genetic approaches of deletion mutants (Suresh *et al.* 2015). These findings suggest that the quantity of germ cell components may be post-transcriptionally regulated by the balance of the ubiquitin-ligase and deubiquitinase activities *in vivo*.

It has been reported that the *Drosophila* deubiquitinating enzyme fat facets (Faf) is expressed in the germ cell progenitors (pole cells) in fly embryos

(Fischer-Vize *et al.* 1992). Later, it was revealed that Faf interacts with a DEAD-box RNA helicase Vasa, one of the widely conserved factors for gametogenesis (Lasko 2013), and protects Vasa from degradation. Indeed, a loss of Faf function displayed defective oogenesis (Liu *et al.* 2003). The mammalian homologues of Faf are two ubiquitin-specific peptidase genes located on sex chromosomes, *Usp9x* (ubiquitin-specific peptidase 9, X chromosome, also known as *Fam*) and *Usp9y* (ubiquitin-specific peptidase 9, Y chromosome). The *USP9Y* gene was previously shown to be dispensable for spermatogenesis and male fertility in humans (Luddi *et al.* 2009). The murine *Usp9y* gene also appears to be not essential, at least for haploid germ cell formation, because *XO^{Sry/Elf2s3y}* male mice with a complete lack of *Usp9y* produced offspring in assisted reproduction (Yamauchi *et al.* 2014). In contrast, murine *Usp9x* is considered to be a functional orthologue of *faf*, since it is able to functionally replace *faf* in *Drosophila* embryos (Chen *et al.* 2000). *USP9X* expression was previously shown in mouse primordial germ cells (PGCs) after 10.5 days post coitum (dpc) (Noma *et al.* 2002). However, the *in vivo* function of *USP9X* in mammalian gametogenesis remains unclear.

In this study, in order to clarify the *in vivo* role of *USP9X* in mouse testicular germ cells, we examined the expression patterns of *USP9X* in mouse spermatogenesis and the phenotype of the *Usp9x*-null spermatogenic cells by using a germ cell-specific conditional knockout (cKO) approach with *Vasa-Cre* mice.

Materials and methods

Animals

C57BL/6 mice (Japan SLC Inc., Hamamatsu, Japan) were used for immunohistochemical analysis. *Usp9x^{loxP/loxP}* female mice (Stegeman *et al.* 2013) were crossed with *Vasa-Cre* male mice (Gallardo *et al.* 2007). *Vasa-Cre* males induce recombination in germ cells between 15 dpc and 18 dpc (Cre efficiency was confirmed by crossing with Cre reporter mice (Supplementary data and Supplementary Fig. 1, see section on supplementary data given at the end of this article)), and deletes *Usp9x*. As *Usp9x* is located on the X chromosome, the male offspring from this cross that inherited the *Vasa-Cre* allele lacked *Usp9x* (*Usp9x* cKO). Cre-negative males were used as controls (Cnt). Mice were collected at newborn, 1-week-old, 2-week-old, 4-week-old and young and aged adult stages (from 7-week-old to 1-year-old ages). As for the male fertility, the *Usp9x* cKO and Cnt males (5 week old; 6 males in each genotype) were housed with 2–3 wild-type C57BL/6 females for 7 weeks, and the numbers of wild-type females impregnated by males were calculated.

The animal experiments were carried out in accordance with the Guidelines for Animal Use and Experimentation of the University of Tokyo (approval IDs: P13-764, P14-876, PM15-085, and PH16-084) and the Tokyo Medical and Dental

University (approval IDs: 0140007A, 0150259C2, 0160024C2 and 0170248C2).

Histology and immunohistochemistry

For histopathological analysis, testes and epididymides were fixed in Bouin's solution or 4% paraformaldehyde (PFA) and then routinely embedded in paraffin. Sections (5 µm thickness) were subjected to hematoxylin–eosin (HE) or periodic acid–Schiff (PAS) staining. For immunohistochemical staining, testes were fixed in 4% PFA and routinely embedded in paraffin or OCT compound. The deparaffinized sections (5 µm thickness) were incubated for 12 h at 4°C with anti-Hsc70t (1:2000 dilution; Tsunekawa *et al.* 1999), anti-Ki-67 (1:100 dilution; Leica Microsystems GmbH), anti-promyelocytic leukemia zinc finger (PLZF; formally known as ZBTB16; 1:100 dilution; Calbiochem) and anti-synaptonemal complex protein 3 (SCP3; 1:100 dilution; Santa Cruz Biotechnology) antibodies. The cryosections (10 µm thickness) were also incubated with anti-GDNF family receptor alpha-1 (GFRA1; 1:100 dilution; R&D Systems), anti-kit oncogene (KIT; 1:100 dilution; R&D Systems), anti-phospho-histone H2AX (g-H2AX; 1:1000 dilution; Upstate), anti-SCP3 (1:100 dilution; Santa Cruz Biotechnology), anti-SOX9 (1:1,000 dilution; Millipore) and anti-*USP9X* (1:200 dilution; Kanai-Azuma *et al.* 2000) antibodies. The immunoreactive signals were visualized by using biotin-conjugated secondary antibodies in combination with the Elite ABC Kit (Vector Laboratories, CA, USA) or using Alexa-488/594-conjugated secondary antibodies (Molecular Probes) in combination with the nuclear counterstaining of 4',6-diamidino-2-phenylindole (DAPI). For negative controls, the sections were incubated with normal IgG (Wako Pure Chemical Industries) instead of the primary antibody, in which any non-specific signals were not detected inside the seminiferous tubules (Supplementary Fig. 2). The paraffin sections were also used for TUNEL assay by using an Apoptotic Detection Kit (Takara). All of the stained samples were analyzed using an Olympus fluorescence microscope (BX51N-34-FL-2; Tokyo, Japan) and Leica TCS SP8 (Leica Microsystems GmbH) confocal laser microscope as described previously (Aiyama *et al.* 2015).

Morphometry

To assess the incidence of the abnormal seminiferous tubules, only circular seminiferous tubules in transverse sections were used and calculated. SCP3-positive, TUNEL-positive and PLZF- and Ki-67-positive cell numbers were counted in 50 circular tubules in each paraffin transverse sections. KIT-positive and SOX9-positive cells were counted in cryosections and the number of KIT-positive cells was divided by the number of SOX9-positive cells in the same section.

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed as described previously (Aiyama *et al.* 2015). In brief, the PFA-fixed seminiferous tubules were incubated for 12 h at 4°C

with anti-GFRA1 antibody (1:100 dilution; R&D Systems). The signals were visualized with Alexa-594-conjugated secondary antibody. All the stained samples were analyzed using an Olympus fluorescence microscope (BX51N-34-FL-2).

Statistical analyses

Quantitative data are represented as the mean $\pm$ standard error (S.E.). Data were analyzed by Student's *t*-test.

Results

USP9X expression in the spermatogonia of postnatal and adult testes

The present immunostaining using anti-USP9X antibody allowed us to detect considerable signal in the cytoplasm of the spermatogenic cells from newborn to adult stages (Fig. 1). The signal quality and antibody specificity were confirmed by a complete loss of the signals in the spermatogenic cells of *Usp9x* conditional knockout (cKO) testes (*Vasa-Cre; Usp9x^{loxP/Y}*) (Fig. 1B and G).

In the newborn testes, USP9X-positive gonocytes were surrounded by Sertoli cells (Fig. 1A and A'), and they were then relocated to the basal compartment of the seminiferous tubules by 1 week old (Fig. 1C). At 2 week old, USP9X-positive signals were weakly detectable in the early spermatocytes located in the central region (red arrows in Fig. 1D) that become positive for anti-SCP3 staining (a synaptonemal complex marker specific for the spermatocytes (Yuan *et al.* 2000)), as well as in the spermatogonia in the basal compartment (white arrows in Fig. 1D). At 4-week-old and adult stages, USP9X-positive signals were restricted to the spermatogonial population located within the basal compartment (Fig. 1E and F), but not in the pachytene spermatocytes, round spermatids and elongated spermatids (Fig. 1F'). Double staining of USP9X and GFRA1 (a specific marker for undifferentiated spermatogonia including spermatogenic stem/progenitor cells (Hara *et al.* 2014)) or KIT (a marker for differentiating spermatogonia (Zhang *et al.* 2011)) revealed that USP9X-positive signals were found in both GFRA1-positive and KIT-positive populations of the spermatogonia in the adult testes (Fig. 1H and I). These data indicate that USP9X is expressed predominantly in the spermatogonia and weakly in early spermatocytes, but not in the pachytene spermatocytes and later stage spermatogenic cells.

Small-sized testes with almost complete lack of epididymal spermatozoa, leading to the complete infertility in *Usp9x* germ cell-specific conditional knockout males

In order to clarify the *in vivo* role of *Usp9x* in spermatogenic cells, the *Vasa-Cre; Usp9x^{loxP/Y}* (cKO)

Figure 1 Expression of USP9X during spermatogenesis. Anti-USP9X (green) and anti-SCP3 (D)/GFRA1 (H)/KIT (I) (red) double staining of cryosections in each stage (A, B: newborn, C: 1-week, D: 2-week, E: 4-week, F, G, H and I: adult). DNA was stained by DAPI. A, C, D, E, F, H and I are sections of wild-type mice (Wt). A' and F' are the higher magnified images in newborn and adult stages respectively. B and G are sections of *Usp9x* conditional knockout mouse (cKO). In the inset of D, white and red arrows indicate SCP3-negative and positive spermatogenic cells respectively. In D, H and I, insets are the higher magnified images indicated by broken rectangles. Broken line, outline of seminiferous tubule; *, signals outside the seminiferous tubules; arrow, nucleus of germ cell; arrowhead, nucleus of Sertoli cell. Scale bars, 20 μ m.

Figure 2 Decreased size of testes and low sperm production in 12-week-old *Usp9x* conditional knockout mice. (A) Gross appearance of Cnt and *Usp9x* cKO mouse testes. (B) The value of testis weight $\times 100$ /body weight (%) of Cnt and *Usp9x* cKO mice. $*P < 0.05$ ($n = 3$ mice) (C) PAS-stained paraffin sections of Cnt and *Usp9x* cKO mouse epididymides. Scale bars, 5 mm in A; 50 μ m in C.

and *Usp9x*^{loxP/y} (Cnt) male founders were produced by the crossing between *Vasa-Cre* males and *Usp9x*^{loxP/loxP} females, and then, their reproductive phenotypes were examined. The testes of 12-week-old *Usp9x* cKO mice were significantly decreased in weight, as compared to those of the Cnt males (Fig. 2A and B). Few spermatozoa were observed in the caudal epididymides of the adult *Usp9x* cKO (Fig. 2C). As for their fertility, the *Usp9x* cKO males were able to properly make the vaginal plugs in wild-type females, but these females did not have any signs of pregnancy and produced no offspring ($n = 6$ males). This is in contrast to the 2.2 ± 0.3 pregnant females with a normal number of offspring produced following mating to the Cnt males. These data indicate that *Usp9x* cKO males are infertile presumably due to few epididymal spermatozoa.

Defective spermatogenesis with reduced numbers of spermatocytes and subsequent failure of spermiation in *Usp9x* conditional knockout testes

Histopathological examination revealed aberrant spermatogenesis with reduced number of spermatogenic cells in 12-week-old *Usp9x* cKO mouse testes (Fig. 3A). Such histopathological phenotypes were similar to those observed in 24-week-old and 1-year-old *Usp9x* cKO testes (Fig. 3B and C), suggesting certain defects in meiotic/post-meiotic germ cells rather than the defective maintenance of spermatogonial stem/progenitor cells in the *Usp9x* cKO testes. By the morphometric analyses using the transverse tubular sections of the 12-week-old

testes, aberrant seminiferous tubules lacking some spermatogenic cells were $85.0 \pm 5.1\%$ of transverse sections of seminiferous tubules. In these abnormal tubules, $3.3 \pm 1.7\%$ of the tubules contained both round and elongated spermatids but completely lacked the spermatocytes; $68.8 \pm 7.7\%$ of the tubules included a few spermatocytes and also a few spermatids; and $13.0 \pm 2.1\%$ of the tubules showed the almost complete lack of the meiotic and post-meiotic spermatogenic cells in the adluminal compartment (Fig. 3D).

Anti-SCP3 immunostaining revealed that the relative number of spermatocytes in *Usp9x* cKO testes was significantly reduced to three-fourths of the control testes (SCP3-positive cell number per tubule: 70.7 ± 3.4 in cKO vs 93.0 ± 3.9 in Cnt; $n = 3$), albeit of no appreciable differences in their signal intensities between the *Usp9x* cKO and control testes (Fig. 3E). Immunostaining of γ -H2AX (the phosphorylated form of histone H2AX; localized at sites of meiotic recombination that occur during leptotene stage and later at XY body during pachytene stage (Mahadevaiah *et al.* 2001, Fernandez-Capetillo *et al.* 2003)) revealed that the spermatocytes, which passed through meiotic differentiation and remained in the affected seminiferous tubules showed no appreciable defects in recombination and formation of XY body (Fig. 3F). Immunostaining of Hsc70t (a specific marker for the cytoplasm of elongating spermatids (Tsunekawa *et al.* 1999)) showed proper luminal distribution and intensities of Hsc70t-positive signals in some seminiferous tubules of the *Usp9x* cKO testes (Fig. 3G). However, some affected spermatids showed aberrant condensed Hsc70t-positive signals as a residual body-like structure in the *Usp9x* cKO seminiferous tubules (arrowheads and inset 'a' in Fig. 3G), together with the mislocation of the fully elongated spermatids attached with Hsc70t-positive signals near the basal compartment (inset 'b' of Fig. 3G). Histopathological analysis using PAS-stained sections confirmed that some fully completed spermatozoa were abnormally retained in the seminiferous tubules containing the elongating spermatids at step 9–10 (Fig. 3H), suggesting some defects in the processes of spermiogenesis and/or spermiation of the *Usp9x* cKO testes. Moreover, some elongated spermatids were degenerating and occasionally misaligned near the basement membrane (circles in right panel of Fig. 3H), suggesting Sertoli cell phagocytosis of aberrant spermatids.

Increased apoptotic cell death in the early spermatocytes of *Usp9x* conditional knockout testes

Next, in order to examine the initial defects of *Usp9x*-null germ cells, we spatiotemporally observed HE-stained paraffin sections. No appreciable defects were observed until 1-week-old stage (data not shown), but in 2-week-old stage, coinciding with the appearance of early spermatocytes in postnatal

Figure 3 Defective spermatogenesis in *Usp9x* conditional knockout mice. (A, B, C and D) PAS-stained paraffin sections of 12-week-old (A, D), 24-week-old (B) and 1-year-old (C) Cnt and *Usp9x* cKO mouse testes. Stars in A, B and C indicate aberrant seminiferous tubules lacking some spermatogenic cells. (E) Anti-SCP3 (brown)-stained paraffin sections of 12-week-old Cnt and *Usp9x* cKO testes. Insets are the higher magnified images of the cells indicated by arrowheads. (F) Anti-g-H2AX (red)-stained cryosections of 12-week-old Cnt and *Usp9x* cKO testes. DNA was stained by DAPI. Arrows show XY body of pachytene spermatocytes and arrowheads show leptotene spermatocytes. (G, H) Anti-Hsc70t (brown; G) and PAS (H) stained paraffin sections of 12-week-old Cnt and *Usp9x* cKO testes. Each arrowhead shows an aberrant condensed residual body-like structure that is positive for Hsc70t staining. Insets are the higher magnified images indicated by broken rectangles. Step 9 and step 10 spermatids are shown with Arabic numerals, and the mislocation of fully elongated spermatids and spermatozoa is indicated by circles in H. Scale bars, 50µm in A, B, C, E, G; 20µm in D, H; 10µm in F.

development, apoptotic cells were frequently detected in *Usp9x* cKO mouse seminiferous tubules (Fig. 4A). Moreover, TUNEL analysis showed no differences

of TUNEL-positive cell number between the *Usp9x* cKO and Cnt mice in the newborn and 1-week-old stages, but the *Usp9x* cKO testes showed a significant

Figure 4 Spatiotemporal patterns of apoptotic cell death in *Usp9x* conditional knockout testes. (A) HE-stained paraffin sections of 2-week-old Cnt and *Usp9x* cKO mouse testes. Arrows show apoptotic cells. Inset is the higher magnified image indicated by broken rectangle. (B, C) TUNEL-stained paraffin sections of 2-week-old (B) and 12-week-old (C) mouse testes. TUNEL-positive cells (arrows) were found in the sections. B' and C' are the higher magnified images indicated by broken rectangles in *Usp9x* cKO of B and C respectively. (D) The numbers of TUNEL-positive cells per tubule ($n=2$ (newborn), $n=3$ (1-week-old, 4-week-old), $n=5$ (2-week-old), $n=6$ (adult)). NS, not significant; $*P<0.05$; $**P<0.01$. Scale bars, 50 μm in A, B and C; 20 μm in B', C'.

increase of TUNEL-positive spermatogenic cells at 2-week-old and later stages (Fig. 4B, C and D). At 2-week-old stage, clusters of several TUNEL-positive spermatogenic cells were frequently observed in the luminal region nearest to the basal compartment (i.e., Sertoli cell nuclei) in the *Usp9x* cKO testes throughout the stages from 2- to 12-week-old (arrows in Fig. 4B and C), suggesting apoptotic cell death around early spermatocyte stages. These data clearly agree with the present histological observation showing the frequent loss of the spermatocytes in the *Usp9x* cKO testes (Fig. 3A, B, C, D and E).

Proper maintenance of the Usp9x-null spermatogonia in the basal compartment of the seminiferous tubules

Next, we examined the phenotype of the *Usp9x*-null spermatogonia that are located in the basal compartment of the seminiferous tubules. Whole-mount staining of anti-GFRA1 antibody, a specific marker for the spermatogonial stem/progenitor cells (Hara *et al.* 2014), showed proper localization of GFRA1-positive spermatogonia in the basal compartment even in 1-year-old cKO testes (Fig. 5A), suggesting no appreciable defects in the spermatogonial stem/progenitor cells in *Usp9x* cKO testes. This is consistent with the partial spermatogenic patches containing spermatocytes and/or spermatids in some areas of *Usp9x* cKO testes even at 1-year-old stage (Fig. 3C). The numbers of undifferentiated spermatogonia were quantitatively examined by using anti-PLZF staining, another marker for undifferentiated spermatogonia (Costoya *et al.* 2004). At 12-week-old, there was no significant difference in the number of PLZF-positive spermatogonia between *Usp9x* cKO testes and the Cnt testes of the littermates (PLZF-positive cell number per tubule: 9.2 ± 1.2 in cKO vs 10.8 ± 1.0 in Cnt; $n=3$). Moreover, double staining of PLZF and Ki-67 (cell proliferation marker (Whitfield *et al.* 2006)) revealed no appreciable defects in Ki-67-positive cell ratio per PLZF-positive cells in cKO testes ($73.1 \pm 1.6\%$ in cKO vs $75.5 \pm 2.8\%$ in Cnt; $n=3$; Fig. 5B). Immunostaining using anti-KIT (a specific marker for differentiating spermatogonia (Zhang *et al.* 2011)) and anti-SOX9 (a marker for Sertoli cells (Kent *et al.* 1996)) antibodies showed no appreciable defects in the KIT-positive spermatogonia in the basal compartment of *Usp9x* cKO testes (KIT-positive cell number per SOX9-positive Sertoli cell: 0.84 ± 0.08 in cKO vs 0.97 ± 0.12 in Cnt; $n=3$; Fig. 5C). Taken together, these data suggest that *Usp9x* may not be essential for the maintenance and proliferation of the spermatogonia in mouse spermatogenesis, despite its predominant expression in them.

Figure 5 Proper maintenance of spermatogonia in *Usp9x* conditional knockout mice. (A) Anti-GFRA1 (red) whole-mount immunostaining of seminiferous tubules of 1-year-old Cnt and *Usp9x* cKO mice. Insets are the higher magnified images indicated by broken rectangles. (B) Anti-PLZF (green) and anti-Ki67 (red) double staining of paraffin sections of 12-week-old Cnt and *Usp9x* cKO mouse testis. PLZF single positive cells (arrows) and PLZF, Ki67 double positive cells (arrowheads) are observed in the seminiferous tubules. Insets are the higher magnified images indicated by the same alphabets. (C) Anti-KIT (red) and anti-SOX9 (not shown) double staining of cryosections of 12-week-old Cnt and *Usp9x* cKO mouse testis. DNA was stained by DAPI. Insets are the higher magnified images indicated by broken rectangles. (D) Schematic representation of the expression of USP9X and phenotypes of conditional knockout mice. Broken line, outline of seminiferous tubule in A and B, outline of interstitium in C; *, signals outside the seminiferous tubules; Spg, spermatogonium; Spc, spermatocyte; rSpt, round spermatid; eSpt, elongated spermatid. Scale bars, 20 μ m.

Discussion

The present study detected considerable amount of USP9X-positive signals in the cytoplasm of gonocytes and spermatogonia during newborn to adult stages (Fig. 1). On the other hand, the meiotic and post-meiotic germ cells were USP9X negative, except for early spermatocytes in the 1st wave of spermatogenesis. Such a loss of USP9X expression soon after the meiotic entry may be caused by meiotic sex chromosome inactivation characterized by a specialized nuclear territory known as the XY body (Handel 2004), in which the *Usp9x* locus undergoes the transcriptional silencing at least from leptotene spermatocytes in mouse spermatogenesis (Mahadevaiah *et al.* 2008). Taken together, these data suggest the potential function of USP9X in the testicular germ cells mainly before the early phase of meiosis. In addition, no appreciable USP9X-positive signals were previously shown to be detected in the spermatogenic

cells in postnatal and adult testes (Noma *et al.* 2002, Sato *et al.* 2004). This discrepancy may be due to the lower sensitivity of the previous antibody to detect the USP9X-positive signals.

A conditional deletion of *Usp9x* in testicular germ cells showed a partial loss of spermatocytes and subsequent defective spermiogenesis in *Usp9x* cKO testes (Figs 3 and 4). The first aberrant phenotypes at early spermatocyte stage were observed continuously from the immature, young adult to aged adult testes (from 2-week to 1-year-old). Moreover, TUNEL-positive apoptotic spermatogenic cells were observed in the adluminal area nearest to the basal compartment throughout the postnatal to adult stages. On the other hand, the maintenance of undifferentiated GFRA1-positive/PLZF-positive and differentiating KIT-positive spermatogonia appeared to be normal, despite the dominant expression of USP9X in these spermatogonia.

Therefore, these data indicate that a loss of function of spermatogonial USP9X leads to the defective transition from the mitotic to meiotic phases and/or defective maintenance of early meiotic phase in *Usp9x* cKO testes. This further suggests that the potential targets of USP9X may be involved in these early meiotic phases by protecting their degradation in the spermatogonia and early spermatocytes.

Recently, it becomes clear that USP9X is involved in various normal developmental processes and some abnormal disease conditions via stabilizing their target molecules (Murtaza *et al.* 2015). The previous studies showed that USP9X is able to stabilize ASK-1/DLK and protect them from proteasomal degradation in the pro-apoptotic JNK signaling cascade (Nagai *et al.* 2009, Huntwork-Rodriguez *et al.* 2013), in addition to the activation of anti-apoptotic factor, MCL1 and survivin (Vong *et al.* 2005, Schwickart *et al.* 2010). Moreover, β -catenin is one of the famous targets of USP9X (Taya *et al.* 1999). β -catenin has been reported to have important roles in germ cell differentiation (Chang *et al.* 2011) and maintenance of spermatogonia via regulation of the canonical Wnt signaling pathway (Takase & Nusse 2016). It is possible that the imbalance in these signaling pathways may contribute to the present phenotypes showing increased apoptotic cell death at early spermatocyte stages, together with subsequent aberrant spermiogenesis in the *Usp9x* cKO testes.

One of the potential targets of USP9X may be VASA (formally known as DDX4 or MVH in mice), since Vasa is demonstrated as a target of *Drosophila* Faf, the functional orthologue of USP9X (Liu *et al.* 2003). Moreover, VASA proteins are well known to be a germ cell-specific component with high sequence conservation from *Drosophila* to mammals (Fujiwara *et al.* 1994). Mouse VASA is specifically expressed in germ cell lineage at stages from the PGCs in embryonic gonads and from spermatogonia to round spermatids during postnatal spermatogenesis (Toyooka *et al.* 2000). Mouse VASA is considered to be important in silencing retrotransposons and protecting germ cells (Kuramochi-Miyagawa *et al.* 2010). In fact, a deletion of mouse VASA leads to severe defects in spermatogenesis, in which almost all of premeiotic germ cells cease their differentiation at early spermatocyte stage and undergo apoptotic cell death (Tanaka *et al.* 2000). These meiotic defects in Vasa-null germ cells are reminiscent of the apoptotic cell death in early spermatocytes of the *Usp9x* cKO testes, which further suggests the potential roles of USP9X in the spermatogenesis through the VASA stabilization. To elucidate the mechanisms of the exact actions of USP9X in these cellular events, the identification of the exact target molecules of USP9X including VASA must be required in future studies.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0184>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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