Protein Phosphatase 4 (PPP4) Prevents Female Reprogramming in the Mouse Testis After Sex Determination and Protects Male Fertility

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Research Article

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Abstract

Background: Impairment of lineage specification and function of gonadal somatic cells can lead to disorders of sexual development (DSDs) and fertility defects in humans. However, little is known about the function of protein phosphatases in testis development.

Results: We showed that protein phosphatase 4 (PPP4) could maintain SOX9 expression in Sertoli cells and play an essential role in Sertoli cell lineage maintenance and male fertility. Conditional deletion of *Ppp4c,* a PPP4 catalytic subunit gene, caused the reprogramming of Sertoli cells to granulosa-like cells postnatally by inducing ectopic expression of FOXL2, which in turn led to testicular BTB structure damage, germ cell loss and ultimate testis to ovary-like gland transformation.

Conclusion: Reprogramming of Sertoli cells due to absence of PPP4 may help explain the etiology of disorders of sexual differentiation and male infertility.

Introduction

In mammals, the testes in males or the ovaries in females are derived from the bi-potential gonads. Development of a testis or ovary is dependent on the differentiation of gonadal somatic cells, which is regulated by sex-determining genes. The *Sry* gene is transiently expressed in the somatic cells of undifferentiated XY gonads from E10-E12 in mice. This gene activates SOX9 and is essential for directing Sertoli cell differentiation in bi-potential gonads and testis formation (ref. 1, 2). In XX gonads, which lack SRY expression, the somatic cells differentiate into granulosa cells (ref. 3–5), which is regulated by FOXL2 (ref. 6, 7) and R-spondin1 (RSPO1)/WNT4-β-catenin (CTNNB1) signaling pathway (ref. 8–10). It is especially noteworthy that Sertoli (or granulosa) cell fate, once specified, is not permanent but instead needs to be constantly reinforced. For example, loss of FOXL2 function in the adult ovary allows granulosa cells to transdifferentiate into Sertoli cells (ref. 6), while loss of DMRT1 in Sertoli cells of the adult testis allows their transformation into granulosa cells (ref. 11). Proper differentiation of these cell types defines the somatic cell environment that is essential for germ cell development, hormone production, and establishment of the reproductive tracts. Impairment of lineage specification and function of gonadal somatic cells can lead to DSDs in mammals including humans (ref. 12).

The maintenance of differentiated Sertoli cell fate is critical for the formation of testicular cord and testis development. As the precursor of seminiferous tubules, testis cord formation begins with the aggregation of Sertoli cells. These Sertoli cells change their surface matrix, recognize and adhere to each other, and surround germ cells to form the testis cord (ref. 13). Then, Sertoli cells enter into the differentiation process, which includes a cessation of proliferation, alterations in protein expression and transcription, and functional maturation (ref. 14). Mature Sertoli cells create the blood-testis barrier (BTB) to provide microenvironments for spermatogenesis and secrete many functional products to nourish germ cells and organize the events of spermatogenesis (ref. 15, 16). Thus, any abnormality in the population and function of Sertoli cells will result in aberrant spermatogenesis and eventually infertility.

Sertoli cells are a central target for the regulation of spermatogenesis. In mammals, spermatogenesis employs an elaborate regulatory mechanism, which is controlled by a multitude of regulators, including hormones, growth factors, endotoxins, and proinflammatory cytokines (ref. 11–13, 17–19). These regulators induce a series of alterations into appropriate receptors that are transduced into the interior of the cells by means of post-translational modifications, mainly (de)phosphorylations (ref. 20). Protein phosphorylation is accomplished by the concerted action of protein kinases and protein phosphatases that insert or remove, respectively, the γ-phosphate of ATP into the target amino acid, making protein phosphorylation a reversible process (ref. 21). The importance of protein kinases in spermatogenesis is reflected in infertility phenotypes observed when knocked out, for example SRC family kinases (SFKs) (ref. 22), ATR kinase (ref. 23), serine/threonine kinase STK33 (ref. 24), MAPKAP kinase 2 (MK2)(ref. 25), CDK16 (ref. 26, 27), Aurora kinase B and C (ref. 28), CIB1 (ref. 29) and other kinases. However, little is known about the function of protein phosphatases in spermatogenesis.

Protein phosphatases 4 (Ppp4/PP4/PPX) belongs to the phosphoprotein phosphatases (PPPs) superfamily including seven members (PP1, PP2A, PP2B, PP4, PP5, PP6, and PP7). PPPs dephosphorylate serine and threonine residues, which are responsible for more than 90% of all the dephosphorylations that occur in a cell. PPP4 regulates many cellular functions independently of other related protein phosphatases in the PPP family (ref. 30). PPP4 often functions as a heterotrimeric complex consisting of one evolutionarily conserved catalytic subunit (PPP4C) that associates with two types of regulatory subunits, which are ubiquitously expressed in all cell types (ref. 31). Emerging evidence indicates that PPP4 may play a role in the DNA damage response and regulation of chromatin activities. Several cellular signaling routes, including NF-kappaB JNK pathway, apoptotic signaling and the target of rapamycin (TOR) pathways appear to be regulated by PPP4 (ref. 32–37). However, the physiological role of PPP4 in mammalian gonads remains unclear.

In the present study, we investigated the function of the PPP4 during testis development in mice. We found that constitutive inactivation of PPP4, by deletion of its catalytic subunit *Ppp4c* exon 3 in Sertoli cells after sex determination, caused the reprogramming of Sertoli cells to granulosa-like cells postnatally, which in turn led to dysgenesis of the testes, testes to ovary-like gland transformation and male infertility. These findings report the critical role of PPP4 in preventing female reprogramming in the mammalian testis after sex determination and protecting male fertility.

Results

Generation of *Ppp4c* conditional knockout (*Ppp4c*^{flox}) mouse strain

Using CRISPR/Cas9 technology, a *Ppp4c* allele in which exon 3 is flanked by *lox*P sites (Fig. 1A-D) was introduced into the mouse germ line. Expression of Cre recombinase results in deletion of exon 3 and frame-shift mutation (Fig. 1E) and generates an allele (*Ppp4c*$^{\Delta}$) leading to inactivation of PPP4C protein.

Germ-line generation of the *Ppp4c*$^{\Delta}$ allele was achieved by using the oocyte-specific *Zp3-Cre* transgene. We found that *Ppp4c*$^{+/\Delta}$animals from *Ppp4c*$^{+/\Delta}$ × *Ppp4c*$^{+/\Delta}$ crosses were viable and phenotypically similarly normal, however, *Ppp4c*$^{\Delta/\Delta}$ embryos died during the embryo stage (data not shown), indicating that PPP4 may play an essential role in embryogenesis.

Conditional knockout of *Ppp4c* in Sertoli cells results in impaired testis development and infertility in mice

To address the role of PPP4 in Sertoli cells and male reproduction, we introduced the Sertoli cell-expressed *Amh-Cre* transgene into the *Ppp4c*^{flox} strain to obtain *Ppp4c*$^{flox/flox}$*,Amh-Cre* males (*Ppp4c Amh-cKO*). A previous study reported that Cre recombinase was specifically expressed in Sertoli cells in *Amh-Cre* mice at E14.5 (ref. 38).

We found that *Ppp4c*$^{flox/flox}$*,Amh-Cre* animals were fully viable. No gross abnormalities of external genitalia were observed in 2-month-old *Ppp4c*$^{flox/flox}$*,Amh-Cre* males (Fig. 2*A*). However, the testes size from the *Ppp4c*$^{flox/flox}$*,Amh-Cre* was dramatically smaller (Fig. 2*B, C*), and testes weight was only ≈4% of that of control littermates (Fig. 2*D*). The rest of the reproductive tract including vas deferens, epididymis, and seminal vesicles was observed in the mutants (Fig. 2*B*). Thus, the regression of the Müllerian duct system and Wolffian duct differentiation were largely normal. Although the body weight was not changed between mutant and control mice (Fig. 2*E*), the ratio of testes weight/body weight was significantly reduced in *Ppp4c*$^{flox/flox}$*,Amh-Cre* males (Fig. 2*F*). Then, we evaluated the impact of *Ppp4c*-deficiency in Sertoli cells on male fertility using a successive breeding assay. Adult *Ppp4c*$^{flox/flox}$*,Amh-Cre* or littermate control male mice were mated with wild-type female mice. The probability of observing a vaginal plug was not obviously different between the two groups of mice (data not shown). However, after a total of 87 matings, control mice sired 12 pups per litter, whereas the *Ppp4c*$^{flox/flox}$*,Amh-Cre* male mice did not produce any progeny (Fig. 2G and H). These results indicated that *Ppp4c*-deficiency in Sertoli cells could injure testes development and induce male infertility in mice.

Aberrant histology of testes and non-obstructive azoospermia (NOA) of *Ppp4c*$^{flox/flox}$*,Amh-Cre* males

To investigate the functions of PPP4 in spermatogenesis, the histology of testes from control and *Ppp4c*$^{flox/flox}$*,Amh-Cre* mice was examined. Histologically, *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes from 2-month-old animals bore no resemblance to age-matched control testes. Mutant testes completely lacked the normal tubular architecture observed in control testes (Fig. 3*A, B*and *C*) and consisted primarily of scattered cells and only some tubular architecture (Fig. 3D*, E* and*F*). In addition, mature spermatozoa with crescent-shaped heads were identified in the control epididymis (Fig. 3G). In contrast, no spermatozoa were found in the *Ppp4c*$^{flox/flox}$*,Amh-Cre* epididymis (Fig. 3*H*). This aberrant histology exhibited in

Ppp4c$^{flox/flox}$*,Amh-Cre* males was very similar to human NOA, which is one of the major causes of male infertility in humans.

Ectopic expression of FOXL2 protein in the testes of *Ppp4c*$^{flox/flox}$*,Amh-Cre* mice

To examine the cell types of aberrant *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes, the expression of several germ and somatic cell marker proteins was examined by immunostaining and western blotting. Germ cell-specific marker MVH was detected in the germ cells of the control testes (Fig. 4A, arrow), whereas no MVH signal was detected in the *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes (Fig. 4B). This result indicated that the germ cell was completely lost in the PPP4C-deficient testes. In normal testes, steroidogenic enzyme 3β-HSD was specifically expressed in Leydig cells with a strong signal (Fig. 4C, arrow). However, in *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes, partial cells showed strong signals of 3β-HSD (Fig. 4D, arrow), but more disorganized cells showed weak signals (Fig. 4D, arrowhead). Normally, SOX9 (Fig. 4E, arrow) and WT1 (Fig. 4G, arrow) proteins are specifically expressed in Sertoli cells of seminiferous tubules. However, almost no SOX9 signals were detected in the *Ppp4c*$^{flox/flox}$*, Amh-Cre* testes except for some remnant tubular architecture (Fig. 4F). In comparison, more weak signals of WT1 were observed in the mutant testes (Fig. 4H, arrow).

In mammalian females, 3β-HSD shows a low level in granulosa cells and oocytes of fetal mouse ovaries, but a higher level after theca cell recruitment and formation of the first antral follicles (ref. 12). Additionally, WT1 is initially expressed in somatic cells of bi-potential gonads. And in adults, its expression is maintained in ovarian granulosa cells and testicular Sertoli cells. However, SOX9 is exclusively expressed in Sertoli cells of testes, not in somatic cells of the ovary. Considering the above facts, we surmised that the WT1-positive or 3β-HSD-positive cells may be granulosa-like cells or theca-like cells.

To test these hypotheses, we first examined gonads of *Ppp4c*$^{flox/flox}$*,Amh-Cre* males for the presence of FOXL2, a female-specific transcription factor expressed in the nuclei of granulosa cells and theca cells(ref. 11, 39, 40), two somatic cell types of the ovarian follicle (Fig. 4K). No FOXL2 signals were found in control testes (Fig. 4I). However, abundant cytoplasmic FOXL2-positive cells were present within mutant testes (Fig. 4G, arrow). Additionally, western blot results also showed FOXL2 expression in *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes with a larger size compared to that of the normal ovary (Fig. S1). Previous studies reported that post-translational regulation (including phosphorylation) of FOXL2 could change its subcellular localization from normal nuclear distribution to cytoplasmic mislocalization (ref. 41-44). So cytoplasmic location and larger amount of FOXL2 protein may be a result of its phosphorylation. Besides, western blot results showed that aromatase protein CYP11A1 was strongly expressed in mutant gonads, which was robustly expressed in granulosa cells of the ovary (Fig. S1). These results indicated that loss of the PPP4C in mouse Sertoli cells activated *FOXL2* and reprogrammed testicular somatic cells into granulosa-like cells and theca-like cells.

PPP4C maintains SOX9 and suppresses FOXL2 expression in postnatal Sertoli cells

Next, we examined the time course of this aberrant testis development and the timing of FOXL2 induction. At postnatal day 7 (P7), although seminiferous tubules *were present in Ppp4c$^{flox/flox}$,Amh-Cre* testes, partial tubules *showed* damaged architecture (Fig. S2). PPP4C and SOX9 double staining experiments showed that all SOX9-positive Sertoli cells in *Ppp4c$^{flox/flox}$,Amh-Cre* testes were PPP4C-negtive, indicating specific deletion of PPP4C in Sertoli cells of mutant mice (Fig. S3). In **control testes** at P7**, Sertoli cells strongly expressed SOX9, whereas FOXL2 was undetectable (**Fig.5**A-D, arrow).** Surprisingly, *in Ppp4c$^{flox/flox}$,Amh-Cre* testes at P7, we found some intratubular cells with typical Sertoli cell features including tripartite nucleoli, co-expressed SOX9, and weak FOXL2 (Fig.5 E-H, arrow), or lacked SOX9 and weakly expressed FOXL2 (Fig.5 E-H, arrowhead). Besides, FOXL2 expression was also observed in interstitial cells between seminiferous tubules, but not in Leydig cells of control testis. Meanwhile, granulosa cells and theca cells in the control ovary expressed FOXL2 but lacked SOX9 (Fig. 5I-L, arrowhead).

By 1-3months *Ppp4c$^{flox/flox}$,Amh-Cre* testes increasingly lost normal tubular architecture and few SOX9-positive cells remained and most remnant cells strongly expressed FOXL2 (Fig. S4). Histological analysis of mutant gonads is shown in Fig. S2. These results showed that foetal loss of PPP4C caused postnatal Sertoli cells to lose the male-promoting SOX9 and instead express the female-promoting FOXL2.

Ppp4c deletion in Sertoli cells results in damaged formation of BTB and functional change of Sertoli cells

The above experiments found partially damaged architecture in *Ppp4c$^{flox/flox}$,Amh-Cre* testes at P7. One of the major functions of Sertoli cells is to form the structure of the BTB, which, when disrupted, results in germ cell death and spermatogenic defects. To test whether the formation of BTB was damaged in *Ppp4c*-deficient testes, we detected the expression of gap junction protein Connexin 43 (CX43) which is a BTB-constituted protein that not only modulates the BTB integrity (ref. 45), but also maintains the homeostasis of the BTB via its effects on tight junction reassembly (ref. 16). Here, in the seminiferous epithelium of control males, CX43 was found to be immunolocalized between Sertoli cells and spermatogonia/primary spermatocytes (Fig. 6A-C, G-I, arrow). In contrast, no immunostaining at all was detected in seminiferous tubules from *Ppp4c$^{flox/flox}$, Amh-Cre* mice at P7, only few interstitial cells displaying weak CX43 signals (Fig. 6D-F, arrowhead), indicating that neither Sertoli cells nor spermatogonia were able to synthesize CX43 protein. Besides, at 2-month-mutant testes, although CX43 signals were found in remnant cells, they displayed several non-continuous punctate dots signals like that in the control (Fig. 6J-L, arrow). Western blot results also showed that expression of CX43 was decreased in mutant testes compared with the control at 2 months (Fig. S1). In addition, we also found that stem cell factor (SCF), a paracrine growth factor normally produced by Sertoli cells, showed aberrant

increased secretion in intratubular cells and interstitial cells of mutant testes compared with the control (Fig. S5). These results indicated that damaged formation of BTB and functional change of Sertoli cells emerged in *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes after PP4 inactivation.

Ppp4c deletion in Sertoli cells results in partial Sertoli cell apoptosis and further massive germ cell death

We considered that integrity disruption of the BTB would result in germ cell death and spermatogenic defects, then, cellular apoptosis in the testes was analyzed with TUNEL staining. The apoptotic cells were increased significantly in the testes of *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes at P7 (Fig. 7D, arrow), compared with the control (Fig. 7A, arrow). However, no obvious apoptotic difference was observed between the testes of *Ppp4c* mutant (Fig. 7E and F) and control (Fig. 7B and C) at 1-2 months. Furthermore, we also detected another apoptosis marker protein cleavage of caspase 3 (Fig. S6). We found that apoptotic signals in mutant testes were dramatically increased relative to the control at P7. Next, we analyzed the cell types of abundant apoptotic cells existing in *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes at P7. Co-staining experiments showed that a few TUNEL-positive cells expressed Sertoli cell-specific protein SOX9 (Fig. S7), and most TUNEL-positive cells expressed germ cell marker protein MVH compared with the control (Fig. S8). These results suggested that *Ppp4c* deletion in Sertoli cells at an early stage of development would result in partial Sertoli cell apoptosis, further massive germ cell death and spermatogenic defects.

CTNNB1 is not involved in Foxl2 expression in *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes

Our previous study showed that, by constitutive activation of Ctnnb1 in Sertoli cells, accumulation of CTNNB1 protein in the nuclei of Sertoli cells led to the transformation of testis Sertoli cells to ovarian granulosa-like cells by inducing Foxl2 expression (ref. 8). Here we found that CTNNB1 protein was localized at the plasma membrane, not the cell nucleus, of Sertoli cells and germ cells in *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes (Fig. 8D-F, arrow) at P7, similar to control testes (Fig. 8A-C, arrow). At 2 months, although CTNNB1 protein was dramatically decreased in *Ppp4c*-mutant testes (Fig. 8J-L, arrow) compared with the control (Fig. 8G-I, arrow), only weak signals were found at the plasma membrane of scattered cells, suggesting that CTNNB1 protein may play a cell adherent role in *Ppp4c*-mutant testes. These findings indicated that the transformation of testis Sertoli cells to ovarian granulosa-like cells by inducing Foxl2 expression in *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes is not a result of the dysregulation of CTNNB1 expression.

Phosphorylation modification of histone protein is altered in *Ppp4c*$^{flox/flox}$*,Amh-Cre* testes

PPP4 belongs to the phosphoprotein phosphatases (PPPs) superfamily and evidence indicates that PPP4 may play a role in the regulation of chromatin activities. Here, western blot results showed that the histone H3 and H2A protein levels were decreased in the *Ppp4c*$^{flox/flox}$*;Amh-Cre* testes compared with the control testes (Fig. S1). Surprisingly, the expression of pH3 and γH2AX was completely absent in the mutant testes, indicating that protein phosphorylation modifications of histones were changed. These results suggested that PPP4 may be involved in the aberrant testis development and the transformation of testis Sertoli cells to granulosa-like cells through regulation of chromatin activities.

Discussion

Gonadal somatic cells initiate sex-specific differentiation during gonadal sex determination with the specification of supporting cell lineages: Sertoli cells in the testis vs granulosa cells in the ovary. The supporting cell lineages then facilitate the differentiation of the steroidogenic cell lineages, Leydig cells in the testis and theca cells in the ovary. Impairment of lineage specification and function of gonadal somatic cells can lead to DSDs and fertility defects in humans(ref. 12).

According to previous findings from human DSD patients and mouse models, expression of a majority of key genes is involved in testis development including transcription factors SRY (ref. 46, 47), SOX9 (ref. 48–50), SOX3 (ref. 9), SOX8 (ref. 51), DMRT1 (ref. 11), GATA4 (ref. 52)), signaling molecules DHH (ref. 53, 54), FGF9 (ref. 55)), nuclear receptor NR0B1 (ref. 56), and kinase MAP3K4 (ref. 57). Their mutations or deficiency would result in XY gonadal dysgenesis. However, the function of protein phosphatases during gonad development remains unclear.

Here we show that sexual fate was regulated by protein phosphatases in the testis: loss of the catalytic subunit of protein phosphatases 4 (PPP4C) in mouse Sertoli cells activates FOXL2 and reprograms Sertoli cells into granulosa-like cells. PPP4-deficiency in Sertoli cells caused the dysfunctions of Sertoli cells including the loss of expression of Sertoli cell-specific genes, damage of BTB formation, and dysgenesis of the testes. In this environment, theca cells form from Leydig cells probably due to effect of reprogrammed granulosa cells, germ cells appeared feminized but ultimate lose. Thus, PPP4 was essential for maintaining mammalian testis determination, and competing regulatory networks maintain gonadal sex long after the fetal choice between male and female.

It is reported that adhesion and transcription factor CTNNB1 regulates differentiation of granulosa cells in the absence of SRY, and overactivation of CTNNB1 in the presence of SRY leads to granulosa formation prior to sex determination (ref. 58). And our previous study found CTNNB1 overactivation in Sertoli cells after sex determination can also direct the transformation of testis Sertoli cells to ovarian granulosa-like cells by inducing FOXL2 expression (ref. 8). In the *Ctnnb1*-activating mouse model, CTNNB1 protein was accumulated in the nuclei of Sertoli cells. However, in *Ppp4c*-mutant testes, CTNNB1 protein was not localized at cell nuclei but at the plasma membrane of Sertoli cells and germ cells similar to control testes, and the expression of CTNNB1 protein was also not affected at P7. These findings

suggested that the transformation of testis Sertoli cells to ovarian granulosa-like cells by inducing FOXL2 expression in *Ppp4c*$^{flox/flox}$;*Amh-Cre* testes was not a result of the dysregulation of CTNNB1 expression.

In addition, the phenotype of DMRT1-deficiency in Sertoli cells mice (ref. 11) is reminiscent of the phenotype in *Ppp4c*$^{flox/flox}$;*Amh-Cre* mice. However, in the PPP4C-deficient mouse model, the FOXL2 expression was identified earlier than in the DMRT1-deficient mice and the tubular structure was completely disrupted at 1 month. The phenotype was more severe than that of the DMRT1-deficient mouse model. Thus, the dysgenesis of the testes in *Ppp4c Amh-cKO* mice was also probably not related to DMRT1.

Inactivation of PPP4 causes the reprogramming of Sertoli cells to granulosa-like cells, which may result from the PPP4C or PPP4 molecule itself suppressing SOX9 expression directly or indirectly and in turn inducing FOXL2 expression in Sertoli cells. Besides, evidence indicates that PPP4 may play a role in the regulation of chromatin activities. Correspondently, we found that the histone H3 and H2A protein levels were decreased in the *Ppp4c*$^{flox/flox}$;*Amh-Cre* testes. Even the expression of pH3 and γH2AX was completely absent in the mutant testes, indicating that protein phosphorylation modifications of histones were changed. Therefore, another mechanism of this reprogramming in PPP4c-deficient mice was probably related to protein phosphorylation modification of PPP4 on key genes involved in testis development and sex reversal. Therefore, the downstream target genes through which the PPP4 or PPP4C acts to regulate the lineage transition between Sertoli and granulosa-like are still unclear and require further investigation.

Conclusion

The analysis presented here demonstrates that protein phosphatases are essential for Sertoli cell lineage maintenance and male fertility. Protein phosphatases PPP4 could maintain SOX9 expression in Sertoli cells directly or indirectly. Deletion of PPP4 in Sertoli cells after sex determination causes the reprogramming of Sertoli cells to granulosa-like cells postnatally, which in turn leads to testicular BTB structure disruption, germ cell loss and ultimate testes to ovary-like gland transformation. Moreover, because many genes implicated in this study are evolutionarily conserved, similar antagonism between PPP4 and FOXL2 for control of gonadal sex may therefore be extended to other mammals. Reprogramming due to inactivation of PPP4 also may help explain the etiology of disorders of sexual differentiation and male infertility.

Materials And Methods

Mice

All animal work was carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee at the Institute of Zoology, Chinese Academy of Sciences (CAS). All mice were

maintained in a C57BL/6;129/SvEv mixed background. DNA isolated from tail biopsies was used for genotyping. Genotyping was performed by PCR as described previously (ref. 59).

Tissue collection and histological analysis

Testes were dissected from *Ppp4c-Amh cKO* and control mice immediately after euthanasia and fixed in 4% paraformaldehyde for up to 24 h, stored in 70% ethanol, and embedded in paraffin. Five-micrometer-thick sections were cut and mounted on glass slides. After deparaffinization, the sections were processed for hematoxylin-eosin (H&E) staining, immunohistochemistry (IHC) or immunofluorescent analysis.

Antibodies

Mouse anti-3β-HSD (sc-515120) antibody was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Goat anti-PPP4C antibody (NBP1-50579) was purchased from Novus Biological (Littleton, CO, USA). Rabbit anti-PPP4C (ab70623), rabbit anti-PPP (PP4) (ab16475), rabbit anti-CYP11A1 (ab175408), rabbit anti-SCP3 (ab15093), rabbit anti-3β-HSD (ab65156), rabbit anti- MVH/DDX4 (ab13840), rabbit anti-β-catenin (ab6302), goat anti-FOXL2 (ab5096), rabbit anti- WT1 (ab89901), rabbit anti-c-Kit (ab32363), rabbit anti-Connexin 43 (ab11370), rabbit anti-SCF (ab64677), rabbit anti-Histone H2A (ab18255), rabbit anti-Histone H2B (ab52484) antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-Phospho-Histone H3 (Ser10) (9701S), rabbit anti-Cleaved Caspase-3 (9661), mouse anti-γH2AX (80312s), rabbit anti-γH2AX (9718) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-Histone H3 (17168-1-AP) antibody was purchased from Proteintech Group (Wuhan, China). Mouse anti-β-Actin (AF0003), rabbit anti-α-Tubulin (AF0001) antibodies were purchased from Beyotime Biotechnology (Shanghai, China). Rabbit anti-SOX9 (AB5535) antibody was purchased from Merck Millipore (Billerica, MA, USA)

Immunofluorescence (IF), immunohistochemistry (IHC) and hematoxylin-eosin staining (H&E)

The IF, IHC and H&E assays were performed as previously described (ref. 59). The IF images were taken immediately using an LSM 780/710 microscope (Zeiss, Oberkochen, Germany) or SP8 microscope (Leica, Wetzlar, Germany). The IHC and H&E images were acquired using a Nikon 80*i* inverted microscope equipped with a CCD camera (Nikon, Tokyo, Japan).

Western blot analysis

The Western blot assays were performed as previously described (ref. 59). The protein lysates (15 μg) were separated via SDS-PAGE and electro transferred to a nitrocellulose membrane. The membrane was scanned using the ODYSSEY Sa Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

Experiments were repeated at least three times. Three to five control or mutant testes were randomly used for immunostaining or western blot at each time point. The quantitative results are presented as the

mean ± SEM. Statistical differences were calculated by two-tailed unpaired t-test for two datasets. *P* values < 0.05 (*) or 0.01(**) were considered to be significant.

Abbreviations

DSD: disorders of sexual development

BTB: blood-testis barrier

NOA: non-obstructive azoospermia

IF: immunofluorescence

IHC: immunohistochemistry

H&E: hematoxylin-eosin staining

Declarations

Ethics approval and consent to participate

All mouse experiments and procedures were approved by the Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences.

Consent for publication Not applicable. **Availability of data and materials**

The datasets generated/analyzed during the current study are available the corresponding author on reasonable request.

Competing interests

All authors declare that they have no conflict of interest and agree to publish these data.

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Authors' contributions

Qing-Yuan Sun, Xiao-Fang Sun and Zhen-Bo Wang designed the experiments, Ming-Zhe Dong produced the flox mice, Feng Han, Ming-Zhe Dong and Wen-Long Lei conducted experiments and wrote the manuscript draft, Fei Gao and Heide Schatten made discussion and helped editing the manuscript.

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Figures

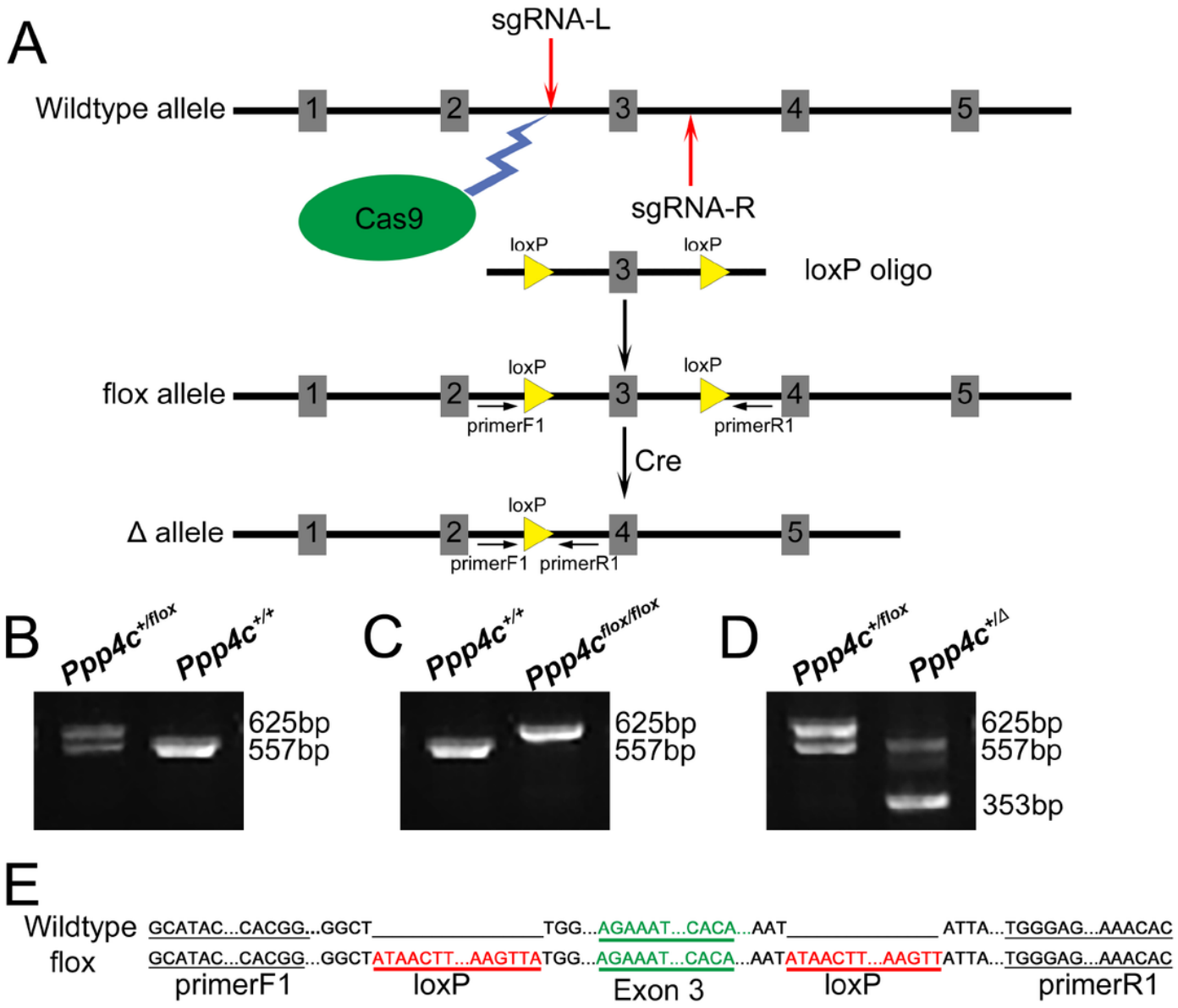

Figure 1

Generation of the Ppp4cflox mouse strain. (A) Scheme for generating animals carrying the conditional knockout allele (Ppp4cflox) and the recombined allele (Ppp4cΔ). (B), (C) and (D) PCR genotyping of wild-type, Ppp4cflox and Ppp4cΔ allele using primers primerF1 and primerR1. In wild-type mice, 557bp band was amplified. In Ppp4cflox/flox mice (as control mice), only 625bp bands were detected. A 557bp and a 625bp bands were amplified in Ppp4c+/flox mice. And in Ppp4c +/Δ mice, A 557bp and a 353bp bands were detected. (E) Genome DNA information of transgenic mouse showed an inserted loxP sequence at each side of Ppp4c gene exon3, learned from genomic sequencing.

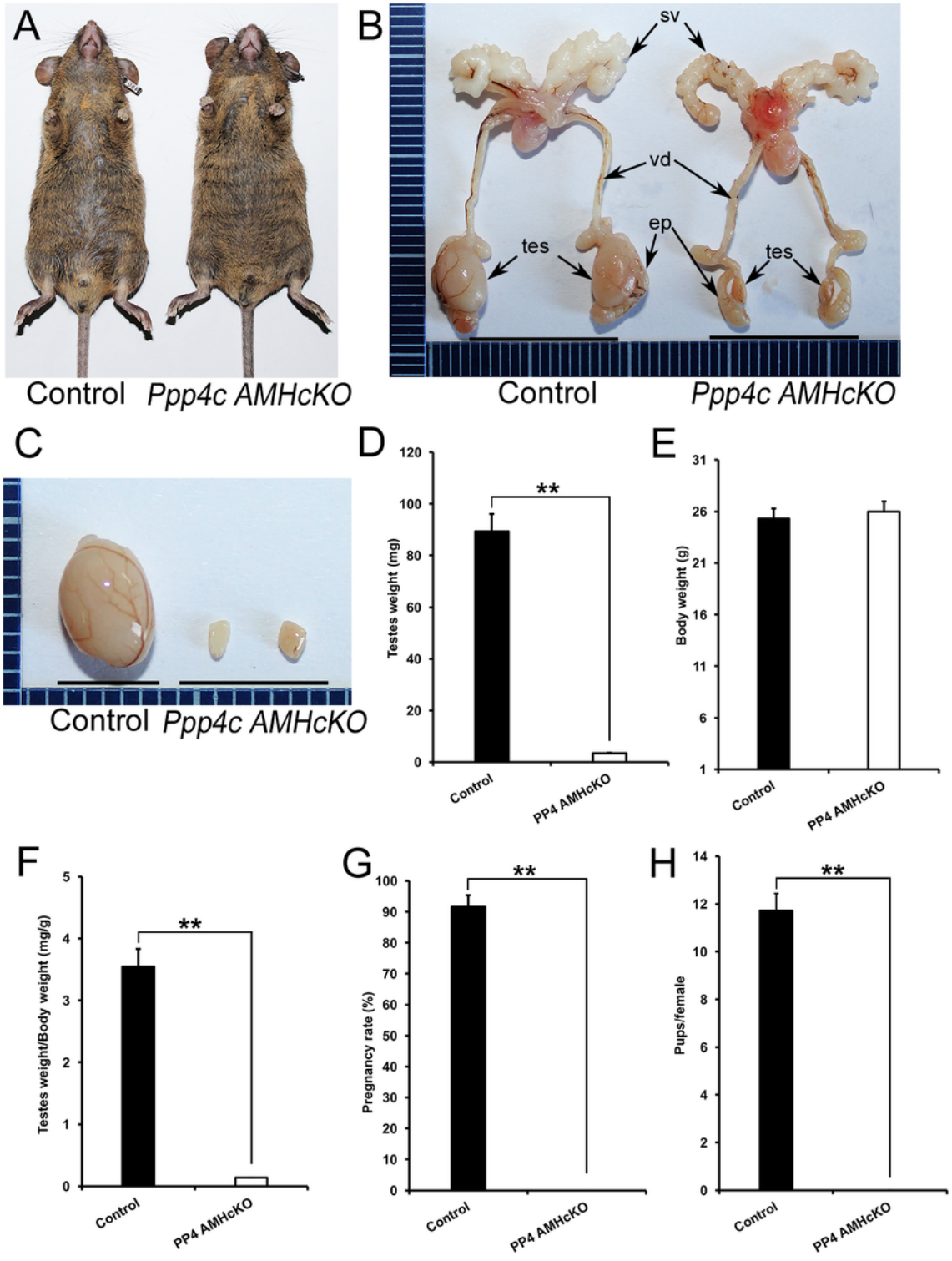

Figure 2

Gross phenotype of 2-months-old Ppp4cflox/flox, Amh-Cre males. Normal external genitalia of Ppp4cflox/flox, Amh-Cre males (A), reproductive tracts from control male and mutant male displaying reduced testes sizes but normal development of both vas deferens, seminal vesicle and epididymis in mutant (B and C), normal body weight (E) but severely reduced weight of testes (D) and ratio of testes/body weight (F) from Ppp4cflox/flox, Amh-Cre males (N≥5). Using a successive breeding assay,

control mice sired 12 pups per litter, whereas the Ppp4cflox/flox, Amh-Cre male mice did not produce any progeny (G and H) (N=5). Ep, epididymis, tes, testis, vd, vas deferens, sv, seminal vesicle. All statistical data are represented as means ± SEM. **p<0.01.

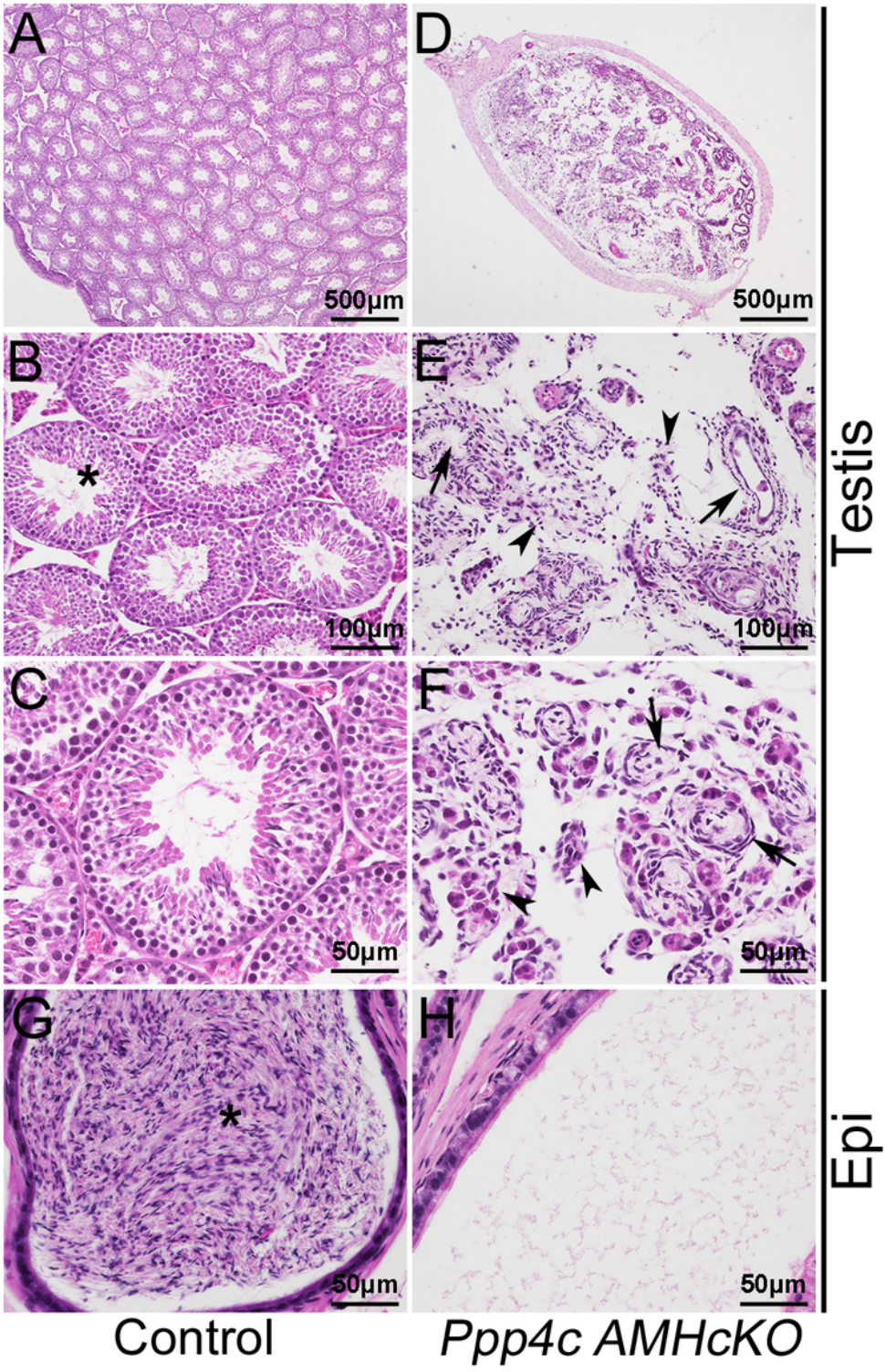

Figure 3

Aberrant histology of testes and NOA in Ppp4cflox/flox,Amh-Cre males at 2 months. Testis sections stained with H&E showing normal tubular architecture (asterisk) and spermatogenic cells in control testes

(A, B and C), while mutant testes almost completely lacked the normal tubular architecture (D, E and F) and consisted primarily of scattered cells (D and F, arrowhead) and only some tubular architecture (D and F, arrow). Corresponding sections of cauda epididymidis showed that sperm could only be detected in control (G, asterisk), but not in Ppp4cflox/flox,Amh-Cre males (H).

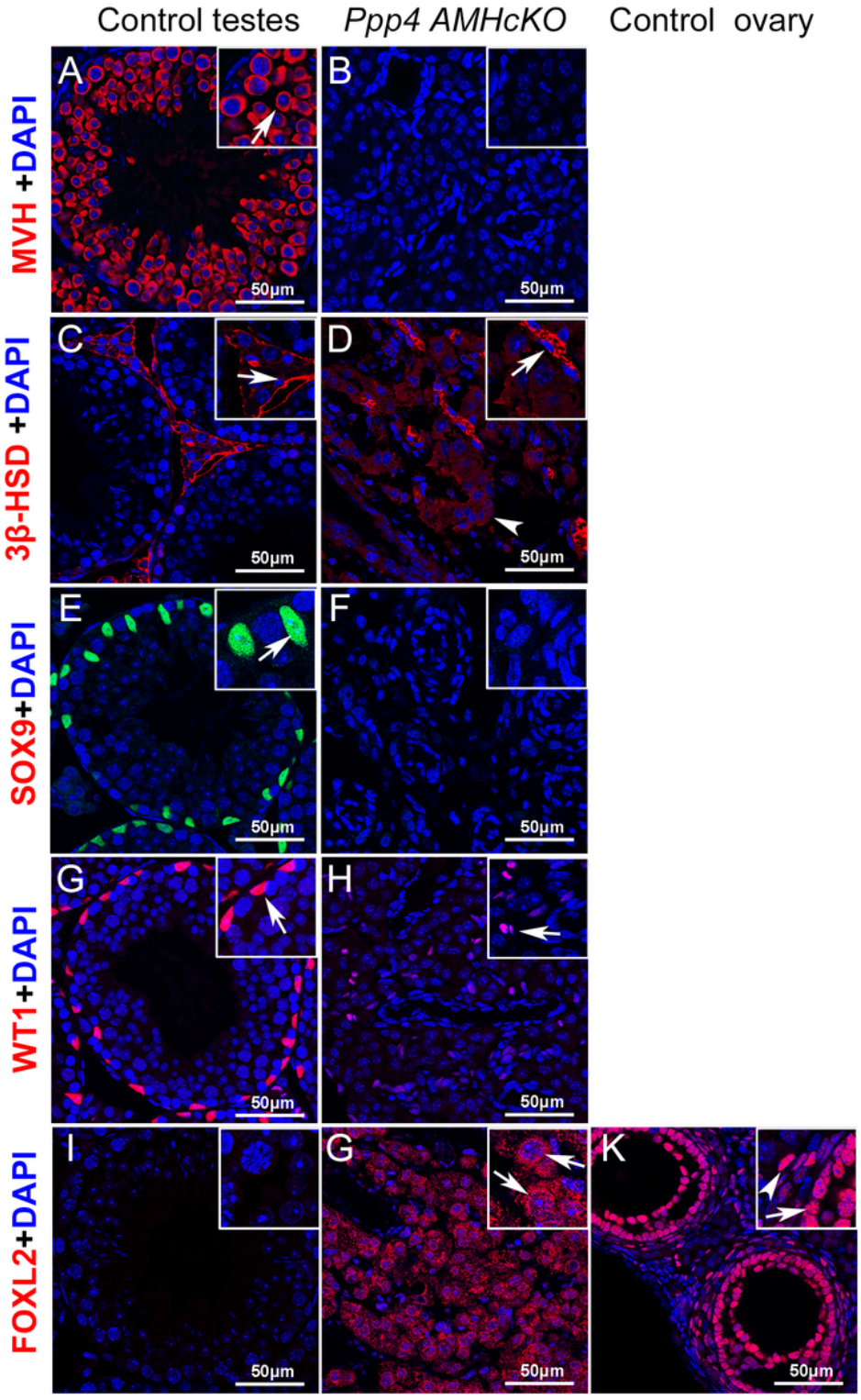

Figure 4

Identification of remnant cells and ectopic expression of FOXL2 protein in the testes of Ppp4cflox/flox, Amh-Cre mice at adulthood. Germ cell-specific marker MVH was observed in the testes of control mice (A, arrow), whereas no MVH signal was found in the Ppp4cflox/flox, Amh-Cre testes (B). In normal testes, steroidogenic enzyme 3β-HSD was specifically expressed in Leydig cells with a strong signal (C, arrow). Although, in mutant testes, partial cells showed strong signals of 3β-HSD (D, arrow), more disorganized cells showed weak signals (D, arrow head). SOX9 (E, arrow) and WT1 (F, arrow) proteins were specifically expressed in Sertoli cells of seminiferous tubules at normal levels. No SOX9 signal was detected in the Ppp4cflox/flox, Amh-Cre testes (F). However, several weak WT1 signals were observed in the mutant testes (H, arrow). In normal adult ovary, FOXL2 protein was expressed in nuclei of granulosa cells (K, arrow) and theca cells (K, arrowhead), which did not exist in control testes (I). However, abundant cytoplasmic FOXL2 signals were observed within remnant cells of adult mutant testes (G, arrow).

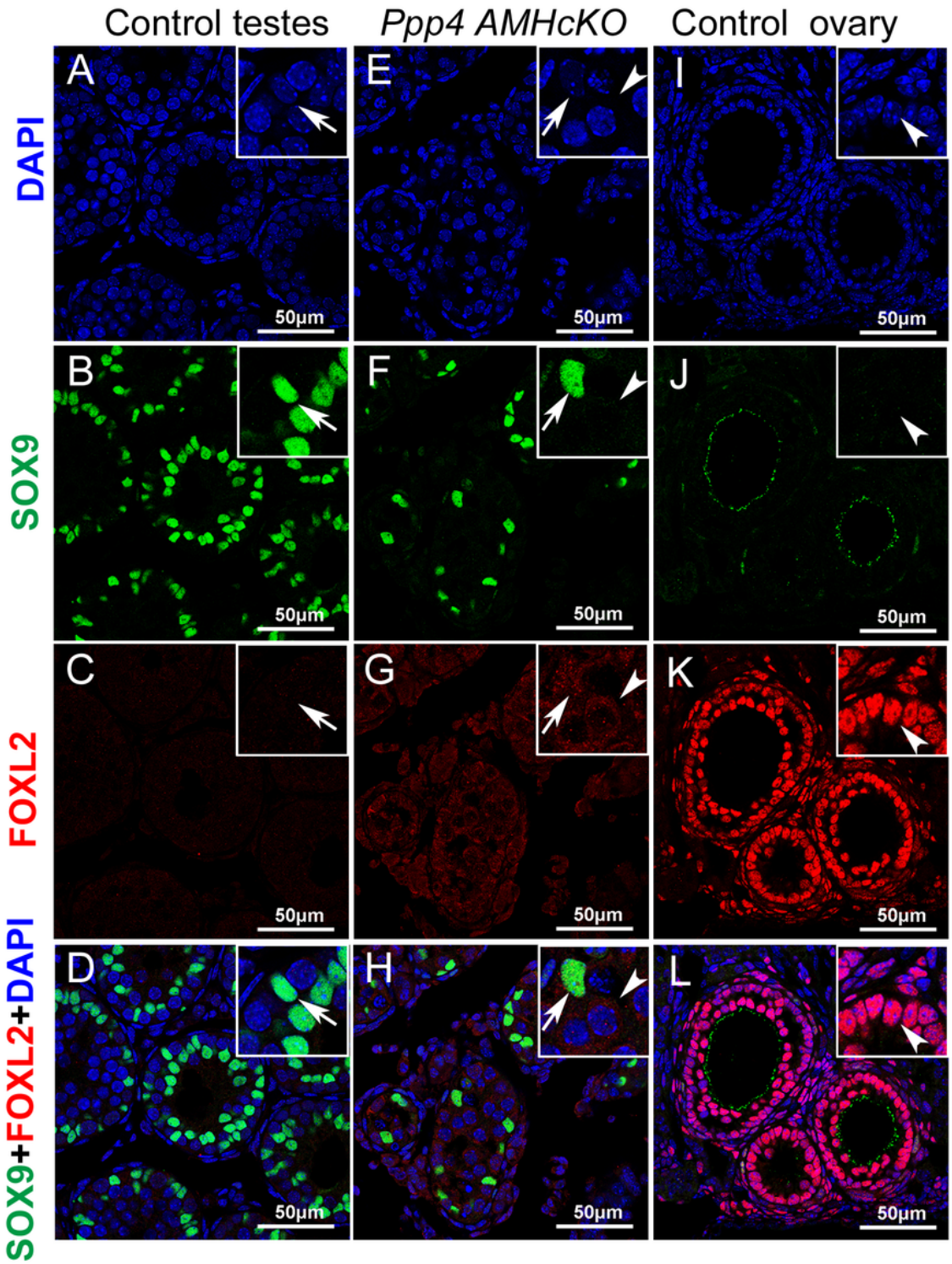

Figure 5

PPP4C maintains SOX9 and suppresses FOXL2 expression in postnatal Sertoli cells at P7. SOX9 and FOXL2 double staining experiment was performed on P7 Ppp4cflox/flox, Amh-Cre testes, control testes and ovary (A-L). Sertoli cells of control testes strongly expressed SOX9, whereas FOXL2 was undetectable (A-D, arrow). FOXL2 but not SOX9 expression was observed in adult granulosa and theca cells of control ovaries (I-J, arrowhead). However, SOX9-positive cells obviously decreased in PPP4c mutant testes

compared with controls (A-H, arrow). In addition, cells expressing FOXL2 or FOXL2 and SOX9 (E-H) were present in Ppp4cflox/flox, Amh-Cre testes.

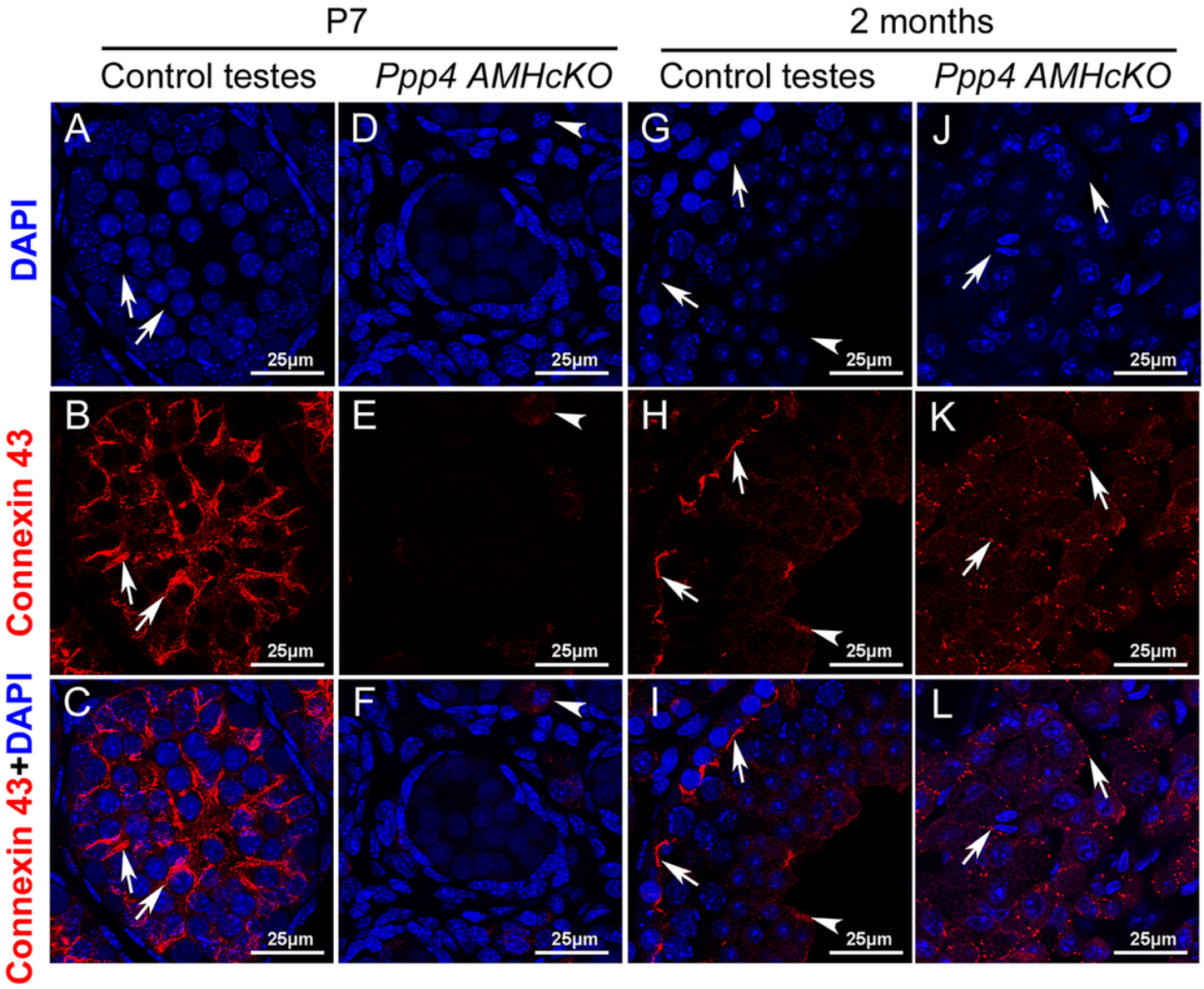

Figure 6

Differential CX43 expression between the testes obtained from control and Ppp4cflox/flox, Amh-Cre mice at P7. In the seminiferous epithelium of control males, CX43 was found to be immunolocalized between Sertoli cells and between Sertoli cells and spermatogonia/primary spermatocytes at P7 (A-C, arrow). In contrast, no immunostaining at all was detected in seminiferous tubules from Ppp4cflox/flox, Amh-Cre mice at P7, only few interstitial cells displaying weak CX43 signals (D-F, arrowhead). In the testes of control males at 2 months, CX43 expression was more predominant at the basal ES (ectoplasmic specialization) region of seminiferous epithelium (G-I, arrow). In the testes of at 2-month-mutant testes, although CX43 expression was found in remnant cells, they displayed several discontinuous punctate signals (J-L, arrow) different from that of the control.

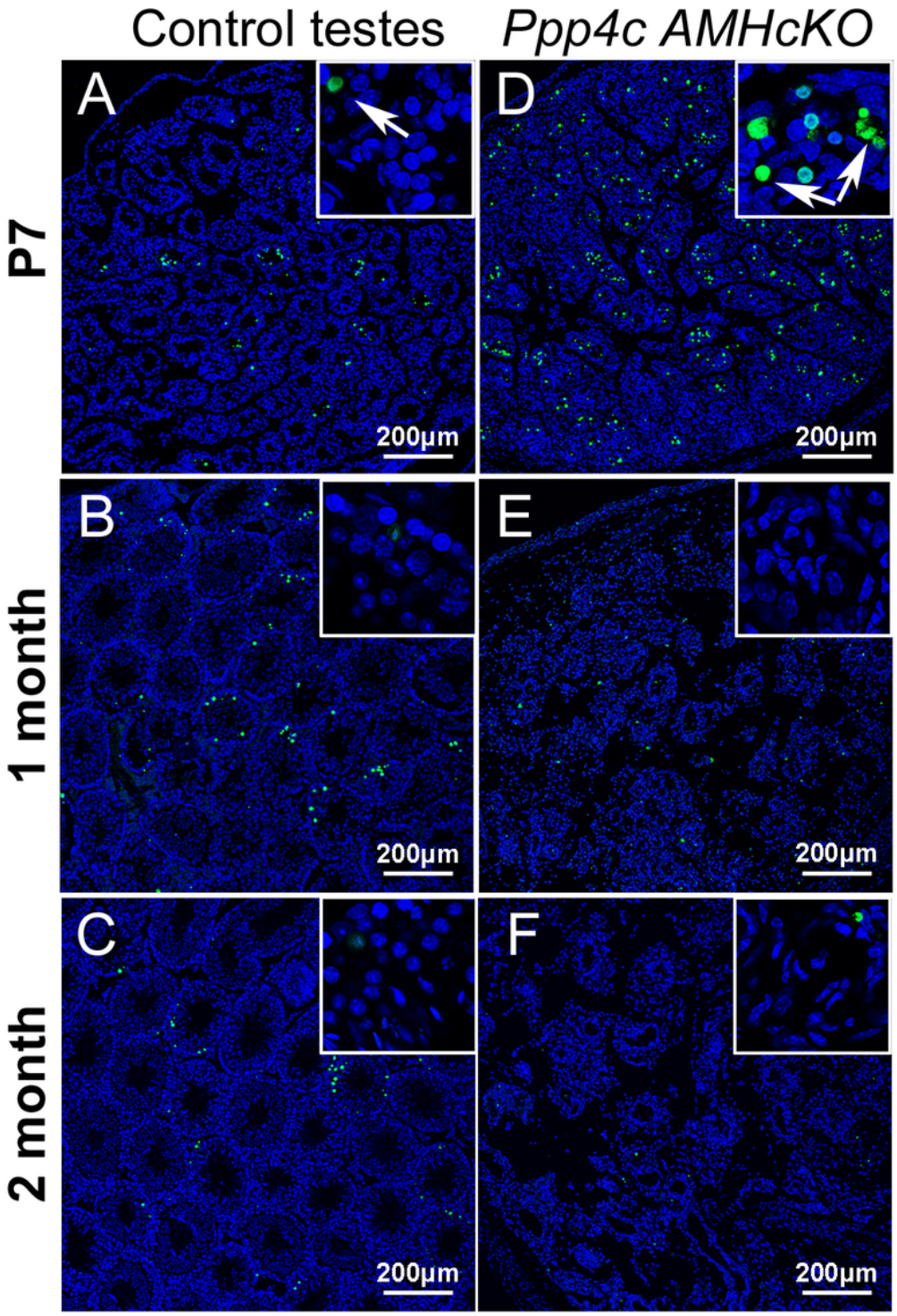

Figure 7

PPP4c deletion in Sertoli cells results in cell apoptosis in the mutant testes by TUNEL staining. Apoptotic cells in the seminiferous epithelium were detected by TUNEL staining (positive cells are green). The apoptotic cells were increased significantly in the testes of Ppp4cflox/flox, Amh-Cre testes at P7 (D, arrow), compared with controls (A, arrow). However, no obvious apoptotic difference was observed between the testes of ppp4c mutant (E and F) and control testes (B and C) at 1month and 2 months.

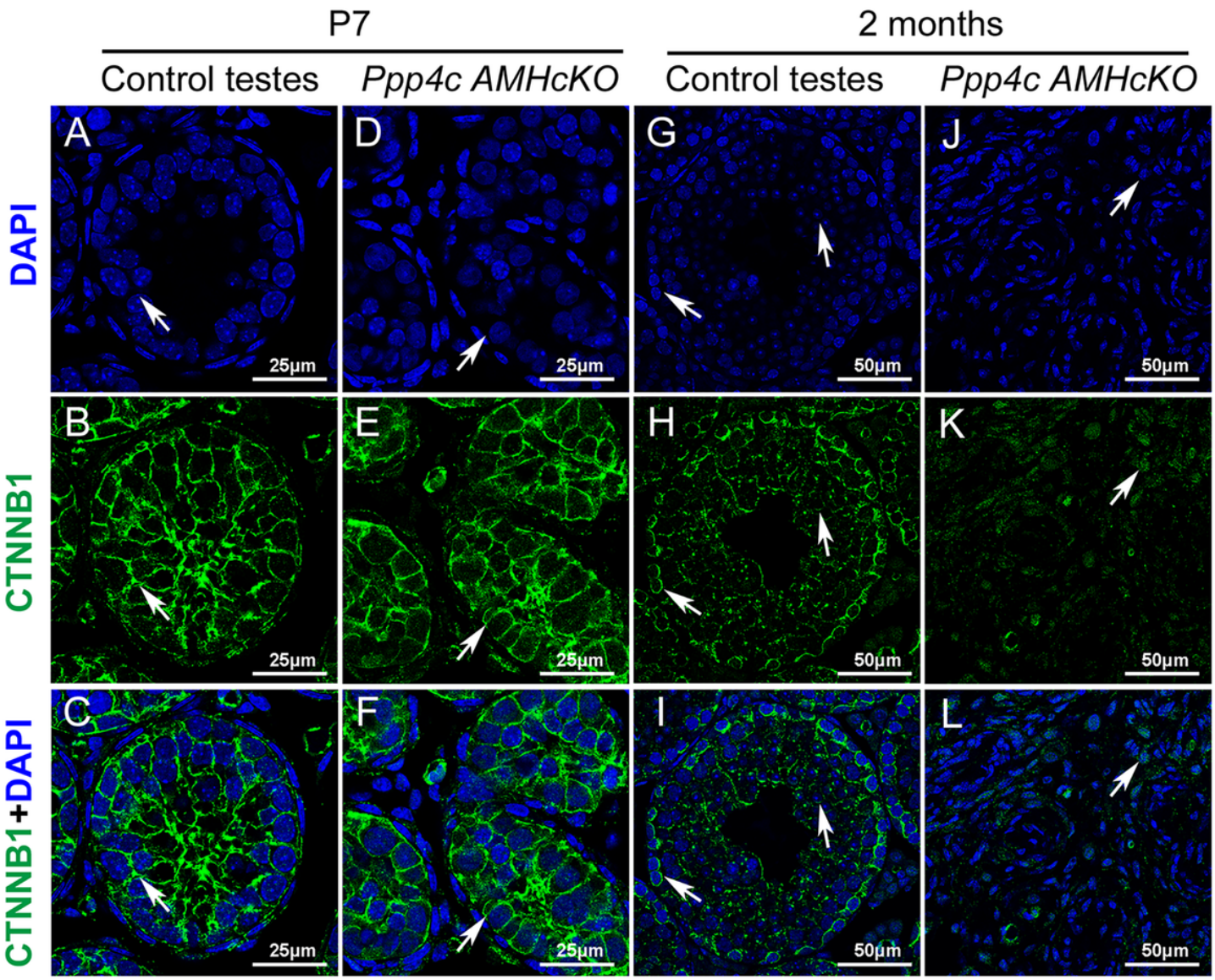

Figure 8

CTNNB1 is not involved in Foxl2 expression in Ppp4c mutant testes. CTNNB1 expression detected by immunofluorescence at the plasma membrane of Sertoli cells and germ cells of Ppp4c mutant testis at P7 (B and C, arrow), similar to control testes (E and F, arrow). At 2 months, CTNNB1 protein was dramatically decreased in PPP4c mutant testes compared with the control (H and I, arrow), and only weak signals were found at the plasma membrane of scattered cells (K and L, arrow).

Supplementary Files

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