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Wt1 negatively regulates β-catenin signaling during testis development

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Abstract

β-Catenin, as an important effector of the canonical Wnt signaling pathway and as a regulator of cell adhesion, has been demonstrated to be involved in multiple developmental processes and tumorigenesis. β-Catenin expression was found mainly on the Sertoli cell membrane starting from embryonic day 15.5 in the developing testes. However, its potential role in Sertoli cells during testis formation has not been examined. To determine the function of β-catenin in Sertoli cells during testis formation, we either deleted β-catenin or expressed a constitutively active form of β-catenin in Sertoli cells. We found that deletion caused no detectable abnormalities. However, stabilization caused severe phenotypes, including testicular cord disruption, germ cell depletion and inhibition of Müllerian duct regression. β-Catenin stabilization caused changes in Sertoli cell identity and misregulation of inter-Sertoli cell contacts. As *Wt1* conditional knockout in Sertoli cells causes similar phenotypes to our stabilized β-catenin mutants, we then investigated the relationship of *Wt1* and β-catenin in Sertoli cells and found *Wt1* inhibits β-catenin signaling in these cells during testis development. *Wt1* deletion resulted in upregulation of β-catenin expression in Sertoli cells both in vitro and in vivo. Our study indicates that Sertoli cell expression of β-catenin is dispensable for testis development. However, the suppression of β-catenin signaling in these cells is essential for proper testis formation and *Wt1* is a negative regulator of β-catenin signaling during this developmental process.

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Keywords

β-Catenin; *Wt1*; Testis; Sertoli cell; Mouse

INTRODUCTION

The testis is essential for the continuation of mammalian species: it secretes hormones and produces sperm. Abnormalities of the testes can lead to various diseases, including infertility, disorders of sexual development (DSD) and cancer, most of which are thought to result from defects that occur during embryonic development. Testis development is unique because it derives from the bipotential gonad primordium, which has the ability to develop as either testis or ovary. The sex-determining gene on the Y chromosome (*Sry*), along with its potential downstream gene *Sox9*, specify Sertoli cells and cause the XY gonad to develop as a testis (Chaboissier et al., 2004; Gubbay et al., 1990; Koopman et al., 1991; Lovell-Badge and Robertson, 1990; Vidal et al., 2001). *Sry* is expressed only during a short window of time in the XY gonad, from E10.5 to E12.5 (Hacker et al., 1995; Koopman et al., 1990). However, *Sox9* expression in the XY gonad starts from E11.5 and is maintained throughout embryogenesis (Kent et al., 1996; Kobayashi et al., 2005; Morais da Silva et al., 1996). After the testis is determined, hormones secreted by Sertoli cells and other differentiated cell types in the XY gonad drive sex differentiation and make XY embryos morphologically very different from XX embryos. Sertoli cells secrete anti-Müllerian hormone (AMH), also called Müllerian-inhibiting substance (MIS) to induce the regression of Müllerian ducts, the precursor of the female reproductive tract (Behringer et al., 1994). Testosterone, produced by Leydig cells, promotes Wolffian duct differentiation into male reproductive tract organs and virilizes the external genitalia. Insulin-like 3, also produced by Leydig cells, together with AMH and testosterone, mediate transabdominal testicular descent into the scrotum (Kobayashi and Behringer, 2003; Nef and Parada, 1999; Nef and Parada, 2000; Zimmermann et al., 1999).

β-Catenin is an intracellular protein that plays important roles in both intercellular adhesion and the canonical Wnt signaling pathway (Morin, 1999; Peifer and Polakis, 2000). It has been demonstrated to be involved in multiple developmental processes and in tumorigenesis. In intercellular adhesion, β-catenin is a component of the cadherin/catenin complexes, which mediate Ca^{2+}-dependent homophilic interactions (Aberle et al., 1996). In the canonical Wnt signaling pathway, β-catenin is a central player involved in the transduction of extracellular signals to the nucleus. In the absence of Wnts, cytoplasmic β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK3β) and casein kinase I (CKI) bound to the adenomatosis polyposis coli (APC) complex and is targeted for degradation through the ubiquitination pathway (Aberle et al., 1997; Morin, 1999; Peifer and Polakis, 2000). Activation of the Wnt pathway inhibits GSK3β, resulting in the stabilization and cytoplasmic accumulation of β-catenin. The stabilized β-catenin translocates into the nucleus and forms a heterodimeric complex with the Tcf/Lef family of DNA-binding proteins to regulate the transcription of downstream target genes, such as *Myc* and *cyclin D1* (He et al., 1998; Tetsu and McCormick, 1999). β-Catenin was previously reported to be expressed in the plasma membrane and cytoplasm of germ cells during testis development. Suppression of Wnt/β-

catenin signaling is necessary for the normal development of primordial germ cells because stabilization of β-catenin in germ cells causes delayed cell cycle progression and results in germ cell deficiency (Kimura et al., 2006). The expression of β-catenin and its potential function in other cell types during testis development has not been studied.

In the present study, we determined the spatiotemporal expression pattern of β-catenin during testis development and found that β-catenin was also expressed in Sertoli cells starting from E15.5. β-Catenin protein was found mainly on the Sertoli cell membrane, but was undetectable in the nucleus. We then used the Cre-*lox*P system to either knockout β-catenin or to express a constitutively active form of β-catenin in Sertoli cells to determine the function of β-catenin during testis formation. We found that deletion of β-catenin in Sertoli cells did not affect testis formation, whereas stabilization of β-catenin in Sertoli cells caused testicular cord disruption, germ cell depletion and inhibition of Müllerian duct regression. β-Catenin stabilization in Sertoli cells caused Sertoli cell identity changes that probably altered inter-Sertoli cell contacts, leading to cord disruption. Our data suggest that inhibition of β-catenin signaling is essential for Sertoli cell and testicular cord maintenance and for germ cell survival during testis development. Interestingly, these phenotypes are similar to Sertoli-specific deletion of Wilms tumor 1 (*Wt1*). We show that Sertoli-specific deletion of *Wt1* causes β-catenin accumulation. These studies provide in vivo experimental evidence that *Wt1* acts as a negative regulator of β-catenin signaling.

MATERIALS AND METHODS

Mice

AMH-Cre$^{tg/+}$ mice and *Catnb*$^{lox(e3)/lox(e3)}$ mice were maintained on a C57BL/6; 129/SvEv mixed genetic background. *Catnb*$^{fx/fx}$ mice were maintained on a C57BL/6 congenic background. Male *AMH-Cre*$^{tg/+}$ mice were mated with female *Catnb*$^{fx/fx}$ or *Catnb*$^{lox(e3)/lox(e3)}$ mice to produce offspring with the genotype of *Catnb*$^{fx/+}$*; AMH-Cre*$^{tg/+}$ or *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$. *Wt1*$^{+/-}$ mice were maintained on a C57BL/6 congenic background. *Wt1*$^{fx/fx}$ mice and *CAGG-CreER*$^{tg/+}$ mice were maintained on a C57BL/6; 129/SvEv mixed genetic background. *Wt1*$^{fx/fx}$ female mice were mated with male mice carrying a *Wt1* null allele and a *CAGG-CreER* transgene to obtain offspring with the genotype of *Wt1*$^{fx/-}$*; CAGGCre-ER*$^{tg/+}$. Genotyping was performed by PCR as described (Brault et al., 2001; Gao et al., 2006; Harada et al., 1999; Hayashi and McMahon, 2002).

Mouse crosses to generate *Sox9*$^{flox/-}$*; AMH-Cre*$^{tg/+}$ mice

Male studs were heterozygous for the *Sox9* flox allele (Chaboissier et al., 2004) and also hemizygous for a Sertoli cell-specific Cre transgene, *AMH-Cre*. Female mice were homozygous for the *Sox9* flox allele and also hemizygous for an oocyte-specific Cre transgene, *Zp3-Cre* (de Vries et al., 2000). Expression of the *Zp3-Cre* transgene in oocytes of *Sox9* flox/flox females leads to recombination of the *Sox9* flox alleles into deletion alleles. When crossed to male studs, mice with the genotype of *Sox9*$^{flox/-}$*; AMH-Cre*$^{tg/+}$, *Sox9*$^{+/-}$*; AMH-Cre*$^{tg/+}$, *Sox9*$^{flox/-}$*;* +/+, and *Sox9*$^{+/-}$*;* +/+ were generated, regardless of their *Zp3-Cre* genotype.

Primary Sertoli cell isolation

A modified method was used to isolate primary Sertoli cells from the testes of 3-week-old animals (van der Wee et al., 2001). Testes were decapsulated under the dissection microscope. The seminiferous tubules were pooled and washed with phosphate-buffered saline (PBS) three times. The tubules then were incubated with 2 mg/ml collagenase I (Sigma) and 0.5 mg/ml DNase I (sigma) in DMEM for 30 minutes at 37°C on a shaker. The tubules were then washed twice with DMEM and further digested with 2 mg/ml collagenase I, 0.5 mg/ml DNase I and 1 mg/ml hyaluronidase type III (Sigma) for 20–30 minutes at 37°C on a shaker. The tubules were allowed to settle and were washed twice with DMEM. The tubules were further digested with 2 mg/ml collagenase I, 0.5 mg/ml DNase I, 2 mg/ml hyaluronidase, 1 mg/ml trypsin for 40–60 minutes at 37°C on a shaker. This final digestion step resulted in a cell suspension containing primarily Sertoli cells and type A spermatogonia. The dispersed cells were then washed twice with DMEM and placed into culture dishes in DMEM containing 10% fetal calf serum and incubated at 37°C and 5% CO_2. Spermatogonia were unable to attach to the dish and were removed after the medium change on the next day. 4OH-Tamoxifen (Sigma, H7904) was dissolved in ethanol to generate a 1 mM stock solution and further diluted to appropriate concentrations prior to use. Recombination was initiated by adding 4OH-TM to cultured Sertoli cells at a final concentration of 1 μM. After 3 days culture, total RNA and protein were extracted as described below.

Western blot analysis

Sertoli cells were washed with ice-cold PBS and lysed at 4°C by shaking robustly in radioimmunoprecipitation assay buffer [RIPA: PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/l EDTA (pH 8.0), 1 mmol/l sodium orthovanadate (pH 10.0), 2 mmol/l phenylmethylsulfonyl fluoride and protease inhibitor cocktail tablet]. Lysates were centrifuged at 16,000 ***g*** for 10 minutes, and the supernatants were subjected to protein content determination using a detergent compatible protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. An equal volume of 2× SDS sample loading buffer containing 100 mmol/l Tris-HCl (pH 6.8), 10% glycerol, 4% SDS, 0.05% Bromophenol Blue and 5% β-mercaptoethanol was added to cell lysates before heating at 95°C for 5 minutes. Aliquots of 20 μg total cell protein were loaded onto 10% SDS-polyacrylamide gels. Proteins were separated by electrophoresis at constant voltage (80–110 V) and electrotransferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C in PBST containing 5% nonfat dried milk and incubated at room temperature for 1 hour with two anti-β-catenin antibodies (a rabbit anti-β-catenin antibody 1:1000, Sigma C 2206, recognizes amino acids 768–781 of human or mouse β-catenin; a mouse monoclonal anti-active β-catenin antibody 1:1000, Upstate #05–665, recognizes the active form of β-catenin dephosphorylated on Ser37 or Thr41) and a mouse anti-β-actin monoclonal antibody (1:10,000, Sigma, A1978). Membranes were washed with PBST buffer three times and incubated at room temperature for 1 hour with an IRDye 800CW conjugated goat anti-rabbit IgG (LI-COR, 926–32211) and an Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes, A21058) at dilutions of 1:2500. After washing, the blots were visualized by scanning using Li-Cor Odyssey Imager (Li-Cor, Lincoln, NE).

Nucleic acid isolation and quantitative reverse transcription-PCR

Total RNA was extracted from cultured Sertoli cells or testes of E14.5 *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ (*n*=3) and *Catnb*$^{lox(e3)/+}$*;* +/+ (*n*=3) animals using a Qiagen RNeasy kit in accordance with the manufacturer's instructions. To quantify gene expression, real-time SybrGreen assays were performed with the isolated RNA. Gene expression was quantified relative to the expression of the gene for *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase). The primers used were: Wt1 (forward, 5′ CCAGTGTAAAACTTGTCAGCGAAA 3′; reverse, 5′ ATGAGTCCTGGTGTGGGTCTTC 3′), β-catenin (forward, 5′ CCGTTCGCCTTCATTATGGA 3′; reverse, 5′ GGGCAAGGTTTCGAATCAATC 3′), Gata4 (forward, 5′ TCCAGTGCTGTCTGCTCTGAAG 3′; reverse, 5′ CTGGCCTGCGATGTCTGAGT 3′), Sf1 (forward, 5′ CTGTGCGTGCTGATCGAATG 3′; reverse, 5′ GCCCGAATCTGTGCTTTCTTC 3′), Dhh (forward, 5′ CCCAACTACAACCCCGACATAA 3′; reverse, 5′ CTTTGCAACGCTCTGTCATCAG 3′), Fgf9 (forward, 5′ CAGCTGTACTGCAGGACTGGATT 3′; reverse, 5′ CGAAGCGGCTGTGGTCTTT 3′), Dax1 (forward, 5′ AGACCCTGCGCTTTGTCAAG 3′; reverse, 5′ CTCCGGGATCTCCATCATCTC 3′), Wnt4 (forward, 5′ CCGGGCACTCATGAATCTTC 3′; reverse, 5′ CACCCGCATGTGTGTCAAG 3′), Cx43 (forward, 5′ CCACTGAGCCCATCCAAAGA 3′; reverse, 5′ TGGTGAGGAGCAGCCATTG 3′), occludin (forward, 5′ CTGCAGGCACACAGGACATG 3′; reverse, 5′ GCCATTCACTTTGCCATTGG 3′), claudin 11 (forward, 5′ CATGGTAGCCACTTGCCTTCA 3′; reverse, 5′ CCAGTCATTGGTGGACGTTGT 3′) and GAPDH (forward, 5′ TTGTCTCCTGCGACTTCAACA 3′; reverse, 5′ ACCAGGAAATGAGCTTGACAAAG 3′).

Tissue collection and histological analysis

Testes were dissected from at least four mutant males at various time-points. Tissues from three to four control littermates were also collected. Tissues were fixed in 4% paraformaldehyde overnight, embedded in paraffin wax and sectioned at 4 μm. After de-waxing, the sections were stained with Hematoxylin and Eosin for histological analyses.

Immunohistochemistry/fluorescence

Immunohistochemical analysis was carried out using the Vectastain ABC (avidin-biotin-peroxidase) kit (Vector Laboratories) as recommended by the manufacturer. Endogenous peroxidase activity was destroyed using 3% hydrogen peroxide for 20 minutes. Antigen recovery was performed by boiling samples in 0.01 M sodium citrate buffer (pH 6.0) for 20 minutes. Sections were incubated with 5% bovine serum albumin in PBS for 30 minutes at room temperature and then incubated for 1 hour at room temperature with either anti-WT1 antibody (Santa Cruz, sc-192), anti- SOX9 antibody (Chemicon, AB5535), anti-3β-HSD (3β-hydroxysteroid dehydrogenase) antibody (Santa Cruz, sc-30821), anti-AMH antibody (Santa Cruz, sc-6886) or anti-GCNA1 (germ cell nuclear antigen 1) antibody (provided by Dr George Enders) at dilutions of 1:100, 1:200, 1:200, 1:100 and 1:50, respectively. After three washes with PBS, the sections were incubated with biotinylated secondary antibody

(Santa Cruz) at a dilution of 1:250 for 45 minutes at room temperature. After incubation with avidin-biotin-peroxidase complex for 45 minutes, the sections were washed with PBS. The color was developed with 3,3′-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) substrate. Samples were counterstained with Harris Hematoxylin. For β-catenin immunofluorescence, rabbit anti-β-catenin antibody (Sigma, C 2206) was used at a dilution of 1:2000 for 1 hour and washed three times in PBS. An Alexa Fluor 488 nm goat anti-rabbit (Molecular Probes, A11008) was used at a dilution of 1:800 for 1 hour and washed twice with PBS. Sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, H1200).

RESULTS

Dynamic expression pattern of β-catenin during testis development

To better understand the function of β-catenin in the developing testis, we first determined the spatiotemporal expression pattern of β-catenin protein during testis development. Consistent with a previous report (Kimura et al., 2006), we observed β-catenin localized to the plasma membrane and cytoplasm of germ cells at all embryonic stages examined (Fig. 1). We also observed expression of β-catenin in Sertoli cells, starting from E15.5 (Fig. 1G,I,K). β-Catenin was localized mainly on Sertoli cell membranes, but not in the nucleus, as shown by a double staining of β-catenin and Sertoli cytoplasmic protein AMH (Fig. 1H,J,L).

β-Catenin deletion in Sertoli cells does not affect testis development

To determine the essential role of β-catenin in Sertoli cells of the developing testis, we specifically deleted β-catenin in Sertoli cells by crossing mice with a floxed β-catenin conditional null allele (Brault et al., 2001) to *AMH-Cre* mice that express Cre only in Sertoli cells after E13.5 (Lecureuil et al., 2002). In *Catnb*$^{fx/fx}$*; AMH-Cre* male mice at E16.5, no β-catenin expression was detected in Sertoli cells but expression of β-catenin in germ cells were unaffected, suggesting that β-catenin was specifically deleted in Sertoli cells (Fig. 2A). Surprisingly, we observed no gross abnormalities of the testes and reproductive tracts of newborn and adult mutant males. In *Catnb*$^{fx/fx}$*; AMH-Cre* males at P0, the testicular cords were normally formed. Well-organized Sertoli cells and germ cells surrounded by a layer of peritubular myoid cells could be found in each cord. In the testes of the adult mutant mice, the seminiferous tubules contained all of the different stages of germ cells (spermatogonia, spermatocytes, spermatids and spermatozoa), suggesting spermatogenesis occurred normally. Sertoli and Leydig cell identity were confirmed by immunostaining of the Sertoli cell marker SOX9 and the Leydig cell marker 3βHSD (Fig. 2B).

Stabilization of β-catenin in Sertoli cells causes testis malformation

To determine the effect of aberrant activation of β-catenin signaling in Sertoli cells, we stabilized β-catenin in Sertoli cells by crossing the *Catnblox(e3)* mouse line to the *AMH-Cre* mice. The *Catnblox(e3)* mouse line contains floxed exon 3 sequences that encode the phosphorylation sites required for β-catenin degradation. Deletion of exon 3 by Cre recombinase leads to the production of stabilized β-catenin (Harada et al., 1999). In *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ testes, we detected the accumulation of β-catenin protein in

Sertoli cells, starting from E13.5. The accumulation of β-catenin initiated in a few Sertoli cells at E13.5 and was found in almost all Sertoli cells in the testicular cords by E15.5. The stabilized β-catenin was localized in both the cytoplasm and nucleus of Sertoli cells (Fig. 3).

Catnb$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ mice were viable. No gross abnormalities of the external genitalia were observed in seven-week-old *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ males, but testis size was much smaller in all of the mutants than that of control littermates. Fifty percent of the *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ males (10/20) were found to have a fully or partially retained uterus in addition to a vas deferens, epididymis, and seminal vesicles, suggesting that the normal regression of the Müllerian ducts caused by AMH was affected (Fig. 4A). Histological analyses revealed severe abnormalities in the testes of *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ mice. Mutant testes completely lacked normal tubular structures and consisted mainly of masses of eosinophilic cells, which were identified as Leydig cells by staining with anti-3β-HSD antibody. Only a few degenerated tubules were found in the *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ testes. However, spermatogenesis was completely absent in these tubules and only rare clumps of Sertoli cells remained (Fig. 4B).

To determine when defects in the testes first occurred, *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ testes from animals of various developmental stages were examined, from E12.5 to birth. No obvious differences in the structure of the testes were observed before E14.5 (Fig. 5). From E15.5 to P0, control testes exhibited progressive testicular cord maturation and expansion of interstitial spaces, which contribute to the growth of testis size. During the same time period in *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ testes, we observed no obvious growth in testis size and increasingly disrupted tubular structure. In control testes, GCNA-1 positive germ cells were localized in the center of testicular cords, surrounded by well-organized Sertoli cells. 3βHSD-positive Leydig cells were sparsely dispersed in the interstitial spaces. However, in mutant testes, Sertoli cells were disorganized and germ cells were scattered outside of the cords. Masses of Leydig cells were found in the interstitial spaces of the mutant testes. At P0, mutant testes consisted largely of clusters of eosinophilic Leydig cells and a few aberrant cord-like structures. Very few germ cells remained in the cord remnants (Fig. 6).

Stabilization of β-catenin causes loss of SOX9 and AMH expression but does not affect WT1 expression in Sertoli cells

SOX9 is a transcription factor that is expressed in Sertoli cells and essential for testis formation (Chaboissier et al., 2004). *Amh* is a downstream target of SOX9 and responsible for the regression of the Müllerian ducts in males (Behringer et al., 1994; De Santa Barbara et al., 1998). We examined how their expression was affected by stabilization of β-catenin. SOX9 protein was detected in Sertoli cells of both control and mutant testes at E12.5. We observed decreased levels of SOX9 in mutant testes at E13.5 and almost no SOX9 was detected in *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ testes at E14.5. We observed similar changes of AMH expression in Sertoli cells of the mutant mice. Previous study showed that *Wt1* deletion in Sertoli cells causes the loss of both SOX9 and AMH expression (Gao et al., 2006). To determine whether the loss of SOX9 and AMH expression in our *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ mice is a secondary effect due to changes in *Wt1* expression, we performed

immunohistochemical analysis of WT1 and found that the expression of WT1 in Sertoli cells was not affected by β-catenin stabilization (Fig. 7).

Stabilization of β-catenin in Sertoli cells alters testicular gene expression

To elucidate putative mechanisms associated with the development of the phenotypes in *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ mutants, we performed quantitative RT-PCR analysis in the testes of *Catnb*$^{lox(e3)/+}$*;* +/+ and *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ mice at E14.5. Compared with *Catnb*$^{lox(e3)/+}$*;* +/+ control testes, we found that transcripts of several important markers of Sertoli cells, such as *Gata4*, *Sf1*, *Dhh* and *Fgf9*, were not significantly changed in the *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ testes. However, the transcripts of *Dax1* (*Nr0b1* – Mouse Genome Informatics), the X-linked orphan nuclear hormone receptor the precise dose of which is required for proper testis development, were upregulated by 70%. Interestingly, the expression of *Wnt4*, an ovarian somatic marker, was upregulated by 100%. We also compared the expression levels of several molecules that are involved in Sertoli cell-Sertoli cell junction formation and found that the connexin 43 (*Cx43*) transcripts were reduced by 50% in the mutant testes while the expression levels of occludin and claudin 11 were not significantly changed. Consistent with the immunohistochemical analysis data, our real-time PCR analysis showed that *Wt1* expression levels were not changed by the stabilization of β-catenin (Table 1).

Wt1 deletion results in upregulation of β-catenin in Sertoli cells

The phenotypes we observed in mice with stabilized β-catenin in Sertoli cells are similar to those of a previously reported conditional knockout of *Wt1* in Sertoli cells using *AMH-Cre*, including disruption of the testicular cords, depletion of germ cells and Müllerian duct regression defects (Gao et al., 2006). Sertoli cells lose the expression of SOX9 and AMH in both *Wt1* and β-catenin stabilized mutant mice. To determine whether the *Wt1* conditional knockout phenotype is related to misregulated β-catenin signaling, we performed β-catenin immunostaining on testes of *Wt1*$^{fx/-}$*; AMH-Cre*$^{tg/+}$ mice. Although expression of β-catenin was only found mainly on Sertoli cell membrane in control testes, we observed nuclear localization of β-catenin in Sertoli cells of *Wt1* conditional knockout testes (Fig. 8A).

To determine the quantitative changes of β-catenin expression after *Wt1* deletion in Sertoli cells, we isolated primary Sertoli cells from the testes of 3-week-old mice and used an inducible in vitro system to measure expression level changes of β-catenin upon *Wt1* deletion. The *CAGGCre-ER* transgenic mouse line has been shown to be a good inducible Cre line in which Cre-mediated recombination is tamoxifen-inducible and dose dependent (Hayashi and McMahon, 2002). We crossed *Wt1*$^{+/-}$*;; CAGGCre-ER*$^{tg/+}$ males with *Wt1*$^{fx/fx}$ females to obtain *Wt1*$^{fx/-}$*; CAGGCre-ER*$^{tg/+}$ male mice and isolated primary Sertoli cells from their testes. We were able to obtain over 90% purity as assessed by immunostaining for WT1 (Fig. 8B). To determine the efficiency of tamoxifen-induced recombination in the isolated Sertoli cells, we performed quantitative RT-PCR and found *Wt1* mRNA levels were reduced by ~6-fold after 3 days treatment with 1 μM 4OH-tamoxifen (Fig. 8C). To determine the expression level change of β-catenin protein upon *Wt1* deletion, we performed western analysis and found that the level of total β-catenin was upregulated more than twofold in *Wt1*$^{fx/-}$*; CAGGCre-ER*$^{tg/+}$ Sertoli cells after 4OH-tamoxifen treatment. To

exclude the possibility that the increase in β-catenin levels may be due to the effect of 4OH-tamoxifen treatment, we also included $Wt1^{fx/fx}$; +/+ Sertoli cells as a control and found that the level of β-catenin was not affected by the drug treatment (Fig. 8D). To determine whether the increase of total β-catenin protein was due to an increase in the transcription of β-catenin, we performed quantitative RT-PCR and found that β-catenin mRNA levels were not changed upon *Wt1* deletion in Sertoli cells (data not shown). To determine whether the increase of total β-catenin protein in Sertoli cells was due to a decrease in the degradation of β-catenin, we performed western analysis using an antibody specific for the active form of β-catenin and found that the level of active β-catenin was upregulated by about twofold in $Wt1^{fx/-}$; $CAGGCre\text{-}ER^{tg/+}$ Sertoli cells after 4OH-tamoxifen treatment (Fig. 8D). Given that the level of β-catenin is controlled both by protein synthesis and degradation, we reasoned that the increase of β-catenin protein might be due to reduced degradation of β-catenin upon *Wt1* deletion.

DISCUSSION

Suppression of β-catenin signaling during normal testis development

β-Catenin is well known as a key effector of the canonical Wnt pathway and also as an important component of the cadherin/catenin intercellular adhesion complexes (Aberle et al., 1996; Morin, 1999; Peifer and Polakis, 2000). Expression of β-catenin was observed in Sertoli cells starting from E15.5. The β-catenin protein was found mainly on the Sertoli cell membrane, but was undetectable in the nucleus. Despite the expression of β-catenin in Sertoli cells, our data suggest that β-catenin is not essential for the maintenance of Sertoli cell function and testicular structure. Conditional knockout of β-catenin in Sertoli cells using *AMH-Cre* mice caused no obvious abnormalities. Mutant mice developed normal testicular structure and had normal spermatogenesis. However, stabilization of β-catenin in Sertoli cells caused severe testicular cord disruption and germ cell depletion, suggesting that β-catenin signaling must be tightly controlled in Sertoli cells for proper Sertoli cell function and the maintenance of testis structure. Although testes of $Catnb^{lox(e3)/+}$; $AMH\text{-}Cre^{tg/+}$ mice were indistinguishable morphologically from control testes at E13.5 before the expression of the *AMH-Cre* transgene, a dramatic and progressive disruption of testicular cord structure was observed beginning at E15.5 following β-catenin stabilization. With the disruption of testicular cords, germ cells leaked into the interstitial spaces and initiated meiosis (data not shown). In the end, the germ cells all died because of the loss of their niche. Adult mutant testes were much smaller than control testes and consisted of Leydig cells, fibroblast-like cells and a few aberrant tubule remnants. Leydig cells developed normally in mutant testes, except that we observed a high proportion of Leydig cells, most probably owing to the collapse of testicular cords and the depletion of germ cells. Leydig cells in mutant testes did not proliferate excessively because we did not observe a significant increase in the number of Leydig cells positive for the mitotic cell marker H3P from E13.5 to E18.5 (data not shown).

β-Catenin controls Sertoli cell identity

Sox9 is expressed in Sertoli cells as early as E11.5 and is maintained throughout embryogenesis. It is both sufficient and necessary for testis formation (Chaboissier et al.,

2004; Vidal et al., 2001). *Amh* is a direct downstream target gene of *Sox9* (De Santa Barbara et al., 1998). AMH protein is an important secretory protein of Sertoli cells, which is necessary to induce the regression of the Müllerian ducts in male fetuses (Behringer et al., 1994). Stabilization of β-catenin in Sertoli cells caused the loss of both SOX9 and AMH, two important Sertoli cell markers. *Wnt4* is an ovarian somatic marker that acts as a partial anti-testis gene by repressing aspects of male development in the female gonad (Heikkilä et al., 2002; Vanio et al., 1999). Interestingly, the stabilization of β-catenin caused an upregulation of *Wnt4* transcript levels.

A previous study showed that *Wt1* is required to maintain SOX9 and AMH expression in Sertoli cells. Sertoli cell-specific deletion of *Wt1* leads the loss of the expression of both SOX9 and AMH (Gao et al., 2006). Here, we found that stabilization of β-catenin also caused the loss of SOX9 and AMH expression in Sertoli cells. However, the expression of WT1 was not altered. This indicates that the expression of WT1 by itself is not sufficient to maintain SOX9/AMH expression in Sertoli cells. This could be explained as: (1) the expression of SOX9/AMH is regulated positively by WT1 and negatively by β-catenin, independently; or (2) WT1 regulates SOX9/AMH expression through the inhibition of β-catenin. Given the data in which we showed upregulation of β-catenin in *Wt1* knockout Sertoli cells, the latter scenario seems more likely.

Stabilization of β-catenin affects inter-Sertoli cell contacts

We detected no increased apoptosis or decreased cell proliferation in Sertoli cells of $Catnb^{lox(e3)/+}$; $AMH\text{-}Cre^{tg/+}$ mice, suggesting that testicular cord disruption is not a result of programmed cell death or reduced proliferation of Sertoli cells (Fig. 9). As we observed a progressive disruption of well-formed tubules upon the stabilization of β-catenin in mutant testes, we speculate that β-catenin stabilization might cause the dysregulation of genes that are important for Sertoli cell-cell contacts or Sertoli cell-germ cell contacts. A major role of Sertoli cells is to provide a nurturing environment for germ cells. As testicular cords start to form, Sertoli cells are evenly distributed and form a single layer surrounding the germ cells. Mature Sertoli cells form three types of intercellular junctions, cadherin-based adherens junctions, occludin-based tight junctions and connexin-based gap junctions. All of these junctions are involved in forming the blood-testis barrier, which physically divides the seminiferous tubules into basal and apical compartments (Mruk and Cheng, 2004). Claudin 11 and occludin are integral components of tight junctions between Sertoli cells and both are expressed in Sertoli cells early in fetal development (Cyr et al., 1999; Hellani et al., 2000). Claudin 11 knockout male mice and occludin knockout male mice are sterile (Gow et al., 1999; Saitou et al., 2000). In our studies, claudin 11 and occludin mRNA levels were not significantly reduced in mutant testis. Connexin 43 (CX43) is a predominant testicular gap junction protein, which is involved in the formation of gap junctions between adjacent Sertoli cells and between Sertoli and germ cells in the seminiferous epithelium (Batias et al., 1999; Decrouy et al., 2004; Perez-Armendariz et al., 2001). CX43 is essential for the control of Sertoli cell proliferation and maturation. Sertoli cell-specific deletion of *Cx43* causes spermatogenesis defects and the formation of Sertoli cell clumps inside seminiferous tubules (Brehm et al., 2007; Sridharan et al., 2007). We observed a significant downregulation of *Cx43* mRNA expression in $Catnb^{lox(e3)/+}$; $AMH\text{-}Cre^{tg/+}$ testes prior to histological

abnormalities. Testicular cords in the mutant mice were subsequently disrupted. Those few remaining degenerated cords were poorly organized and mainly appeared as clumps of Sertoli cells, which could partially be a result of reduced *Cx43* expression. However, dysregulation of other cell contact molecules must also be involved as Sertoli cell specific deletion of *Cx43* does not cause embryonic testis defects. These changes could be a direct result of the stabilization of β-catenin, but also could be secondary to the misregulation of *Sox9*, *Wnt4* and *Dax1*.

Regulation of β-catenin by *Wt1* in Sertoli cells during testis development

Before our study, there was no in vivo experimental evidence to demonstrate whether *Wt1* can interact with the β-catenin signaling pathway. Only one in vitro study in the MDA-MB-231 breast cancer cell line indicated that *Wt1* could inhibit the transformed phenotype of breast cancer cells and downregulate β-catenin/TCF signaling pathway through destabilization of β-catenin (Zhang et al., 2003). In our study, we combined a *Wt1* conditional null allele with a tamoxifen-inducible Cre allele and investigated the expression level changes of β-catenin upon *Wt1* deletion in isolated primary Sertoli cells. We found that the level of total and active β-catenin was dramatically upregulated in $Wt1^{fx/-}$; $CAGGCre\text{-}ER^{tg/+}$ Sertoli cells upon 4OH-tamoxifen treatment. One caveat of our in vitro study is that Sertoli cells at 3 weeks of age might be different from embryonic Sertoli cells. But, importantly, we found that *Wt1* deletion also caused upregulation of β-catenin in Sertoli cells in vivo. β-Catenin immunostaining on the testes of $Wt1^{fx/-}$; $AMH\text{-}Cre^{tg/+}$ mice showed nuclear localization of β-catenin in the Sertoli cells of *Wt1* conditional knockout testes, whereas the expression of β-catenin was only found on the Sertoli cell membrane in control testes. The negative regulation of β-catenin by *Wt1* in Sertoli cells is further supported by the data that stabilization of β-catenin in Sertoli cells has similar phenotypes to *Wt1* conditional knockout mutants. The negative regulation of β-catenin by *Wt1* is probably achieved by enhancing the degradation of β-catenin protein as we did not observe any significant changes in the levels of β-catenin mRNA upon *Wt1* deletion.

Signals regulate later stages of testis development

It has been shown that *Sox9/Fgf9* and *Wnt4* signaling play antagonistic roles during sex determination (Kim et al., 2006). In undifferentiated XY gonads, *Sry* expression initiates the male pathway by upregulating *Sox9*. *Sox9* upregulates *Fgf9*, which initiates a *Sox9/Fgf9* positive-feedback loop that acts as an antagonist of *Wnt4* and accelerates commitment to the male pathway. In undifferentiated XX gonads, the *Sox9/Fgf9* feedback loop is not established and *Wnt4* commits gonadal development to the female pathway. After sex is determined, the signals required for the maintenance of testis development are relatively unknown. A previous study found that *Wt1* is required for the maintenance of *Sox9* expression in Sertoli cells during later stages of testis development (Gao et al., 2006). In this study, we found stabilization of β-catenin also caused the inhibition of *Sox9* expression. Loss of *Sox9* expression appears in both the *Wt1* deletion and β-catenin stabilized mutants. Surprisingly, we found that *Sox9* is not essential for the testis development after E14.5. We used *AMH-Cre* combined with *Zp3-Cre* to create *Sox9* flox/− Sertoli cell knockout mice and we did not detect any abnormalities in mutant testes at E18.5 (Fig. 10). Mutant Sertoli cells expressed AMH and β-catenin normally suggesting that *Sox9* is not essential for the

maintenance of Sertoli cell identity during later stages of testis development. This could be due to other *Sox* family members, such as *Sox8*, that might act redundantly or compensate for *Sox9* during later stages of testis development (Chaboissier et al., 2004).

In this study, we identified that β-catenin signaling as an important regulator for the later stages of testis development. Upregulation of β-catenin signaling causes not only the loss of the male marker SOX9/AMH expression, but also overexpression of the female marker *Wnt4*, which thus alters Sertoli cell identity and causes the disruption of testicular cords. *Wt1*, which is expressed in Sertoli cells throughout testis development, functions as a negative regulator of the β-catenin signaling pathway to commit XY gonads to the male development pathway. As we used *AMH-Cre* to study the roles of *Wt1* and β-catenin in Sertoli cells after sex determination, it is not clear whether our finding is also applicable for earlier stages of testis development (i.e. testis determination). A previous study found that *R-spondin1* mutation caused female to male sex reversal in humans and it was proposed that *R-spondin1* might function through the stabilization of β-catenin signaling during female sex determination (Parma et al., 2006). Similar experiments using earlier expressed Cre lines (to delete β-catenin in bipotential XX gonads or stabilize β-catenin in bipotential XY gonads) will be required to determine whether β-catenin signaling is also involved in sex determination.

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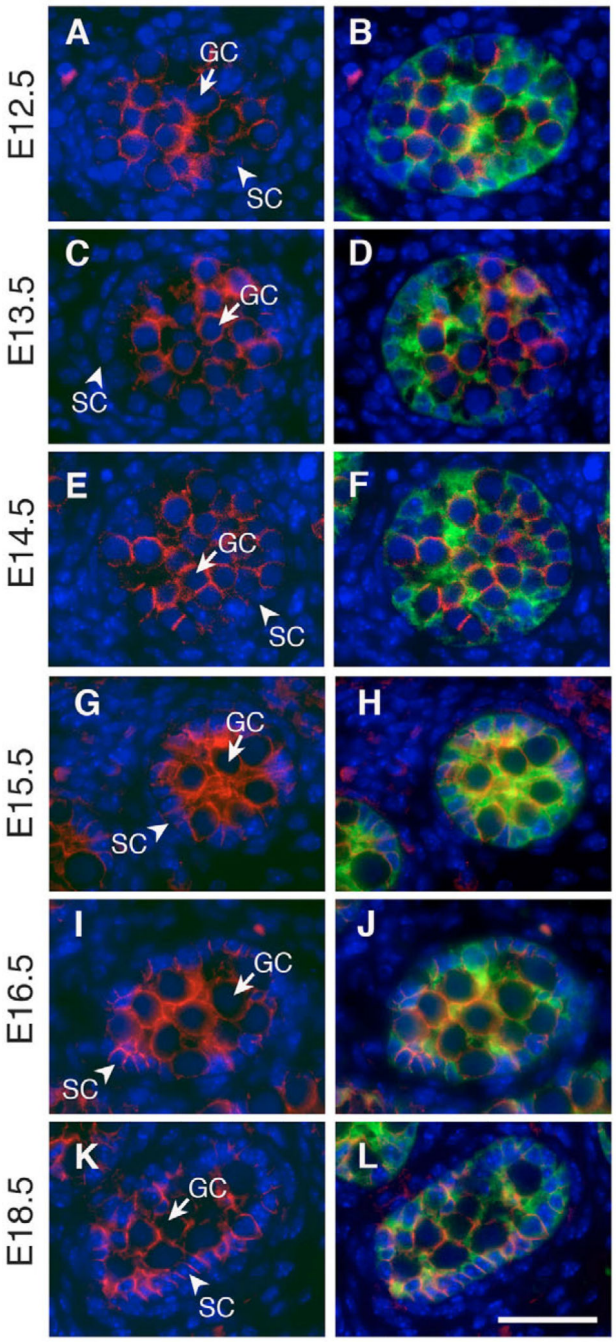

Fig. 1. Expression pattern of β-catenin in the developing testis

(**A–L**) β-Catenin (red)/AMH (green) double immunostaining at different stages. Nuclei were counterstained with DAPI (blue). (A,C,E,G,I,K) β-Catenin channel only and (B,D,F,H,J,L) β-catenin and AMH channels. β-Catenin was detected in the germ cells predominantly in the plasma membrane and cytoplasm at all stages. Sertoli cells started to express β-catenin from E15.5, mainly on the Sertoli cell membrane, but not in the nucleus (G–L). SC, Sertoli cell; GC, germ cell. Scale bar: 20 μm.

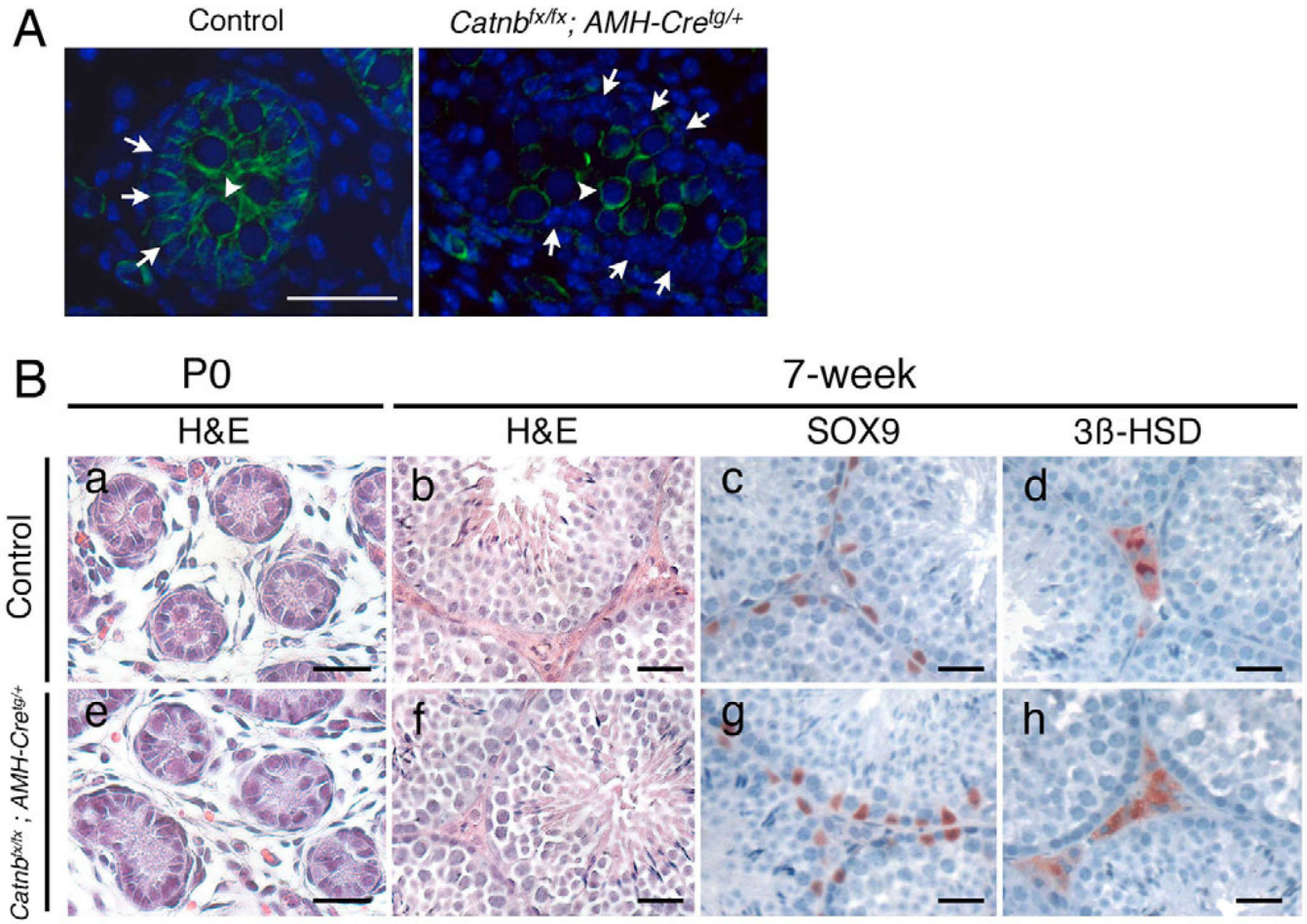

Fig. 2. *Catnb*$^{fx/fx}$; *AMH-Cre*$^{tg/+}$ male mice develop normal testes

(**A**) Sertoli cell-specific deletion of β-catenin in testes of *Catnb*$^{fx/fx}$; *AMH-Cre*$^{tg/+}$ male mice at E16.5. In control testes, β-catenin protein was detected in both germ cells (left panel, arrowhead) and Sertoli cells (left panel, arrows). In *Catnb*$^{fx/fx}$; *AMH-Cre*$^{tg/+}$ testes, β-catenin protein was undetected in Sertoli cells (right panel, arrows) but its expression in germ cells remained unaffected (right panel, arrowhead). β-Catenin protein was stained by an anti-β-catenin antibody (green) and nuclei were counterstained with DAPI (blue). (**B**) Normal histology of *Catnb*$^{fx/fx}$; *AMH-Cre*$^{tg/+}$ testes at P0 (e) and 7 weeks (f) compared with control testes of the same age (a and b, respectively). Adult mutant mice expressed normal Sertoli cell marker SOX9 (g) and Leydig cell marker 3β-HSD (h), when compared with control mice (c and d, respectively). Scale bars: 20 μm.

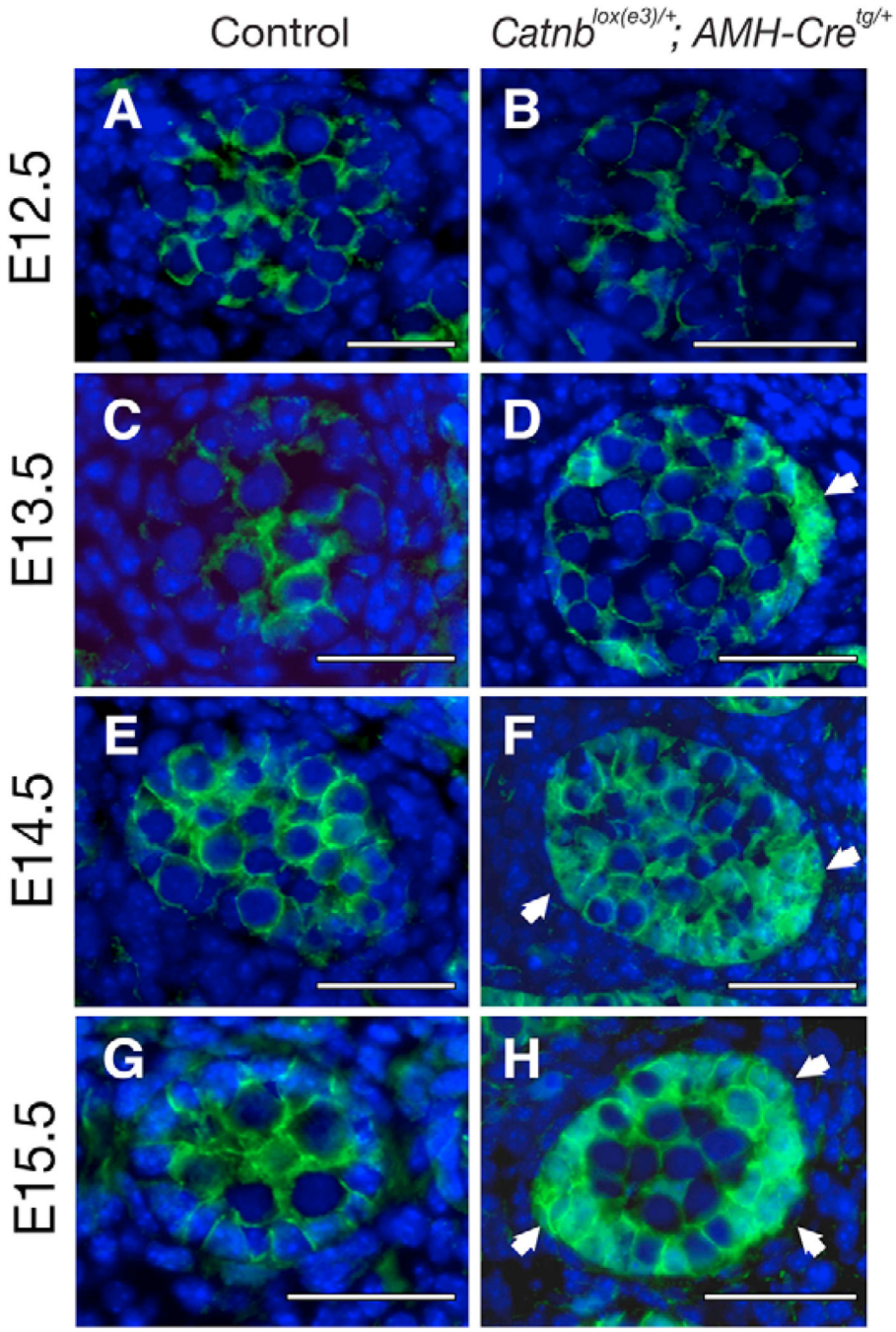

Fig. 3. Stabilization of β-catenin in Sertoli cells of *Catnb*$^{lox(e3)/+}$; *AMH-Cre*$^{tg/+}$ mice

(**A–H**) β-Catenin protein was stained by an anti-β-catenin antibody (green) and nuclei were counterstained with DAPI (blue). The accumulation of β-catenin in Sertoli cells started from E13.5 in a few Sertoli cells (D, arrow) and expanded to more Sertoli cells at E14.5 (F, arrows). At E15.5, almost all Sertoli cells in the testicular cord were affected (H, arrows). The stabilized β-catenin was localized both in cytoplasm and nucleus in Sertoli cells. Scale bars: 20 μm.

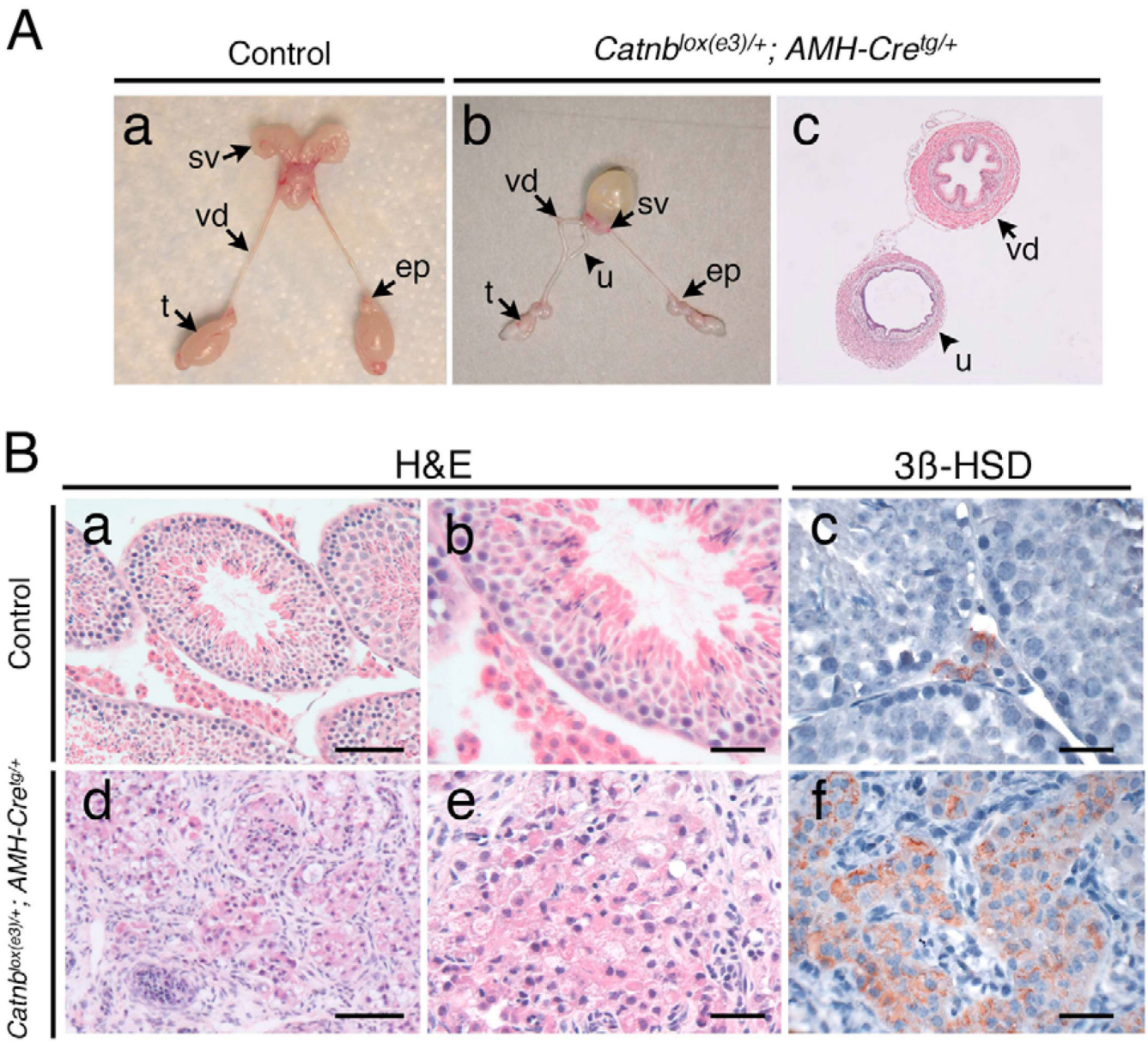

Fig. 4. Phenotype of 7-week-old *Catnb*$^{lox(e3)/+}$; *AMH-Cre*$^{tg/+}$ males

(**A**) *Catnb*$^{lox(e3)/+}$; *AMH-Cre*$^{tg/+}$ males developed severely reduced size of testes and had retained uterus (b, light microscopy; c, Hematoxylin and Eosin staining). ep, epididymis; sv, seminal vesicle; t, testis; u, uterus; vd, vas deferens. (**B**) Testis sections stained with Hematoxylin and Eosin, and anti-3β-HSD antibody showing normal tubular architecture in control testes (a–c) and disruption of tubules in mutant testes (d–f). Scale bars in B: 60 μm in a,d; 20 μm in b,c,e,f.

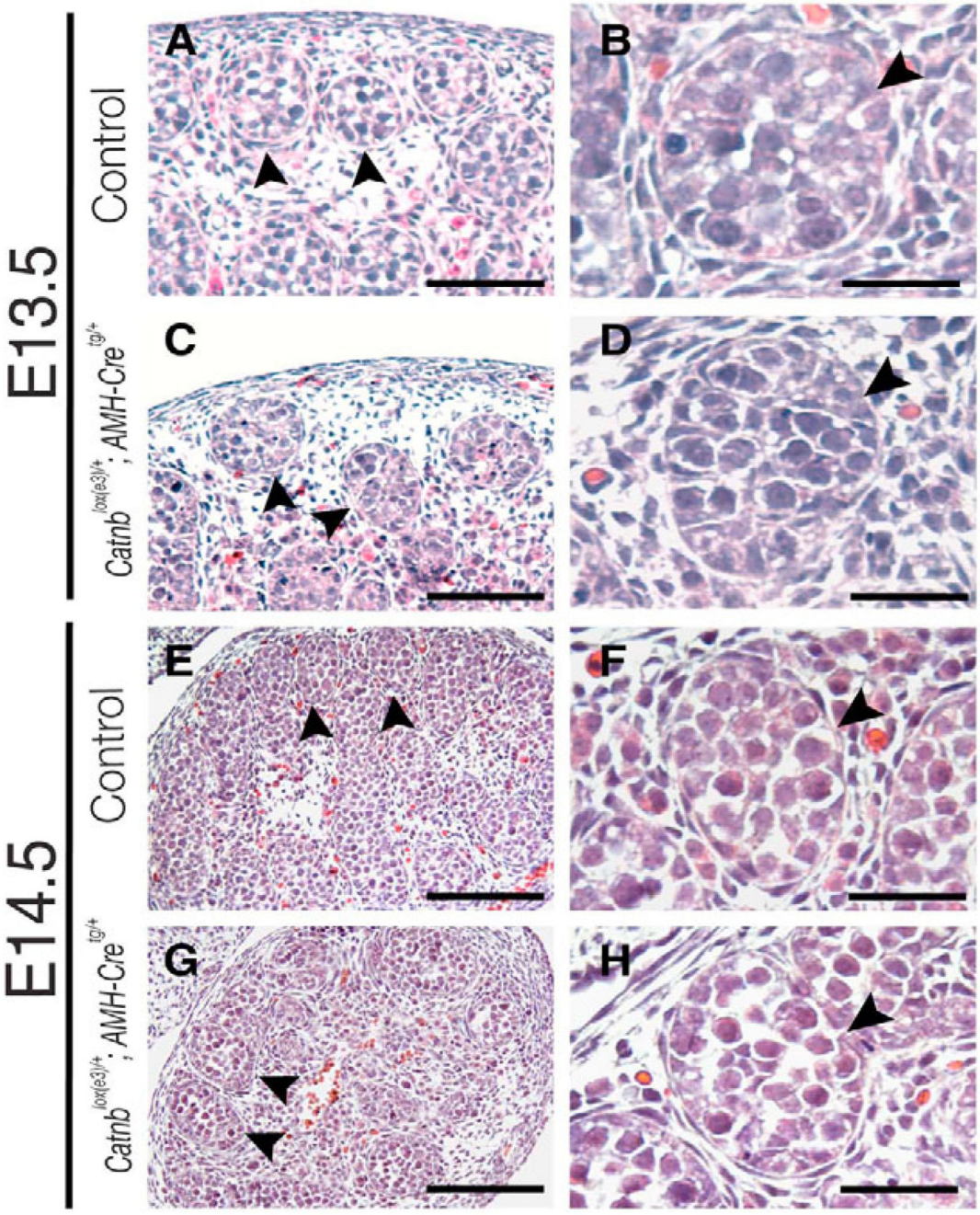

Fig. 5. ***Catnb*** $^{lox(e3)/+}$***; AMH-Cre*** $^{tg/+}$ **testes at E13.5 and E14.5**

(**A–H**) Normal testicular histology with normal tubules (arrowheads) observed in mutant (C,D,G,H) and control (A,B,E,F) testes at low (A,C,E,G) and high (B,D,F,H) magnification. Scale bars: 60 μm in A,C,E,G; 20 μm in B,D,F,H.

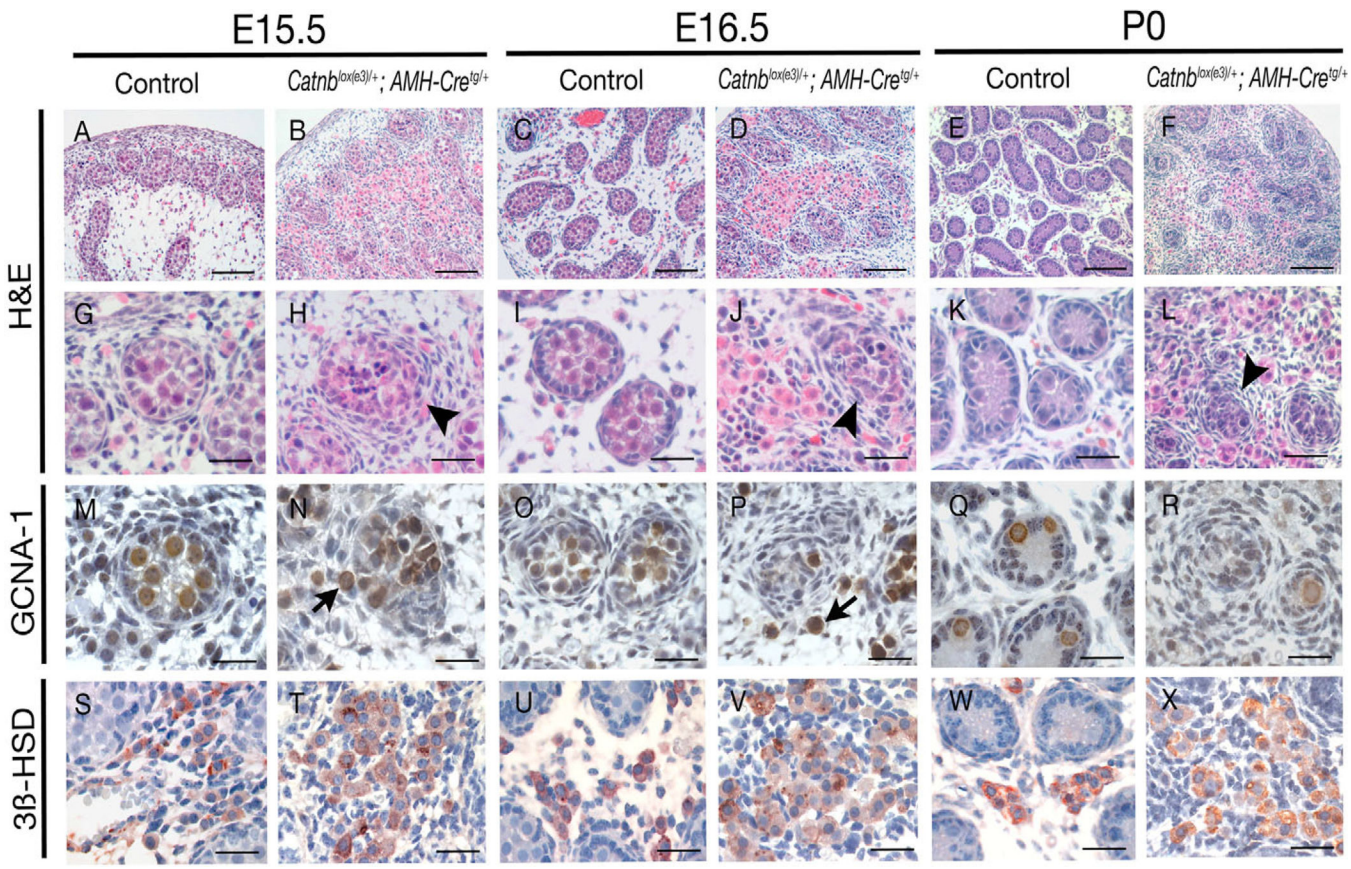

Fig. 6. Disruption of testicular cords and loss of germ cells in *Catnb*$^{lox(e3)/+}$; *AMH-Cre*$^{tg/+}$ testes between E15.5 and P0

(**A–L**) Progressive tubule development in control testes from E15.5 to P0 (low magnification, A,C,E; high magnification, G,I,K), and progressive disruption of cord architecture (arrowheads) in *Catnb*$^{lox(e3)/+}$; *AMH-Cre*$^{tg/+}$ testes (low magnification, B,D,F; high magnification, H,J,L), shown by Hematoxylin and Eosin staining. (**M–R**) Germ cells (GCNA1 positive) located within tubules of control testes (M,O,Q), but located outside of the tubules in *Catnb*$^{lox(e3)/+}$; *AMH-Cre*$^{tg/+}$ testes from E15.5 (arrow, N,P), and very few germ cells remained by P0 (R). (**S–X**) Leydig cells (3β-HSD-positive) scattered in interstitial spaces of control testes (S,U,W); masses of Leydig cells were found in the interstitial spaces of the mutant testes (T,V,X). Scale bars: 60 μm in A–F; 20 μm in G–X.

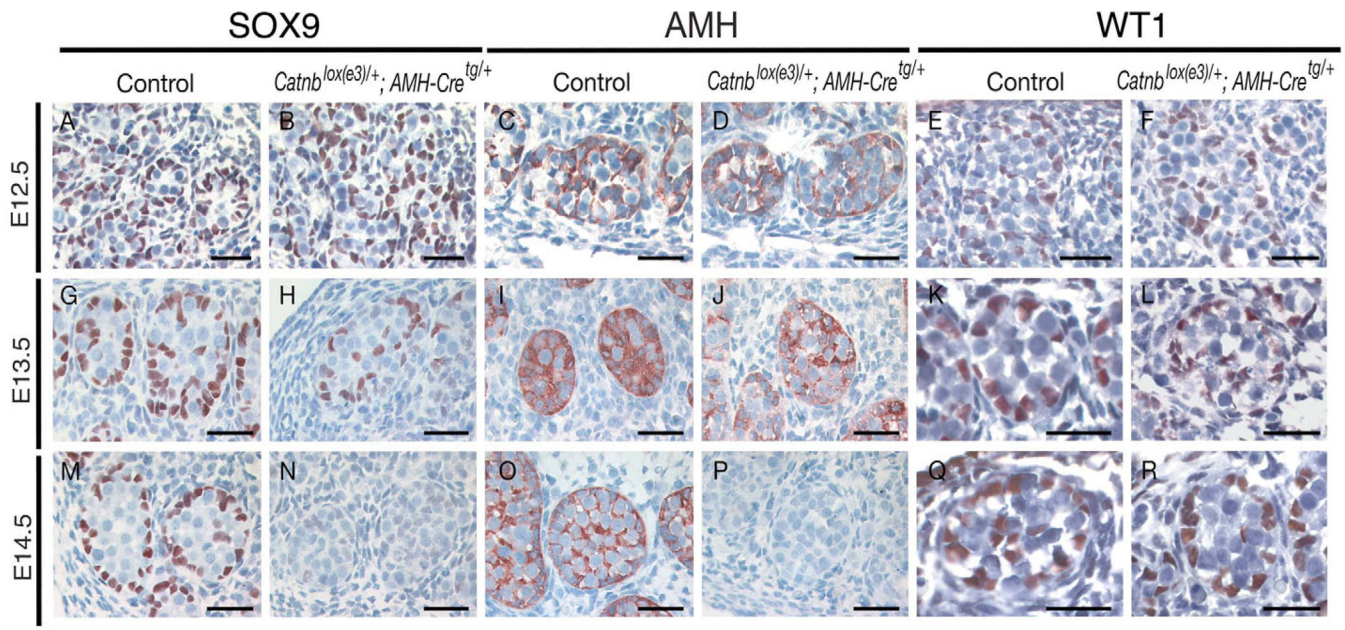

Fig. 7. Loss of SOX9 and AMH expression but unaffected WT1 expression in *Catnb$^{lox(e3)/+}$; AMH-Cre$^{tg/+}$* Sertoli cells

(**A–R**) Control testes show normal SOX9 (A,G,M), AMH (C,I,O) and WT1 (E,K,Q) expression in Sertoli cells at E12.5, E13.5 and E14.5. SOX9 (B) and AMH (D) expression was normal in E12.5 *Catnb$^{lox(e3)/+}$; AMH-Cre$^{tg/+}$* testes, but was almost absent in E14.5 testes (N,P). WT1 expression was not affected in *Catnb$^{lox(e3)/+}$; AMH-Cre$^{tg/+}$* testes (F,L,R). Scale bars: 20 μm.

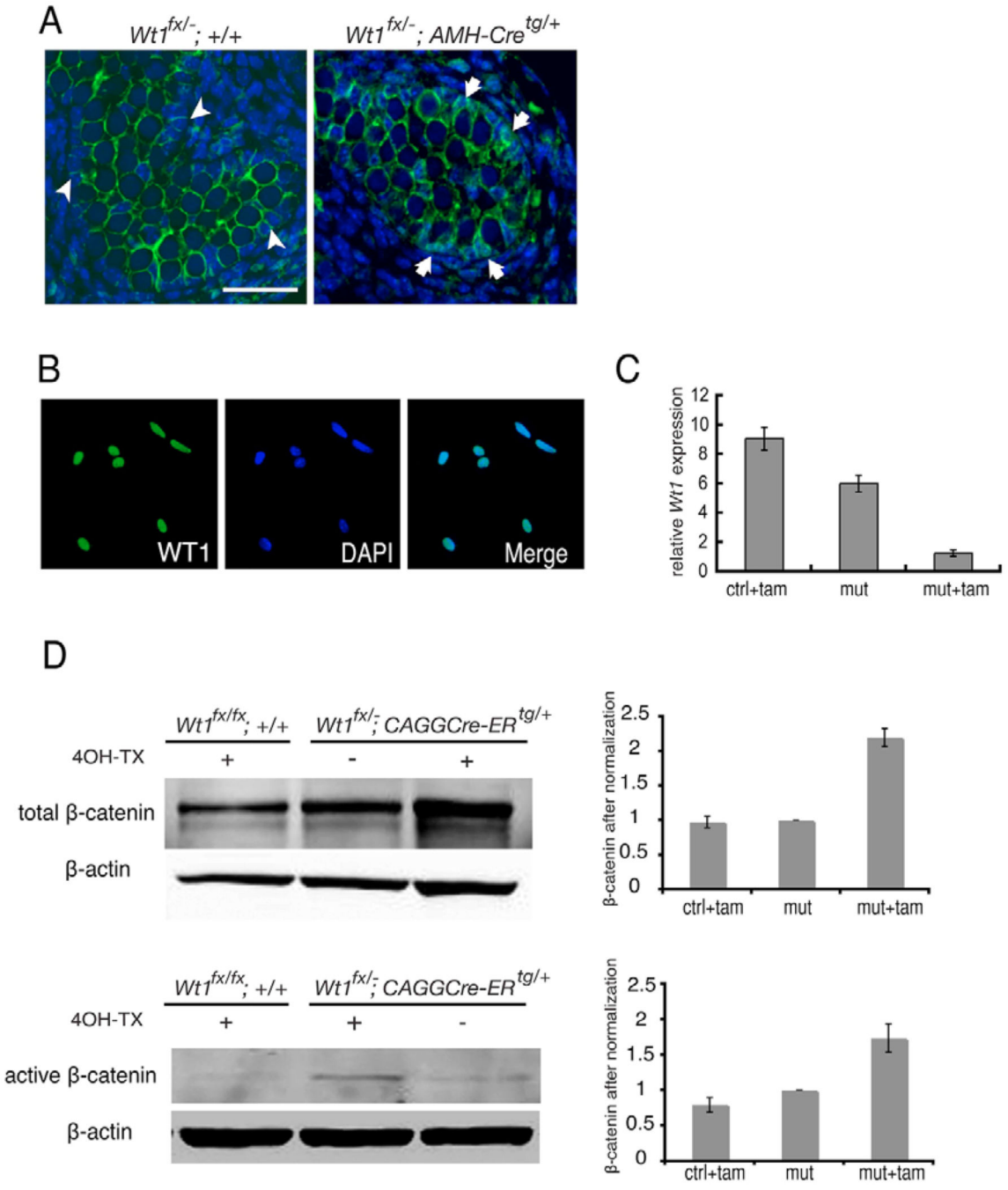

Fig. 8. Upregulation of β-catenin after *Wt1* deletion in Sertoli cells

(**A**) Nuclear localization of β-catenin in Sertoli cells of $Wt1^{fx/-}$; $AMH\text{-}Cre^{tg/+}$ mice at E15.5. β-Catenin protein was stained by an anti-β-catenin antibody (green) and nuclei were counterstained with DAPI (blue). β-Catenin was normally located on Sertoli cell membrane in control testes (left panel, arrowheads). However, β-catenin was detected in Sertoli cell nuclei in testes of *Wt1* conditional knockout mice (right panel, arrows). Scale bar: 20 μm. (**B**) Immunofluorescent images of isolated primary Sertoli cells stained with WT1 (green). Nuclei were counterstained with DAPI (blue). Over 90% of the cells were WT1 positive.

(**C**) Real-time PCR quantification of *Wt1* expression in isolated primary Sertoli cells of mutant and control mice, with or without 4OH-tamoxifen treatment. Numerical data present mean±s.e.m. of relative expression of *Wt1* in three independent experiments. Column 1, $Wt1^{fx/fx}$; +/+ with treatment; column 2, $Wt1^{fx/-}$; $CAGGCre\text{-}ER^{tg/+}$ without treatment; column 3, $Wt1^{fx/-}$; $CAGGCre\text{-}ER^{tg/+}$ with treatment. *Wt1* expression was significantly reduced upon 4OH-tamoxifen treatment in $Wt1^{fx/-}$; $CAGGCre\text{-}ER^{tg/+}$ Sertoli cells. (**D**) Western blot to analyze the expression levels of total β-catenin and the active form of β-catenin in isolated primary Sertoli cells of mutant and control mice, with or without 4OH-tamoxifen treatment. The levels of total β-catenin and the active form of β-catenin were upregulated upon 4OH-tamoxifen treatment in $Wt1^{fx/-}$; $CAGGCre\text{-}ER^{tg/+}$ Sertoli cells (quantification on the right).

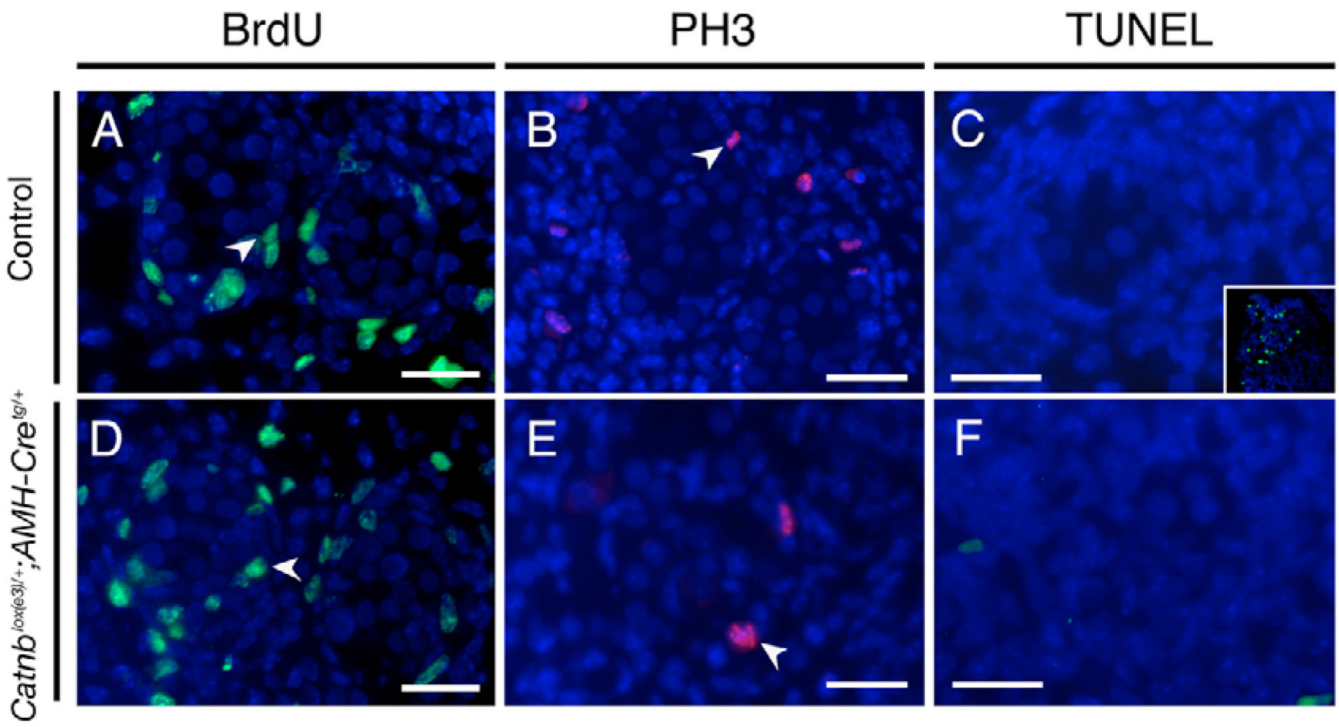

Fig. 9. Cell proliferation and apoptosis in *Catnb*$^{lox(e3)/+}$; *AMH-Cre*$^{tg/+}$ Sertoli cells

Immunostaining with proliferative marker BrdU (**A, D**) and PH3 (**B, E**) identified proliferating Sertoli cells of both control and mutant testes at E15.5 (arrowheads). (**C,F**) TUNEL staining identified no apoptotic cells in either control or mutant testes at E15.5; inset in C shows apoptotic cells in the regressing Müllerian duct as a positive control. Scale bar: 20 μm.

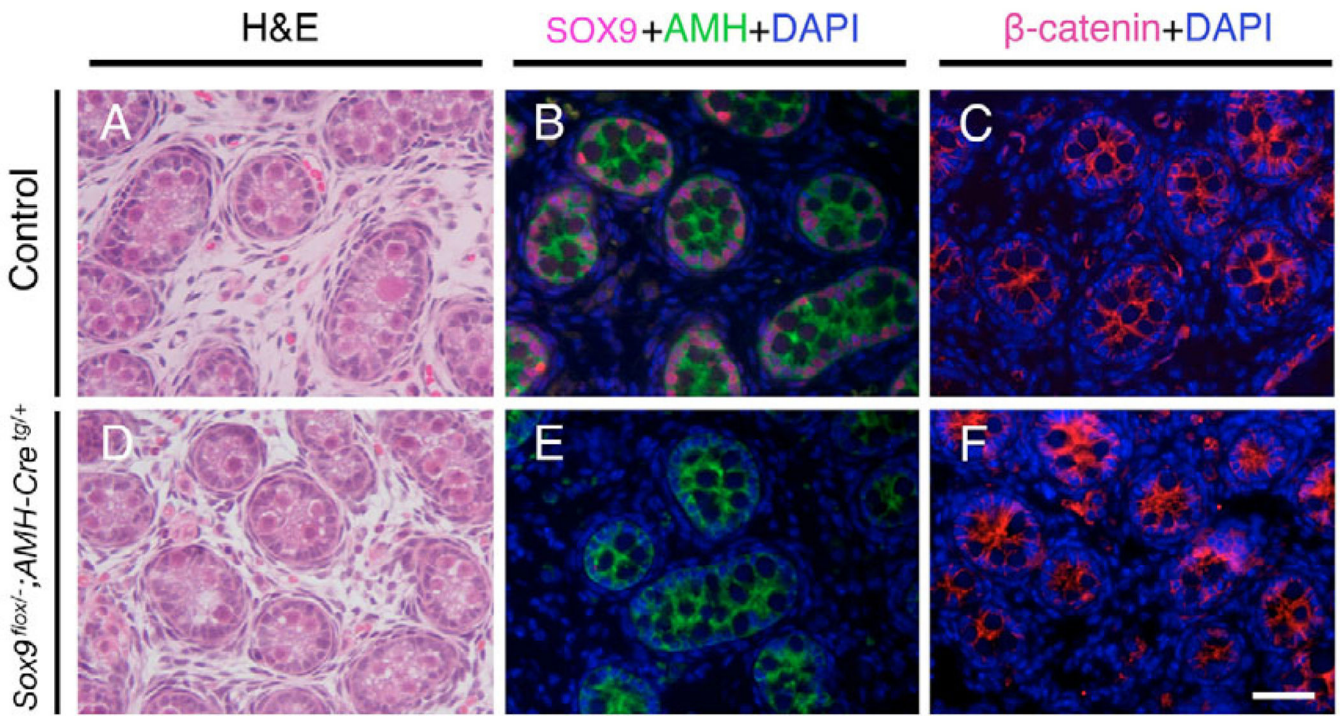

Fig. 10. *Sox9*$^{flox/-}$; *AMH-Cre*$^{tg/+}$ male mice develop normal testes at E18.5

(**A–F**) *Sox9*$^{flox/+}$; *AMH-Cre*$^{tg/+}$ male mice were crossed with *Sox9*$^{flox/flox}$; *Zp3-Cre*$^{tg/+}$ female mice to obtain *Sox9*$^{flox/-}$; *AMH-Cre*$^{tg/+}$ embryos. Hematoxylin and Eosin staining showing normal histology of *Sox9*$^{flox/-}$; *AMH-Cre*$^{tg/+}$ testes (D) when compared with control testes of the same age (A). SOX9 was totally deleted in Sertoli cells of mutant testes (E). However, the expression of AMH and β-catenin in Sertoli cells was unaffected upon SOX9 deletion (E,F). Scale bar: 20 μm.

Table 1

Real-time PCR quantification of gene expression changes in *Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$ mutant testes at E14.5

	***Catnb*$^{lox(e3)/+}$*; +/+**	***Catnb*$^{lox(e3)/+}$*; AMH-Cre*$^{tg/+}$**
Wt1	1.15±0.02	1.09±0.08
Gata4	1.40±0.20	1.43±0.05
Sf1	1.25±0.07	1.07±0.07
Dax1	1.10±0.05	1.58±0.10*
Dhh	1.15±0.06	1.02±0.02
Fgf9	1.19±0.10	1.30±0.06
Wnt4	1.15±0.08	2.39±0.12**
Cx43	2.12±0.03	1.06±0.06*
Occludin	1.09±0.05	1.13±0.05
Claudin 11	1.11±0.04	1.06±0.06

Three mice of each genotype were used. Data are mean±s.e.m. (*P<0.05; **P<0.01).