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Nxf3 is Expressed in Sertoli Cells, but is Dispensable for Spermatogenesis

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SUMMARY

In eukaryotes, mRNA is actively exported to the cytoplasm by a family of nuclear RNA export factors (NXF). Four *Nxf* genes have been identified in the mouse: *Nxf1*, *Nxf2*, *Nxf3*, and *Nxf7*. Inactivation of *Nxf2*, a germ cell-specific gene, causes defects in spermatogenesis. Here we report that *Nxf3* is expressed exclusively in Sertoli cells of the postnatal testis, in a developmentally regulated manner. Expression of *Nxf3* coincides with the cessation of Sertoli cell proliferation and the beginning of their differentiation. Continued expression of *Nxf3* in mature Sertoli cells of the adult is spermatogenesis stage-independent. *Nxf3* is not essential for spermatogenesis, however, suggesting functional redundancy among *Nxf* family members. With its unique expression pattern in the testis, the promoter of *Nxf3* can be used to drive postnatal Sertoli cell-specific expression of other proteins such as Cre recombinase.

Keywords

Sertoli cells; testis; spermatogenesis; *Nxf3*

INTRODUCTION

In 1865, Enrico Sertoli first identified a tree-like cell type in the testis and described it as a "mother" cell of germ cells (Sertoli, 1865). The Sertoli cell supports and regulates virtually every aspect of the germ cell development in the testis, such as spermatogonial proliferation, spermatogonial differentiation, meiosis, and spermiogenesis (Griswold, 1998). During embryogenesis, the expression of the sex determining gene *Sry* in pre-Sertoli cells directs male development of bipotential gonads (Koopman et al., 1990). At birth, Sertoli cells proliferate actively, but cease to divide around puberty (Sharpe et al., 2003). At that time, Sertoli cells undergo a maturation/differentiation process that includes dramatic morphological and functional changes. Sertoli cells establish cell-cell contacts with germ cells; in fact, the cytoplasm of these cells is indented by spermatocytes and spermatids as they emerge. During differentiation, Sertoli cells also establish intercellular junction complexes, including the tight junction, the basal ectoplasmic specialization, the basal tubulobulbar complex, and the desmosome-like junction. These junction complexes

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collectively constitute the blood-testis barrier (Dym and Fawcett, 1970; Wong and Cheng, 2005). Because each Sertoli cell can only support a finite number of germ cells, the number of Sertoli cells ultimately determines the size of testis and, hence, sperm output (Russell and Peterson, 1984). Because Sertoli cells are so intimately involved in spermatogenesis, understanding the molecular mechanisms that control the expression of genes involved in the proliferation and differentiation of Sertoli cells will provide significant insight into regulation of male fertility and will identify likely therapeutic targets for male contraception.

In eukaryotes, mature mRNAs are transported into the cytoplasm by a family of nuclear RNA export factors (NXF). These factors consist of several conserved domains: the leucine-rich repeat (LRR) domain responsible for RNA-binding, the NTF2-like domain that exhibits sequence and structural similarity to the nuclear transport factor 2 (NTF2), and the nucleoporin (NUP)-binding domain at the C-terminal region (Fig. 1A) (Kang and Cullen, 1999). The single *Nxf* gene (Mex67) of yeast is essential for cell viability. Metazoans have multiple *Nxf* genes; in the mouse, four *Nxf* genes, *Nxf1*, *Nxf2*, *Nxf3*, and *Nxf7*, have been identified. NXF1 and NXF2 share identical domain structures, and both function as nuclear exporters of RNA in cultured cells (Sasaki et al., 2005;Tan et al., 2005). A previous study showed that unlike NXF1/NXF2, human NXF3 lacks the C-terminal NUP-binding domain and thus fails to directly interact with components of the nuclear pore complex (Yang et al., 2001). Instead, NXF3 contains a different nuclear export sequence (NES), a leucine-rich Crm1-binding domain, that is absent in NXF1/NXF2 (Fig. 1A) (Yang et al., 2001). The NES of NXF3 recruits the exportin Crm1 and in turn Crm1 carries NXF3 through the nuclear pore complex to the cytoplasm. Therefore, NXF3 and NXF1/NXF2 mediate nuclear RNA export via entirely distinct pathways (Yang et al., 2001). In metazoans, it has been hypothesized that while NXF1 is responsible for nuclear export of bulk mRNAs, other *Nxf* gene products are involved in the nuclear export of tissue- or developmental stage-specific mRNAs (Herold et al., 2000).

Nxf1 is an autosomal gene, whereas, *Nxf2*, *Nxf3*, and *Nxf7* are located on the X chromosome. This X-linked *Nxf* gene family exhibits distinct expression patterns. *Nxf7* is only expressed in embryos (Sasaki et al., 2005). *Nxf2* is specifically expressed in testis and brain (Sasaki et al., 2005; Tan et al., 2005), and its inactivation causes defects in spermatogenesis (Pan et al., 2009). Interestingly, *Nxf3* is testis-specific (Yang et al., 2001). In this report, we show that *Nxf3* is specifically expressed in Sertoli cells in the testis, but is not essential for spermatogenesis.

RESULTS

Nxf3 is expressed in Sertoli cells in the testis

Northern blot and RT-PCR analyses have shown that the *Nxf3* gene is expressed in the testis of both mice and humans (Sasaki et al., 2005; Yang et al., 2001). In order to examine the expression of NXF3 at the protein level, we generated antibodies against two recombinant fusion proteins of mouse NXF3: GST-NXF3 (aa 203-303) and 6xHis-NXF3 (aa 355-553). Both antibodies recognized NXF3, but neither NXF1 nor NXF2 by Western blot (data not shown). Western blot analysis confirmed the testis-specific expression of NXF3 (Fig. 1B).

To determine the specific cell type within the testis in which *Nxf3* is expressed, we performed RNA in situ hybridization (ISH) on adult mouse testis sections. ISH results demonstrated that the *Nxf3* transcript is present only in Sertoli cells, the irregularly shaped cells located at the periphery of seminiferous tubules (Fig. 2A). *Nxf3* transcript was not detected in other cell types, including various types of germ cells or interstitial Leydig cells. This outcome was further confirmed by ISH analysis of testes from sterile XX^{Y*} male mice

(Fig. 2B), whose seminiferous tubules are completely devoid of germ cells and thus contain only Sertoli cells (Hunt and Eicher, 1991).

We next analyzed the expression of the NXF3 protein by immunofluorescence analysis (Fig. 3). To identify Sertoli cells, we performed double immunostaining of testis sections with antibodies against GATA1, which is specifically expressed in Sertoli cells (Yomogida et al., 1994). Consistent with ISH data, we detected the NXF3 protein only in Sertoli cells in the testis (Fig. 3A). Sections of seminiferous tubules in mouse can be categorized into twelve distinct stages associated with progressing spermatogenesis (Russell et al., 1990). The expression of several genes (such as *Gata1* and *Ctsl*) in Sertoli cells is known to be stage-dependent (Fig. 3B) (Charron et al., 2003;Yomogida et al., 1994). In contrast, we detected *Nxf3* in Sertoli cells in all stages of tubules, suggesting that its expression is stage-independent (Fig. 3C).

Expression of *Nxf3* coincides with maturation of Sertoli cells in the developing testes

Western blot analysis on postnatal testes revealed that NXF3 expression is developmentally regulated. NXF3 was first detected at day 10 after birth (Fig. 4A). The abundance of NXF3 increased dramatically at day 14 and this level was maintained in adult testes. As proliferation of Sertoli cells ceases at approximately the same time (postnatal day 12–16 in mice) that immature Sertoli cells begin to differentiate (Hess et al., 2006;Sharpe et al., 2003), the developmental expression of NXF3 appears to coincide with the differentiation of Sertoli cells.

The testes of sterile XX^{Y*} male mice are completely devoid of germ cells and thus contain a higher percentage of Sertoli cells than those of wild type mice (Hunt and Eicher, 1991). As expected for a Sertoli cell-specific protein, the abundance of NXF3 protein was much higher in XX^{Y*} testis than in adult (day 56) wild type testis (Fig. 4A). Western blot analysis showed that NXF3 migrates as two bands at ~60 kD (Fig. 4A). To determine the nature of the two NXF3 protein isoforms revealed by Western blot analysis, we retrieved mouse *Nxf3* expressed sequence tags (ESTs) from GenBank, compared them with its reference cDNA sequence (NM_001024141), and found an alternative exon 2 (exon 2′ in Fig. 4B). We confirmed the splicing pattern for the two *Nxf3* alternative transcripts (referred to as *Nxf3-1* and *Nxf3-2*) by RT-PCR of bulk testis cDNA and sequencing (data not shown). *Nxf3-1* (NM_001024141) apparently encodes a protein 13 amino acids longer than *Nxf3-2* (Fig. 4B). It is highly likely that NXF3-1 and NXF3-2 correspond to the two protein bands revealed by Western blot analysis. Alternatively, the larger NXF3 protein band could be due to post-translational modification (such as phosphorylation) of the smaller species.

Nxf3 is dispensable for spermatogenesis

To elucidate the role of *Nxf3* in spermatogenesis, we generated a floxed-*Nxf3* conditional allele ($Nxf3^{fl}$) in mice using the Cre-*loxP* strategy (Fig. 5A). As expected, both $Nxf3^{fl/Y}$ males and $Nxf3^{fl/fl}$ females were fertile. To disrupt the *Nxf3* gene, $Nxf3^{fl}$ mice were crossed with ACTB-Cre mice, in which Cre recombinase is expressed in all cells of the early embryo (Lewandoski et al., 1997). Western blot analysis confirmed the absence of NXF3 protein in testes of $Nxf3^{-/Y}$ mice (Fig. 5B). The deleted region (exons 2–8) encodes aa 2-271. Therefore, the N-terminal half of NXF3, including the nuclear localization signal (NLS) and the majority of the LRR RNA-binding domain, is deleted (Fig. 1A). A remnant C-terminal fragment of NXF3 (aa 317-553) is likely to be expressed in the mutant testes. However, Western blot analysis with antibody UP1992 (raised against aa 355-553) did not detect truncated NXF3 protein species in $Nxf3^{-/Y}$ testes. In addition, immunofluorescence analysis with the UP1992 antibody showed the absence of NXF3 in $Nxf3^{-/Y}$ testes (Fig. 5C). Collectively, these data strongly suggest that the *Nxf3* mutant allele is null.

Both *Nxf3*$^{-/Y}$ males and *Nxf3*$^{-/-}$ females appeared to be grossly healthy and fertile. These mice were generated on a mixed genetic background. When backcrossed to C57BL/6J strain for seven generations, the *Nxf3*$^{-/Y}$ males were still fertile, with normal testis weight and normal sperm count, in comparison with wild type littermates (Table 1). Histology of *Nxf3*$^{-/Y}$ testes appeared to be normal (data not shown). Previously we reported that *Nxf2*$^{-/Y}$ males from the inbred C57BL/6J strain background displayed a significant reduction (48%) in sperm production (Pan et al., 2009). To test if *Nxf2* and *Nxf3* function synergistically in spermatogenesis, we generated *Nxf2*$^{-/Y}$ *Nxf3*$^{-/Y}$ double mutant males. The sperm count from the double mutant males on a mixed genetic background was only reduced by 32% compared to the wild type littermates, suggesting the lack of compound effects in the double mutant (Table 2). The lower percentage of sperm output reduction in double mutant males (mixed strain background) compared to *Nxf2*$^{-/Y}$ single mutant males (inbred C57BL/6J strain background) was likely due to the hybrid vigor of the mixed genetic background (Eggan et al., 2001). Collectively, these genetic studies suggest that *Nxf3* is not required for spermatogenesis in the mouse.

DISCUSSION

Genetic studies in Sertoli cells have provided a plethora of information about the important role of this unique nurse cell in spermatogenesis, and particularly in the formation of the blood-testis barrier (Wong and Cheng, 2005). For instance, claudin-11 is a tight junction-associated transmembrane protein (Morita et al., 1999). Because claudin-11-deficient male mice fail to form tight junctions between Sertoli cells, spermatogenesis is blocked, resulting in male sterility (Gow et al., 1999). As another example, androgens regulate Sertoli cell proliferation and differentiation, and studies of Sertoli cell-specific androgen receptor (AR) knockout mice have shown that this specific ablation of AR causes spermatogenic arrest in meiosis and increases the permeability of the blood-testis barrier (Chang et al., 2004; De Gendt et al., 2004; Meng et al., 2005). Similarly, disruption of either ETV5 (ets variant gene 5) or connexin 43, proteins involved in transcription and gap junction formation in Sertoli cells, respectively, leads to male sterility (Chen et al., 2005; Sridharan et al., 2007). These genetic studies demonstrate that Sertoli cell function is essential for normal spermatogenesis.

In this report, we have identified *Nxf3* as a Sertoli cell-specific gene in the testis. However, *Nxf3* is not essential for male fertility. *Nxf3* is most likely functionally redundant with *Nxf1*, as *Nxf1* is ubiquitously expressed and might be an essential housekeeping gene (Herold et al., 2000). *Nxf3* function is not likely to overlap with *Nxf2* or *Nxf7*, as *Nxf2* is restricted to germ cells in the testis while *Nxf7* is only expressed in embryonic tissues (Lai et al., 2006; Sasaki et al., 2005; Takano et al., 2007; Tan et al., 2005; Wang and Pan, 2007).

As Sertoli cells switch from proliferation to differentiation, the blood-testis barrier begins to form and germ cells enter meiosis. Given that the developmental expression of *Nxf3* in post-natal testes coincides with the differentiation of Sertoli cells, usage of the *Nxf3* promoter, for example by generation of *Nxf3*-Cre mice, can greatly facilitate research on the formation of blood-testis barrier and regulation of meiotic entry of germ cells by Sertolic cells in the testis.

MATERIALS AND METHODS

Generation of antibodies

Two polyclonal anti-NXF3 antibodies were generated using recombinant proteins. The GST-NXF3 (aa 203-303) fusion protein was expressed in BL21 bacteria using the pGEX-4T-1 vector. The 6×His-NXF3 (aa 355-553) was expressed in M15 bacteria using the

pQE30 vector. Protein purification was performed and used to immunize rabbits as previously described (Pan et al., 2005). For GST-NXF3 (aa 203-303), anti-sera UP2047 and UP2048 were produced. The 6×His-NXF3 (aa 355-553) antigen resulted in anti-sera UP1991 and UP1992. The NXF2 antibody (UP1898) was generated previously (Wang and Pan, 2007).

RNA in situ hybridization

For preparation of RNA probes, a fragment of mouse *Nxf3* cDNA was PCR amplified from bulk testis cDNA with the following primers: 5′-AGCGAGCTCGACCAAGATTTGATGACCC and 5′-AGCCTCGAGTTAGGATCTTGGATCTGTC. The cDNA fragment was cloned into the pBS-KS vector digested with *Sac*I and *Xho*I, resulting in the construct pJP111. Linearized pJP111 plasmid was used for preparation of sense and anti-sense RNA probes. Non-radioactive *in situ* hybridization was performed as described (Berger and Hediger, 2001), using a digoxigenin (DIG)-labeled cRNA probe that contained 1056 bases of *Nxf3* sequence (nucleotides 763-1818). Frozen sections of adult testis (10 μm) were cut in a cryostat and captured onto Superfrost Plus microscope slides (Fisher Scientific). Sections were then fixed and acetylated, and hybridized at 68°C over three nights to the *Nxf3* probe (approximate concentration 100 ng/ml). Hybridized probe was visualized using alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche) and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate (Kierkegard and Perry Laboratories). Sections were rinsed several times in 100 mM Tris, 150 mM NaCl, 20 mM EDTA pH 9.5, and coverslipped with glycerol gelatin (Sigma). Likewise, control sections were hybridized in an identical concentration of the *Nxf3* sense probe.

Western blot analysis

Testes collected from mice at different postnatal days were homogenized in SDS-PAGE sample buffer using a glass homogenizer. Protein lysate (30 μg) was separated on 10% SDS-PAGE gels and electro-blotted onto PVDF membranes. Western blotting was performed using the following primary antibodies: anti-serum UP1898 (anti-NXF2, 1:500), anti-serum UP2048 (anti-NXF3, 1:500), and anti-β-actin (1:5,000, Sigma), followed by HRP-conjugated secondary antibodies (Sigma).

Immunofluorescent analysis

Immunofluorescent analysis was performed as previously described (Yang et al., 2007). NXF3 immunostaining was sensitive to long fixation (> 3 hours), however. Briefly, mouse testes were fixed in 4% paraformaldehyde (PFA) for 3 hours at 4°C, dehydrated in 30% sucrose overnight, submerged in tissue freezing medium, and frozen in dry ice/95% ethanol. Eight-micrometer thick sections were cut at −20°C. NXF3 antibody was affinity-purified from antiserum UP1992 using the immunoblot method (Harlow and Lane, 1998). Rat anti-mouse GATA1 monoclonal antibody (1: 100, sc-265, Santa Cruz Biotechnology) was used for double immunostaining.

Targeted inactivation of the *Nxf3* gene

Gene targeting was performed as previously described (Pan et al., 2009). To generate the *Nxf3* targeting construct, three DNA fragments (2.3 kb, 3.3 kb, and 2.6 kb) were amplified by high-fidelity PCR using an *Nxf3*-containing BAC clone (RPCI23-1A3) as template (Fig. 5A). The CMV-HyTK double selection cassette was flanked by *loxP* sites and enabled hygromycin-positive selection and thymidine kinase-negative selection. Hybrid V6.5 ES cells (C57BL/6 × 129/sv) were electroporated with linearized *Nxf3* targeting construct (pUP98/FspI) and selected for integration in the presence of hygromycin B (120 μg/ml;

Invitrogen). By screening 196 hygromycin-resistant ES cell clones, we identified four *Nxf3*^{3lox} clones that resulted from homologous recombination. Two *Nxf3*^{3lox} ES cell lines were then electroporated with the pOG231 plasmid that transiently expresses Cre recombinase. Two days after electroporation, cells were passaged, and then subjected to selection with gancyclovir (2 μM; Sigma) for removal of the HyTK cassette. Ninety-six colonies were picked for each ES line and screened by PCR. Recombination between the immediate HyTK-flanking *loxP* sites resulted in the *Nxf3*^{fl} allele (Fig. 5A).

Two ES clones harboring the *Nxf3*^{fl} allele were injected into B6C3F1 (Taconic) blastocysts that were subsequently transferred to the uteri of pseudopregnant ICR females. The *Nxf3*^{fl} allele was transmitted through the germline in chimeric mice derived from both clones. To delete the *Nxf3* floxed region, *Nxf3*^{fl} mice were crossed with ActB-Cre mice, in which Cre recombinase is ubiquitously expressed (Lewandoski et al., 1997). All offspring were genotyped by PCR. Wild type (359 bp) and floxed (541 bp; *Nxf3*$^{tm1.1Jw}$) alleles were assayed by touchdown PCR with the primers 5′-CCTCTTGGAGGACCACAGACTTCACCCTCA and 5′-AAAGAGAGATGAGCTTCCAACAGTAGCCCA. The *Nxf3* knockout (389 bp; *Nxf3*$^{tm1.2Jw}$) allele was assayed by PCR with the primers 5′-AAAGAGAGATGAGCTTCCAACAGTAGCCCA and 5′-CAAGCAGAAGAGAGAGTGAAGACTGAGGAA.

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Abbreviations

aa	amino acid
ISH	in-situ hybridization
LRR	leucine-rich repeat
NES	nuclear export sequence
NLS	nuclear localization sequence
NXF	nuclear RNA export factor

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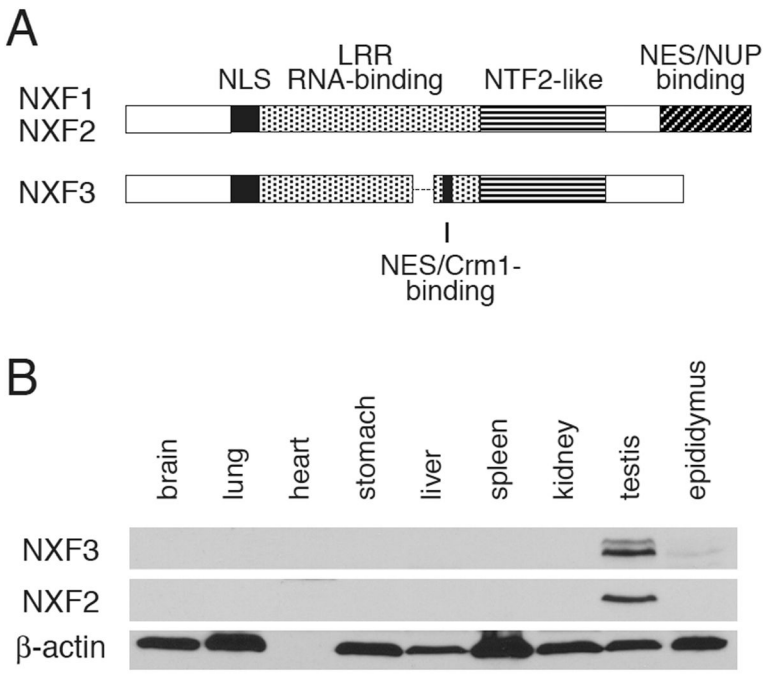

Figure 1.

Comparison of the NXF family members. (**A**) Functional domains of NXF. Unlike NXF1/NXF2, NXF3 lacks the C-terminal NUP (nucleoporin)-binding NES (nuclear export signal), but contains Crm1-binding NES. NLS, nuclear localization signal; LRR, leucine-rich repeat. (**B**) Testis-specific expression of NXF3. Equal amounts (30 μg) of protein extracts from each mouse tissue were used. Blots were probed with anti-NXF3, anti-NXF2, and anti-β-actin antibodies respectively. Note that heart contains little β-actin (a non-muscle cytoskeletal actin).

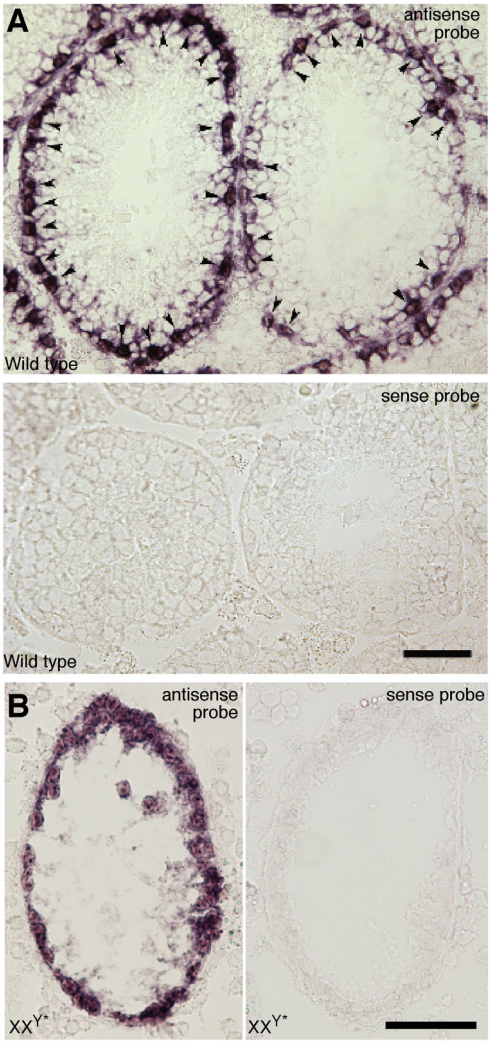

Figure 2.

Nxf3 is expressed in Sertoli cells in the testis. RNA in situ hybridization (ISH) of *Nxf3* was performed on adult mouse testis cryo-sections using a digoxigenin (DIG)-labeled cRNA probe that contained 1056 bases of *Nxf3* sequence (nucleotides 763-1818). (**A**) ISH analysis of *Nxf3* in adult wild type testes. ISH with *Nxf3* anti-sense probe (upper panel) showed that *Nxf3* transcripts were detected only in Sertoli cells (indicated by arrowheads) located at the periphery of seminiferous tubules. *Nxf3* sense control probe produced no signal in testis sections (lower panel). (**B**) ISH analysis of *Nxf3* in adult XX^{Y*} testes. Scale bars, 50 μm.

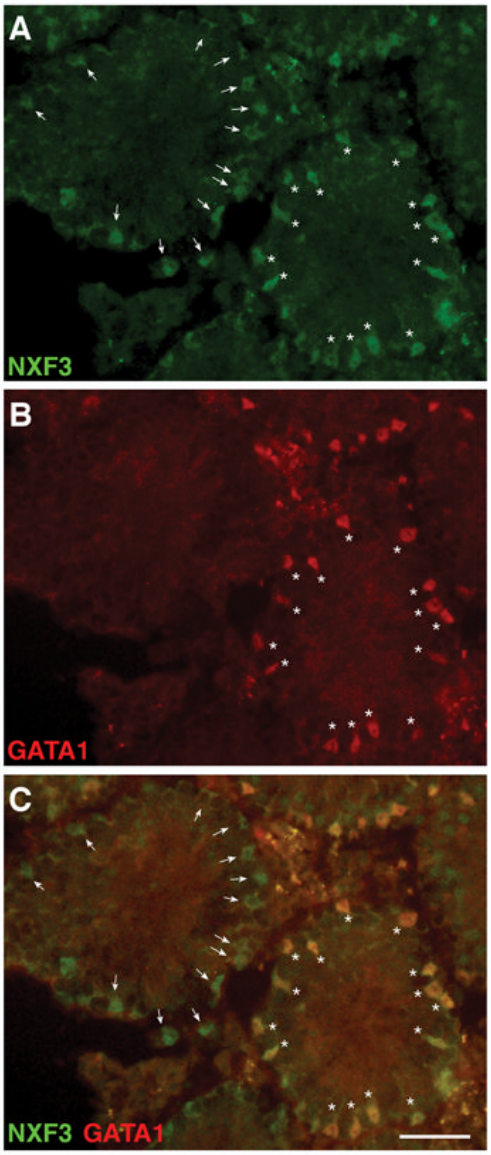

Figure 3.

Immunofluorescent analysis of the NXF3 protein in testes. Sections of wild type adult testes were double immunostained with anti-NXF3 (**A**) and anti-GATA1 (**B**) antibodies. While Sertoli cells in the right tubule (indicated by asterisks) expressed both NXF3 and GATA1, Sertoli cells in the left tubule (indicated by arrows) expressed NXF3 but not GATA1. Scale bar, 50 μm.

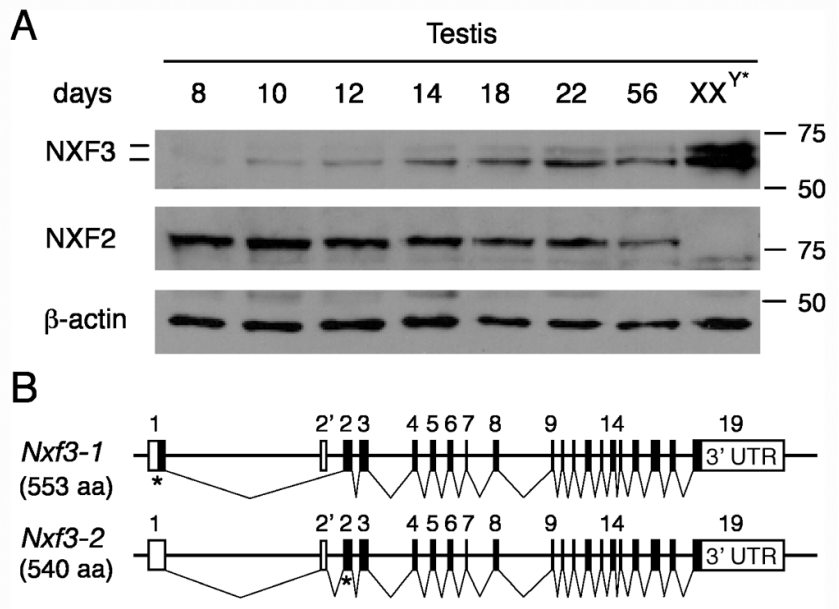

Figure 4.

Temporal expression of NXF3 in developing testes. (**A**) Western blotting analyses on post-natal wild type testes. The number of days after birth is indicated. XX^{Y*}, germ cell-deficient adult testis (Hunt and Eicher, 1991). NXF2, germ cell-specific control (Wang et al., 2001). β-actin, loading control. Note the two closely migrating NXF3 bands, most prominently in XX^{Y*} testis. (**B**) Identification of two alternatively spliced *Nxf3* transcripts. Exons are depicted as rectangles and are designated by numbers shown above. Coding regions are shown in black. Non-coding regions (5′ and 3′ UTRs) are shown in open rectangles. Exon 2′ is an alternative exon that is only present in the *Nxf3-2* transcript. The asterisk (*) indicates the location of start codon.

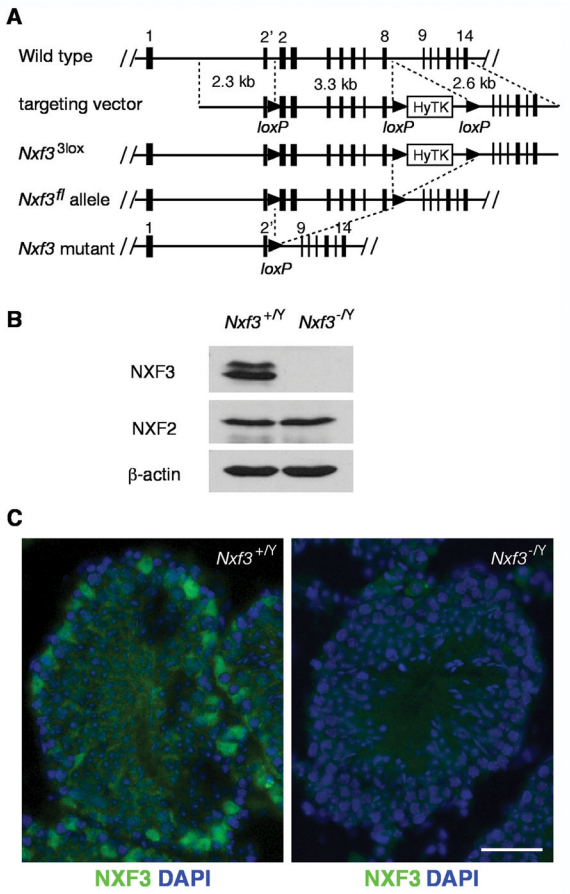

Figure 5.

Inactivation of the *Nxf3* gene in mouse. (**A**) The *Nxf3* targeting construct and various *Nxf3* alleles. In the $Nxf3^{fl}$ allele, one *loxP* site is inserted in intron 1 and one in intron 8. Exons 2–8 encode amino acids 2-271. (**B**) Western blot analysis of adult wild type and $Nxf3^{-/Y}$ testes. The anti-NXF3 antibody UP2048 (raised against aa 203-303) was used. (**C**) Immunofluorescence analysis of NXF3 protein in adult wild type and $Nxf3^{-/Y}$ testes. The anti-NXF3 antibody UP1992 (raised against aa 355-553) was used for immunostaining. Scale bar, 50 μm.

TABLE 1

Testis weight and sperm production in *Nxf3*$^{+/Y}$ and *Nxf3*$^{-/Y}$ mice[a]

	Genotype		
	+/Y (n= 5[b])	−/Y (n= 5[b])	P value[C]
Body weight (g)	23.0±0.9	22.9±1.5	0.90
Testicular weight (mg)	176.0±4.3	172.6±5.3	0.30
Sperm/cauda (10^7)	1.05±0.04	1.01±0.09	0.46

[a] Mice backcrossed to the C57BL/6J strain for seven generations were used at 2 months of age.

[b] 5 mice (n=5) for each genotype were used.

[C] by Student's *t*-test.

TABLE 2

Testis weight and sperm production in wild type (WT) and *Nxf2*$^{-/Y}$ *Nxf3*$^{-/Y}$ (dKO) mice[a]

	Genotype			
	WT (n=3[b])	**dKO (n=3[b])**	**Ratio of dKO to WT**	**P value**
Body weight (g)	25.0±2.1	23.9±0.8	0.96	0.44
Testicular weight (mg)	188.6±2.2	155.0±9.2	0.82	0.0035*
Sperm/cauda (10^6)	10.20±1.75	6.97±0.68	0.68	0.041*

[a] Mice from the mixed genetic background were used at 2 months of age.

[b] 3 mice (n=3) for each genotype were used.

* Values were statistically significant (Student's *t*-test).