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Inactivation of *Nxf2* causes defects in male meiosis and age-dependent depletion of spermatogonia

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Abstract

In eukaryotes, mRNA is actively transported from nucleus to cytoplasm by a family of nuclear RNA export factors (NXF). While yeast harbors only one such factor (Mex67p), higher eukaryotes encode multiple NXFs. In mouse, four *Nxf* genes have been identified: *Nxf1*, *Nxf2*, *Nxf3*, and *Nxf7*. To date, the function of mouse *Nxf* genes has not been studied by targeted gene deletion in vivo. Here we report the generation of *Nxf2* null mutant mice by homologous recombination in embryonic stem cells. *Nxf2*-deficient male mice exhibit fertility defects that differ between mouse strains. One third of *Nxf2*-deficient males on a mixed (C57BL/6 × 129) genetic background exhibit meiotic arrest and thus are sterile, whereas the remaining males are fertile. Disruption of *Nxf2* in inbred (C57BL/6J) males impairs spermatogenesis, resulting in male subfertility, but causes no meiotic arrest. Testis weight and sperm output in C57BL/6J *Nxf2*$^{-/Y}$ mice are sharply reduced. Mutant epididymal sperm exhibit diminished motility. Importantly, proliferation of spermatogonia in *Nxf2*$^{-/Y}$ mice is significantly decreased. As a result, inactivation of *Nxf2* causes depletion of germ cells in a substantial fraction of seminiferous tubules in aged mice. These studies demonstrate that *Nxf2* plays a dual function in spermatogenesis: regulation of meiosis and maintenance of spermatogonial stem cells.

Keywords

NXF2; spermatogenesis; spermatogonia; stem cell; meiosis; mouse

Introduction

In eukaryotes, transport of mRNA from the nucleus to the cytoplasm is mediated by a family of nuclear RNA export factors (NXF) including NXF1. NXF1 (previously known as TAP) was identified as a factor that binds to the constitutive transport element of the retrovirus Mason-Pfizer monkey virus (MPMV) and that is required for nuclear export of incompletely spliced

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MPMV transcripts (Gruter et al., 1998). NXF1 can be UV cross-linked to poly(A)$^+$ RNA and participates in nuclear export of bulk cellular mRNA (Kang and Cullen, 1999; Katahira et al., 1999). Inactivation of Mex67p, the only NXF sequence homologue in yeast, blocks export of poly(A)$^+$ RNA and causes lethality (Segref et al., 1997). Interestingly, co-expression of human NXF1 and a cofactor termed p15 rescues the lethal phenotype of Mex67 yeast mutant, showing functional conservation of the nuclear RNA export pathway during eukaryote evolution (Segref et al., 1997).

Metazoans encode several *Nxf* members. In the mouse genome, four *Nxf* genes have been identified: *Nxf1*, *Nxf2*, *Nxf3*, and *Nxf7* (Sasaki et al., 2005; Tan et al., 2005). These *Nxf* genes exhibit distinct tissue expression patterns: *Nxf1* is widely expressed; *Nxf2* is expressed in testis and brain; *Nxf3* is only expressed in testis; and *Nxf7* is only expressed in embryonic tissues. While *Nxf1* is autosomal, *Nxf2*, *Nxf3*, and *Nxf7* are all X-linked.

In addition to a centrally located RNA-binding domain, NXF1 contains an important C-terminal domain that mediates nuclear export through binding to components of the nuclear pore complex (Bachi et al., 2000; Kang and Cullen, 1999; Katahira et al., 1999). Functionally, NXF2 exhibits the same domain structure as NXF1 and possesses nuclear RNA export activities (Herold et al., 2000; Sasaki et al., 2005; Tretyakova et al., 2005). Notably, NXF3 lacks the C-terminal nuclear pore complex-binding domain that is found in both NXF1 and NXF2, but has evolved a Crm1-binding domain (Yang et al., 2001). NXF7 apparently lacks the nuclear RNA export activity (Sasaki et al., 2005; Tretyakova et al., 2005). These studies suggest that while NXF1, as a housekeeping gene, is responsible for nuclear export of bulk poly(A)$^+$ RNA, the non-ubiquitously expressed NXF factors (NXF2, NXF3, and NXF7) might be involved in nuclear export of a subset of RNA or in translational control.

Identification of NXF2-interacting proteins suggests that NXF2 plays an additional role in the regulation of mRNA stability or trafficking. NXF2 is associated with FMR1 (Fragile X mental retardation syndrome 1), a translational regulator (Lai et al., 2006). Intriguingly, NXF2 and FMR1 appear to destabilize *Nxf1* mRNA in cultured neuronal cells since both are present in *Nxf1* mRNA-containing ribonucleoprotein particles (Zhang et al., 2007). NXF2 interacts with KIF17, a cytoplasmic motor protein (Takano et al., 2007). NXF2 (and NXF1) also interacts with the microtubule-associated proteins such as MAP1B (Tretyakova et al., 2005). Neuronal mRNA granules move along dendrites in a microtubule-dependent manner. Thus, the presence of NXF2 together with KIF17 and MAP1B in neuronal granules indicates a possible role in the cytoplasmic transport and localization of mRNAs.

Although biochemical and cell biological studies have provided tremendous insight into the function of mammalian NXFs, to date, none of the *Nxf* genes have been disrupted in mice. We previously identified *Nxf2* as a germ cell-specific gene from mouse spermatogonia in a cDNA subtraction screen (Wang et al., 2001). Here we report that disruption of *Nxf2* impairs spermatogenesis and provide evidence that *Nxf2* plays a role in male meiosis and maintenance of spermatogonial stem cells.

Materials and methods

Antibody production and Western blot analysis

A GST-NXF1 (aa 200-300) fusion protein was expressed in *E. coli* using the pGEX4T-1 vector. Purified recombinant protein was used to immunize rabbits, resulting in antiserum UP2121. The NXF2 antibody was generated previously (Wang and Pan, 2007). Affinity purified anti-NXF1 (UP2121) and anti-NXF2 (UP1989) antibodies were used for western blotting analysis (1:50). Anti-β-actin was used as a control (1:5,000; Sigma-Aldrich).

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Targeted inactivation of the *Nxf2* gene

To generate the *Nxf2* targeting construct, three DNA fragments (4.2 kb, 2.8 kb, and 2.6 kb) were amplified by high-fidelity PCR using an *Nxf2*-containing BAC clone (RPCI23-65A22) as template (Fig. 1a). 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 *Nxf2* targeting construct and selected for integration in the presence of hygromycin B (120 μg/ml; Invitrogen). By screening 384 hygromycin-resistant ES cell clones, we identified two *Nxf2*^{3lox} clones that resulted from homologous recombination. These two *Nxf2*^{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 *Nxf2*^{fl} allele (Fig. 1a).

Generation and backcross of *Nxf2* mutant mice

Two ES clones (A8 and B2) harboring the *Nxf2*^{fl} allele were injected into B6C3F1 (Taconic) blastocysts that were subsequently transferred to uteri of pseudopregnant ICR females. The *Nxf2*^{fl} allele was transmitted through the germline in chimera mice derived from both clones. To delete the *Nxf2* floxed region, *Nxf2*^{fl} mice were crossed with TNAP-Cre mice (Lomeli et al., 2000). The TNAP-Cre allele was subsequently excluded from *Nxf2* mutant mice by breeding. TNAP-Cre mice were of a mixed (C57BL/6 × 129) genetic background (Kehler et al., 2004). *Nxf2*$^{+/-}$ mutant mice were backcrossed to the C57BL/6J strain (The Jackson Laboratory) for more than ten generations. Experiments were performed on mice of both mixed (C57BL/6 × 129) and inbred (C57BL/6J) strain background. All offspring were genotyped by PCR. Wild type (243 bp) and floxed (433 bp) alleles were assayed by PCR with the primers CTATCAGTGGTTAATGGTGCC and TGATGGCTGCACACTAGTGCT. The *Nxf2* knockout (465 bp) allele was assayed by PCR with the primers TGTTCAGCTCAGTGTGTATTG and CTATCAGTGGTTAATGGTGCC.

Histological, TUNEL, immunofluorescent, and surface spread analyses

For histological analysis, testes were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For TUNEL and immunofluorescent analyses, testes were fixed in 4% paraformaldehyde, dehydrated in 30% sucrose, frozen, and sectioned. TUNEL assays were performed using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon). Immunostaining of testis sections was performed with anti-FMR1 antibody (Cat. No. ab17722, Abcam). Metaphase spread cells were stained with 4% Gurr Giemsa (Invitrogen). Antibodies for immunostaining of surface spread nuclei were described previously (Yang et al., 2008).

Mating test

Each male starting two months of age was housed with two healthy wild type C57BL/6J females. Females were replaced every two months. Cages were observed daily and the litter size was recorded. Six males for each genotype (wild type and *Nxf2*$^{-/Y}$) were tested separately for up to 7 months.

Sperm count and sperm motility analysis

For sperm count, cauda epididymides were dissected in phosphate buffered saline solution. Sperm were squeezed out with fine forceps. Epididymides were minced, pipetted repeatedly, and incubated at room temperature for 10 minutes to allow sperm to disperse. Samples were fixed in 4% paraformaldehyde. Sperm were counted using a hematocytometer.

For motility analysis, sperm were collected from 2-month-old wild type and *Nxf2*$^{-/Y}$ mice by placing minced cauda epididymides in Krebs-Ringer bicarbonate medium without Ca^{2+}, BSA, and $NaHCO_3$ as previously described (Lee and Storey, 1986). The working "complete" medium was prepared by adding $CaCl_2$ (1.7 mM), pyruvate (1 mM), $NaHCO_3$ (25 mM), and BSA (3 mg/ml), followed by gassing with 5% CO_2, 95% O_2 to pH 7.3. Aliquots of each sperm suspension were loaded into a 100 μm-deep chamber, prewarmed at 37°C (Conception Technologies). Sperm motility and concentration were quantified using a computer-assisted semen analysis system (CASA) running IVOS (version 12.2L, Hamilton Thorne Research). At least 1000 sperm per sample were analyzed. For statistical analysis, frequencies of eight motion parameters: motility (%), VAP, VSL, VCL, ALH, BCF, STR, and LIN were determined. For statistical testing, sperm motility measurements of each parameter were pooled for each genotype and for time of observation. Considering the log-normal distribution, Student's *t*-test for independent observations was applied to define differences between wild type and mutant in VAP, VSL, VCL, and BCF means (normalized by natural logarithms). For the same purpose, the nonparametric ALH and STR distributions were tested by Friedman's analysis of variance. Statistical analyses were performed using the InStat program (GraphPad software).

BrdU incorporation assay and spermatogonium count

Adult mice (2-3 months-old) were injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU) (50 mg/kg, Sigma) two hours prior to euthanasia. Testes were decapsulated. Seminiferous tubules were incubated with 1 mg/ml collagenase II (Calbiochem) at 37°C for 30 minutes. The pellet was collected and digested in 0.5% trypsin-EDTA (Invitrogen) with 20 μg/ml of DNase I (Roche) at 37°C for 15 minutes. DMEM medium with 10% FBS was added to terminate the digestion. Cells were pelleted by centrifugation, resuspended in DMEM, and fixed with 10% formalin at 4°C for 30 minutes. After washing with PBS, cells were added to slides. Cells (slides) were treated in 1 M HCl at 37°C for 1 hour. Cells were double stained with rat anti-BrdU antibody (Abcam, Cat. Ab-6326) and anti-TEX17 antibody. Texas red or FITC-conjugated secondary antibodies and antifade mounting medium with DAPI (Vector laboratories) were used.

To determine the number of spermatogonia, postnatal day 4 testes were fixed in fresh 4% paraformaldehyde at 4°C overnight, dehydrated in 30% sucrose, and embedded with TBS tissue freezing medium. Frozen sections were cut using a cryo-microtome. Every fifth section was stained with DAPI for counting of spermatogonia. All cross-sections of seminiferous tubules were examined and the number of spermatogonia within each round tubule was recorded. Spermatogonia in longitudinal tubules were not counted. Four mice of each genotype were analyzed. For each mouse, 200 round tubules were examined.

Microarray analysis

Total RNA was prepared from postnatal day 21-old testes by using TRIzol reagent (Invitrogen) and subsequently purified using an RNeasy kit (Qiagen). Samples were analyzed in triplicates (3 *Nxf2*$^{-/Y}$ and 3 wild type littermates). Five micrograms of total RNA from each sample were used for the generation of biotinylated cRNA. The cRNA samples were hybridized to Mouse Genome 430 2.0 GeneChips (Affymetrix) at the University of Pennsylvania Microarray Core Facility according to the manufacturer's expression analysis technical manual (Affymetrix). We imported microarray data files (.cel) into Partek Genomics Suite software v6.0. GCRMA was applied to calculate log2 transformed probe set signal values. We filtered those values to retain probe sets with values ≥ 5 in at least 2 out of the 6 samples. The filtered list (23,250 genes) was subjected to a two-class unpaired analysis using SAM (Statistical analysis of microarrays), where we calculated q values reflecting FDR (False Discovery Rate) and d scores for every probe set on the list. We identified 342 genes with > 2-fold difference at a 1% FDR

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(Supplementary Table S1). The microarray data have been deposited in the GEO database under the accession number GSE13526.

Results

Disruption of the *Nxf2* gene

Mouse *Nxf2* is an X-linked gene expressed specifically in germ cells in the testis (Sasaki et al., 2005; Tan et al., 2005; Wang et al., 2001; Wang and Pan, 2007). The NXF2 protein is nuclear in spermatogonia but localizes to the nuclear rim in early spermatocytes, suggesting that it might function both in the development of spermatogonia and in meiosis (Wang and Pan, 2007). To elucidate the role of *Nxf2* in spermatogenesis, we generated a floxed *Nxf2* conditional allele ($Nxf2^{fl}$) in mice using the Cre-*loxP* strategy (Fig. 1a). As expected, both $Nxf2^{fl/Y}$ males and $Nxf2^{fl/fl}$ females were fertile. *Nxf2* floxed mice were crossed with TNAP-Cre mice that express Cre recombinase exclusively in primordial germ cells, resulting in $Nxf2^{-/Y}$ male mice that lack exons 3-11 in the germline (Lomeli et al., 2000). Western blot analysis showed that the NXF2 protein was absent in $Nxf2^{-/Y}$ testis. In contrast, the abundance of NXF1 was not affected in $Nxf2^{-/Y}$ testis (Fig. 1b). In addition, real-time PCR analysis revealed that the expression level of *Nxf3* and *Nxf7* in testes was comparable between wild type and $Nxf2^{-/Y}$ 2-month-old inbred C57BL/6J mice (data not shown), indicating that disruption of *Nxf2* did not affect gene expression levels of other *Nxf* genes.

Meiotic arrest in mixed C57BL/6 × 129 $Nxf2^{-/Y}$ males

The *Nxf2* mutant mice were generated in a C57BL/6 and 129 mixed mouse strain. Both $Nxf2^{-/Y}$ males and $Nxf2^{-/-}$ females were viable and appeared to be grossly normal. While $Nxf2^{-/-}$females were fertile, $Nxf2^{-/Y}$ males had incomplete penetrance of sterility (Fig. 1c). Of adult $Nxf2^{-/Y}$ males, 29% (35 out of 121) had sharply reduced testis weight (~ 65 mg). The remaining $Nxf2^{-/Y}$ males had testes of normal weight (~ 160 mg) with apparently normal spermatogenesis and were fertile.

Histological analysis of small *Nxf2*-deficient testes revealed meiotic arrest and abnormal chromosome segregation. While wild type testis contained a full spectrum of spermatogenic cells (Fig. 1d), *Nxf2*-deficient tubules lacked post-meiotic germ cells, showing a block in late meiosis (Fig. 1e). In wild type anaphase spermatocytes, two sets of chromosomes migrated synchronously toward opposite poles (Fig. 1f). However, in *Nxf2*-deficient anaphase cells, chromosome segregation was chaotic (Fig. 1g).

Chromosome mis-segregation in *Nxf2*-deficient spermatocytes

We focused our analysis on the small testes from $Nxf2^{-/Y}$ mice, which always exhibited meiotic arrest. Immunostaining of surface spread nuclei of spermatocytes with anti-SYCP1 and anti-SYCP2 antibodies revealed that chromosomal synapsis appeared to be normal in *Nxf2*-deficient spermatocytes as judged by the formation of the synaptonemal complex (data not shown). We then measured the number of meiotic crossovers in pachytene spermatocytes. MLH1 marks the site of crossovers at the mid-to-late pachytene stage (Baker et al., 1996; Edelmann et al., 1996). We found that the number of MLH1 foci (21.0 ± 3.7, n = 42 nuclei) in *Nxf2*-deficient spermatocytes was close to that (21.3 ± 2.4, n = 34 nuclei) in the wild type and that the distribution of MLH1 foci was normal (Fig. 2a, b). Interestingly, analysis of metaphase I spermatocytes showed that the number of bivalent chromosomes (15.2 ± 2.9 bivalents/cell) in $Nxf2^{-/Y}$ mice was greatly reduced in comparison with wild type (20 bivalents/cell) (Fig. 2c, d). The presence of univalent chromosomes leads to chromosome non-disjunction at the subsequent anaphase I stage and thus triggers the spindle assembly checkpoint (Eaker et al., 2002; Li and Nicklas, 1995). We found abnormal chromosome segregation in spermatocytes (Fig. 1g) and massive apoptosis of germ cells in *Nxf2*-deficient Stage XII tubules (containing

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metaphase I and anaphase I spermatocytes) (Fig. 2f). These studies show that *Nxf2* is not essential for crossover formation but is required for proper chromosome segregation during male meiosis.

Reduced sperm production and impaired sperm motility in *Nxf2*$^{-/Y}$ mice

To dissect the possible effect of the mixed genetic background on the mutant phenotype, we backcrossed the *Nxf2* mutant allele to the inbred C57BL/6J mouse strain for more than ten generations. All subsequent studies were performed with C57BL/6J mice. We found that *Nxf2*$^{-/-}$females were fertile, but *Nxf2*$^{-/Y}$ males exhibited impaired fertility. Six males of each genotype (*Nxf2*$^{-/Y}$ and wild type littermate) were subject to mating test. All six wild type males continued to sire offspring at an expected frequency. In contrast, two *Nxf2*$^{-/Y}$ males never sired any offspring and the other four *Nxf2*$^{-Y}$ males sired one to three litters initially before becoming infertile. The litter size (average ± standard deviation) sired by *Nxf2*$^{-/Y}$ males (4.3 ± 2.0, n=6 litters) was sharply reduced in comparison to that of wild-type littermate controls (7.3 ± 2.3, n=40 litters) ($P < 0.0055$). While the body weight of 2-month-old *Nxf2*$^{-/Y}$ males was similar to that of wild type littermates, the testis weight of *Nxf2*$^{-/Y}$ males was reduced by 30% (Table 1). Cauda epididymides from *Nxf2*$^{-/Y}$ males contained half the number of sperm compared to controls (Table 1). Analysis of sperm motility by computer-assisted sperm analysis revealed that in *Nxf2*$^{-/Y}$ mice, the percentage of motile sperm was significantly decreased compared to wild type (Table 2). Thus, loss of *Nxf2* function impairs sperm function in the C57BL/6J background.

To address whether reduced sperm production in *Nxf2*$^{-/Y}$ males is caused by defects in meiosis, we analyzed spermatocytes from adult (2-month-old) mice by surface spread analysis. The relative percentage of each stage of spermatocytes was comparable between *Nxf2*$^{-/Y}$ and wild type mice (data not shown). Chromosomal analysis of *Nxf2*-deficient metaphase I spermatocytes (50 cells examined) by Giemsa staining revealed no univalent chromosomes. Collectively, these results showed that in the C57BL/6J mouse strain, *Nxf2* is not essential for the progression of meiosis.

FMR1 interacts with NXF2 in testis and both proteins co-localize at the perinuclear region in early germ cells (Lai et al., 2006). We examined the subcellular localization of FMR1 in *Nxf2*-deficient testis (Supplementary Fig. S1). FMR1 was still localized to the perinuclear region in *Nxf2*-deficient germ cells, suggesting that FMR1 localization is independent of NXF2.

Reduced spermatogonial proliferation in *Nxf2*$^{-/Y}$ mice

The *Nxf2* gene was initially cloned from mouse spermatogonia (Wang et al., 2001). The NXF2 protein is abundantly expressed in the nuclei of spermatogonia, suggesting that it might be involved in the proliferation and/or differentiation of spermatogonia (Wang and Pan, 2007). However, spermatogonia were present in the *Nxf2*-deficient testis from young mice, showing that *Nxf2* is not essential for the survival of spermatogonia. We first examined the number of spermatogonia in postnatal day 4 testes, where spermatogonia have characteristically large nuclei with little heterochromatin. The number of spermatogonia was similar between wild type (88.9 ± 19.9 spermatogonia/100 tubule cross-sections) and *Nxf2*$^{-/Y}$ (91.6 ± 10.6) mice at postnatal day 4.

We next performed BrdU incorporation assays to measure the proliferation rate of spermatogonia in adult mice (2-3 months-old). Wild type and *Nxf2*$^{-/Y}$ males were injected intraperitoneally with BrdU two hours prior to euthanasia. Single cells were prepared from testes and were double immunostained with anti-TEX17 and anti-BrdU antibodies. TEX17 is specifically expressed in spermatogonia and thus is a marker of spermatogonia (Wang et al., 2005). We found that the percentage of BrdU-positive spermatogonia (24.9 ± 3.8%) in

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Nxf2$^{-/Y}$ testis was significantly lower than that (33.3 ± 2.0%) of wild type ($p < 0.028$), suggesting that *Nxf2* promotes proliferation of spermatogonia in adult mice.

Histological analysis of testes from 2.5-month-old C57BL/6J *Nxf2*$^{-/Y}$ mice revealed abnormal spermatogenesis (Fig. 3). Consistent with reduced testis weight, the diameter of seminiferous tubules in the *Nxf2*$^{-/Y}$ mice (Fig. 3b) was smaller than that in the wild type (Fig. 3a). The *Nxf2*$^{-/Y}$ testis exhibited no spermatogenetic arrest, evident from the presence of spermatogonia, spermatocytes, and spermatids. However, the number of germ cells in *Nxf2*$^{-/Y}$ tubules was clearly lower than that in the wild type, suggesting that spermatogenesis is impaired in 2.5-month-old *Nxf2*$^{-/Y}$ mice (Fig. 3b).

Loss of spermatogonia in aged *Nxf2*$^{-/Y}$ mice

We next determined the long-term effect of reduced spermatogonial proliferation on spermatogenesis (Fig. 3c-f). In testes of older (9-month old) *Nxf2*$^{-/Y}$ mice, 26% of seminiferous tubules were “Sertoli cell only” (SCO) tubules that are devoid of germ cells (Fig. 3d, f). In contrast, SCO tubules were very rare in testes from older (9 month old) wild type and young (2.5-month old) *Nxf2*$^{-/Y}$ mice (~1%). Age-dependent depletion of spermatogenesis in mutant mice suggests that *Nxf2* promotes the self-renewal and/or survival of spermatogonial stem cells.

Transcript profiling of *Nxf2*$^{-/Y}$ testes

Inactivation of Mex67 in yeast blocks nuclear export of bulk poly(A)$^{+}$ RNA (Segref et al., 1997). To determine whether disruption *of Nxf2* leads to nuclear accumulation of poly(A)$^{+}$ RNA in germ cells, we performed in situ hybridization on *Nxf2*$^{-/Y}$ and wild type testis sections with an oligo(dT)$_{45}$-Cy3 probe (Herold et al., 2001). This analysis did not reveal increased abundance of poly(A)$^{+}$ RNA in the nuclei of *Nxf2*-deficient germ cells (data not shown), indicating that nuclear export of bulk cellular mRNA can occur in the absence of *Nxf2*.

To systematically identify genes with altered transcript abundance in *Nxf2*$^{-/Y}$ testes, we performed microarray analysis of testes from post-natal day 21 mice using Affymetrix Mouse Genome 430 2.0 GeneChips. At post-natal day 21, the weight of *Nxf2*$^{-/Y}$ testes (29.3 ± 4.5 mg) was not significantly different from that of wild type (30.4 ± 5.5 mg). With an expression cutoff of two-fold change or greater, our microarray analysis identified 331 genes that were down regulated in *Nxf2*$^{-/Y}$ testes (Supplementary Table S1). We noted that most genes with altered abundance are specifically or preferentially expressed in spermatids such as *Odf1*, *Txndc8*, and *Hils1*. Real-time PCR analysis showed that these genes are only slightly down regulated in adult *Nxf2*$^{-/Y}$ testes (Supplementary Table S2), suggesting that the dramatically decreased abundance of these genes in post-natal day 21 *Nxf2*$^{-/Y}$ testes could be due to delayed appearance of spermatids in the mutant testes.

Discussion

NXF2 exhibits two distinct localization patterns in germ cells: nuclear localization in spermatogonia and nuclear rim (envelope) localization in early spermatocytes, suggesting that NXF2 might have a dual function in spermatogenesis: development of spermatogonia and regulation of meiosis (Wang and Pan, 2007). In support of this prediction, disruption of *Nxf2* leads to age-dependent loss of spermatogonia and defects in male meiosis.

Since spermatogonial stem cells (SSCs) replenish spermatogenesis, males produce sperm through lifetime (Brinster, 2007; de Rooij, 1998). Spermatogonial stem cells undergo self-renewal, proliferation, and differentiation. Eventually, differentiating spermatogonia enter meiosis to produce sperm. Thus, the number of spermatogonial stem cells and the rate of spermatogonial proliferation affect the ultimate sperm output. Disruption of *Nxf2* in C57BL/

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6J inbred mice resulted in a paucity of germ cells in the testis and reduced sperm output in the epididymis, but no meiotic block. BrdU incorporation experiments showed that the proliferation of spermatogonia in *Nxf2*$^{-/Y}$ testes was significantly reduced. These data suggest that the reduction in testis weight and sperm output in *Nxf2*$^{-/Y}$ mice might be due to a decreased population of spermatogonia – pre-meiotic germ cells. Disruption of genes (for example, *Plzf* and *Bcl6b*) involved in the self-renewal and survival of SSCs causes a progressive loss of SSCs with age (Buaas et al., 2004; Costoya et al., 2004; Oatley et al., 2006). We found that a substantial percentage of seminiferous tubules lacked germ cells in 9-month-old *Nxf2*$^{-/Y}$ mice but not in young adult mutant mice, suggesting that *Nxf2* plays a role in the maintenance of spermatogonial stem cells.

The effect of *Nxf2* ablation on meiosis depends on the mouse strain. In mice of mixed (C57BL/6 × 129) genetic background, nearly one-third of *Nxf2*$^{-/Y}$ males displayed meiotic arrest, whereas the remaining mutant males had normal spermatogenesis and were fertile. However, in C57BL/6J inbred *Nxf2*$^{-/Y}$ males, meiotic arrest was never observed and meiotic progression appeared to be normal. All C57BL/6J inbred *Nxf2*$^{-/Y}$ males had a reduction in testis weight, sperm count, and sperm motility. Variable phenotypic expression of mouse mutants has been observed for many genes such as *Scmh1* and *Dazl* (Lin and Page, 2005; Saunders et al., 2003; Takada et al., 2007). The incomplete penetrance of meiotic defects in *Nxf2*$^{-/Y}$ mice might be attributed to compensatory effects by other *Nxf* genes such as the ubiquitously expressed *Nxf1* gene.

Nxf2 could regulate spermatogenesis through two possible mechanisms: a) nuclear retention/export of specific transcripts and b) RNA trafficking and translational control after nuclear export. Most cellular mRNAs exit the nucleus through the NXF1/Mex67-mediated pathway. However, the identity of mRNA substrates for other NXF homologues in metazoans remains unknown except for *Ce*NXF-2. In *C. elegans*, *Ce*NXF-2 is required for nuclear retention of *tra-2* mRNA in the absence of TRA-1 (Kuersten et al., 2004). TRA-1 and TRA-2 are involved in determination of female cell fate in *C. elegans*. The nuclear export of *tra-2* mRNA is mediated through the TRE element in its 3′UTR in a leptomycin B (inhibitor of Crm1)-sensitive manner. *Ce*NXF-2 and REF-1 specifically bind to the TRE to cause the nuclear retention of *tra-2* mRNA by blocking NXF1-mediated nuclear export. Binding of TRA-1 to the TRE could displace *Ce*NXF-2, resulting in the nuclear export of *tra-2* mRNA via an unknown Crm1-dependent factor. These studies demonstrate that *Ce*NXF-2 together with other proteins influences the choice of nuclear export pathways for *tra-2* mRNA (Kuersten et al., 2004). It is not clear whether *Ce*NXF-2 is the orthologue of mouse NXF2, since *Ce*NXF-2 lacks the nuclear pore complex-binding domain that is present in mouse NXF2 (as well as in NXF1). We predict that given its nuclear localization in spermatogonia, mouse NXF2 could be involved in nuclear retention of certain gene transcripts in spermatogonia.

NXF2 could also be involved in mRNA trafficking and translational control in the cytoplasm through its interaction with various cytoplasmic proteins including the kinesin KIF17, the microtubule-associated protein MAP1B, and the RNA-binding translational regulator FMR1 (Lai et al., 2006; Takano et al., 2007; Tretyakova et al., 2005; Zhang et al., 2007). KIF17 has also been shown to be associated with ACT (activator of CREM in the testis) and TB-RBP (testis brain RNA-binding protein) (Chennathukuzhi et al., 2003; Macho et al., 2002). Macroorchidism (enlarged testis) is a prominent symptom of Fragile X syndrome in humans and the *Fmr1* knockout mice (Bakker et al., 1994). Although FMR1 is expressed in spermatogonia but not in Sertoli cells in the testis, macroorchidism in the *Fmr1* knockout is caused by increased proliferation of Sertoli cells (Slegtenhorst-Eegdeman et al., 1998). NXF2 and its interacting proteins (KIF17, MAP1B, and FMR1) are associated with RNA-containing granules in transfected neurons, indicating a role in translational control.

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Transcript profiling of wild type and *Nxf2*-deficient testes failed to identify specific mRNA substrates for NXF2. Disruption of *Nxf2* might affect the translation of NXF2-regulated mRNA but not the abundance. In this case, the mRNA substrates could be identified by crosslinking and immunoprecipitation with NXF2 antibody followed by microarray profiling or direct sequencing of associated RNAs.

Our genetic study of *Nxf2* in mice has important implications for male infertility in humans. Infertility affects 15% of couples worldwide (Matzuk and Lamb, 2002). The human *NXF2* is also a testis-specific gene located on the X chromosome. A recent study of 65 infertile men with Sertoli cell-only syndrome did not identify mutations in *NXF2* (Stouffs et al., 2008). Disruption of *Nxf2* causes sharply reduced sperm output in mice. Therefore, mutations in the human *NXF2* gene could be found in infertile men with oligozoospermia (reduced sperm count) rather than azoospermia (no sperm in semen).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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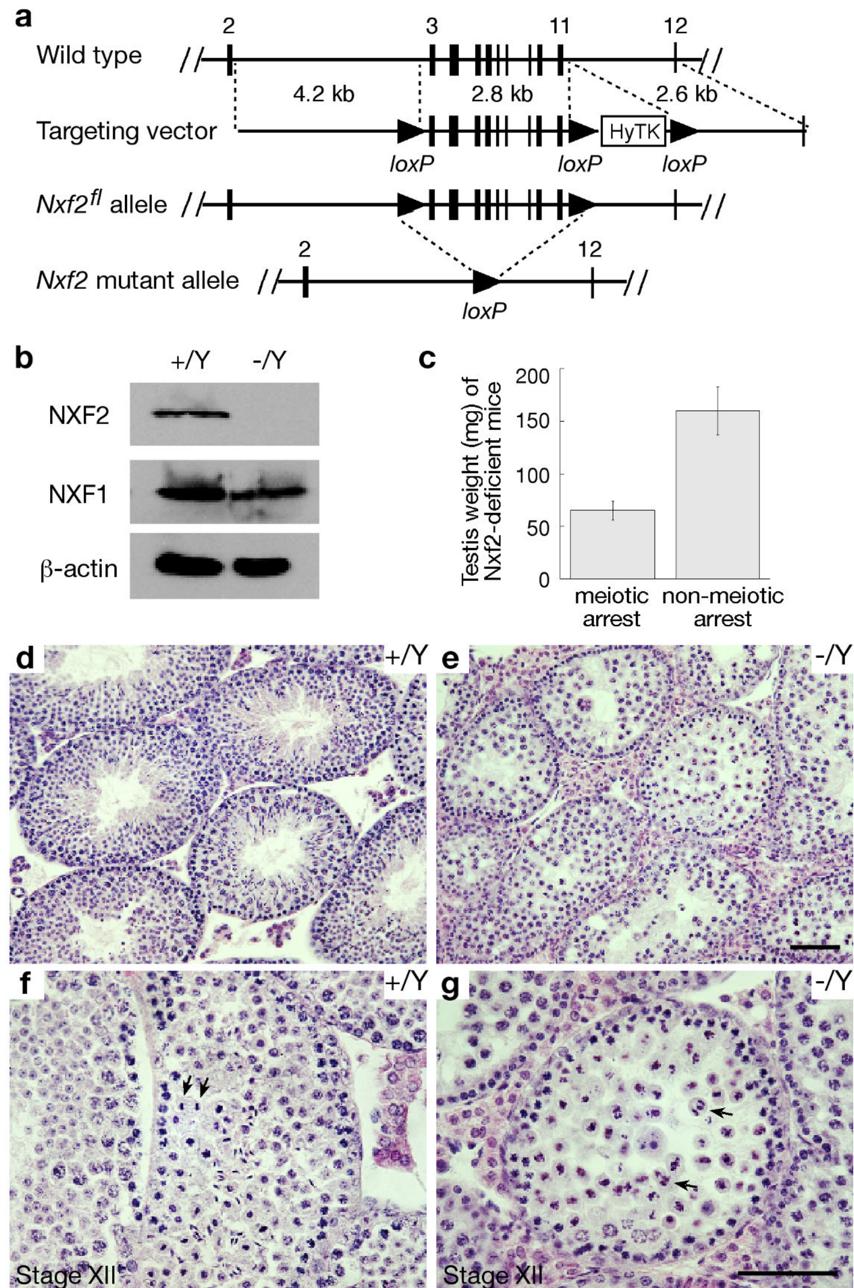

Fig. 1.

Inactivation of *Nxf2* causes male meiotic arrest in mice of mixed (C57BL/6 × 129) backgrounds. (a) The *Nxf2* targeting construct and various *Nxf2* alleles. The mouse *Nxf2* gene consists of 23 exons and spans a 22-kb genomic region on the X chromosome. In the *Nxf2*^{flox} allele, one *loxP* site is inserted in intron 2 and one in intron 11. Exons 3-11 encode amino acids 44-345. (b) Western blot analysis of adult wild type and *Nxf2*$^{-/Y}$ testes. Equal amounts (30 μg) of testis protein extracts were loaded. Three blots were probed with anti-NXF2, anti-NXF1, and anti-β-actin antibodies respectively. The abundance of NXF1 did not differ between *Nxf2*$^{-/Y}$ and wild type testes. (c) Testis weight of adult *Nxf2*$^{-/Y}$ mice. Adult *Nxf2*$^{-/Y}$ males have either small or large testes. The average testis weight ± standard deviation

is shown for each group (22 mice each). (d, f) Histological analysis of testes from adult wild type mice. (e, g) Histological analysis of small testes from 4-month-old *Nxf2*$^{-/Y}$ mice reveals meiotic arrest. Arrows indicate normal chromosome segregation in wild type anaphase I spermatocytes (f) and chaotic chromosome segregation in *Nxf2*$^{-/Y}$ anaphase I spermatocytes (g). Scale bars, 50 μm.

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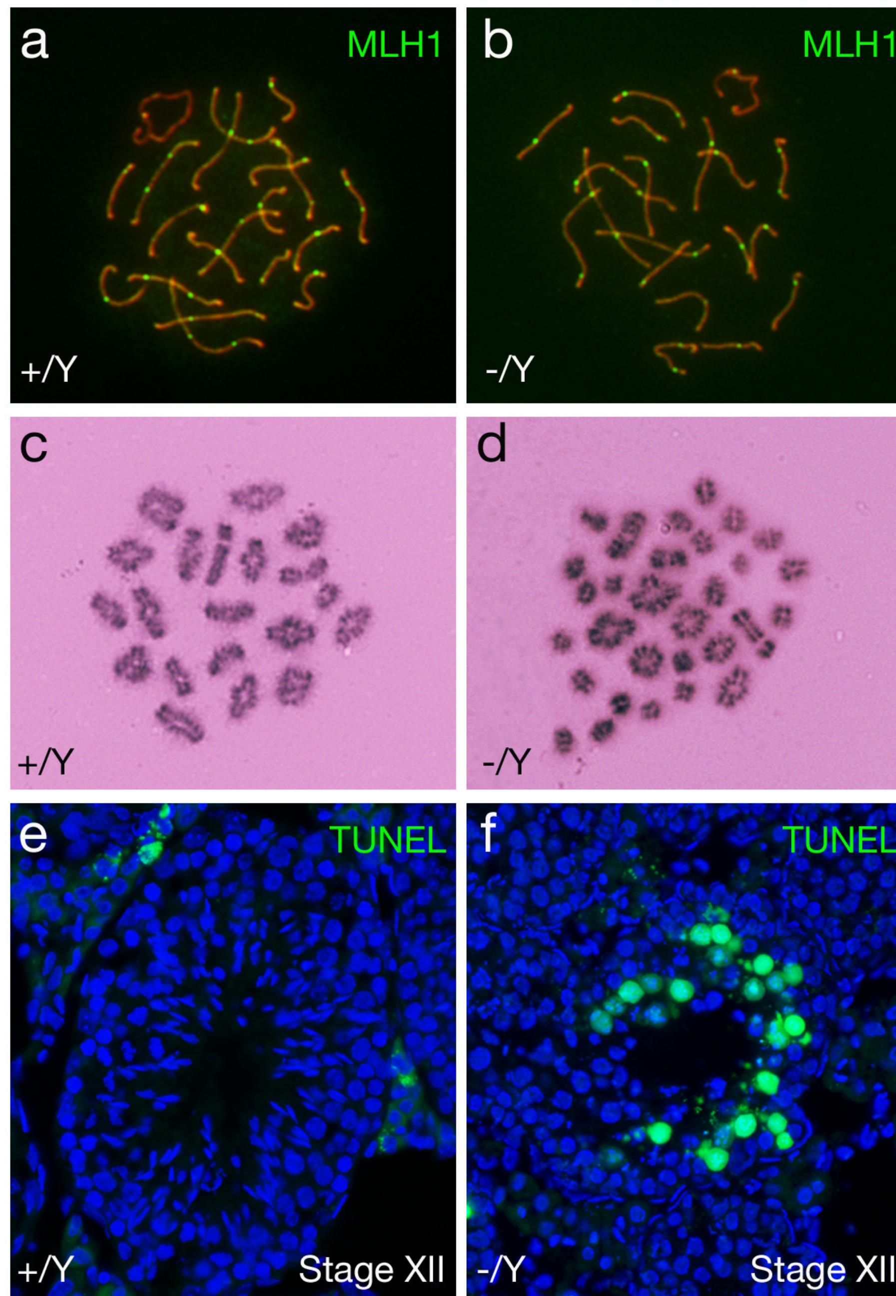

Fig. 2.

Analyses of crossovers, bivalent formation, and apoptosis in *Nxf2*-deficient spermatocytes from mice of mixed (C57BL/6 × 129) backgrounds. Adult *Nxf2*-deficient mice with small testes (~60 mg) were analyzed. (a, b) The number of MLH1 foci is comparable between wild type and *Nxf2*-deficient pachytene spermatocytes. (c) 20 bivalents in wild type metaphase I spermatocytes. (d) Prevalence of univalent chromosomes in *Nxf2*-deficient metaphase I spermatocytes. Of 30 chromatin masses, 10 are bivalent and 20 univalent. (e) TUNEL analysis of wild type tubules. Apoptotic cells are absent in stage XII tubules. (f) Massive apoptosis of germ cells (presumably anaphase I spermatocytes) in *Nxf2*$^{-/Y}$ Stage XII tubules.

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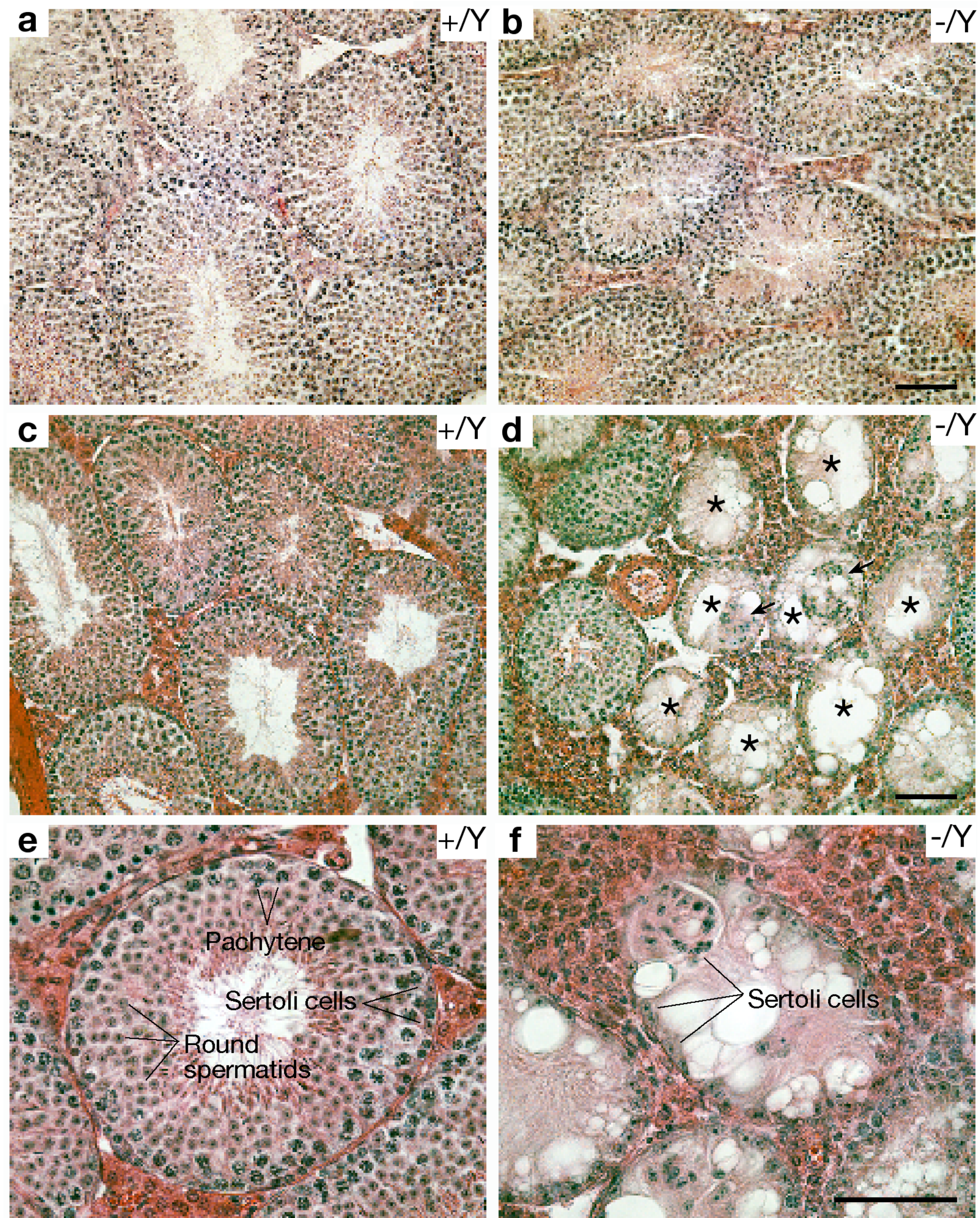

Fig. 3.

Age-dependent loss of spermatogonia in C57BL/6J *Nxf2*$^{-/Y}$ mice. (a, b) Histological analysis of testes from 2.5-month-old mice. The diameter of seminiferous tubules is smaller in *Nxf2*$^{-/Y}$ mice (b) than in wild type (a). (c, d) Histological analysis of testes from 9-month-old mice at medium magnification (200×). Sertoli cell-only (SCO) tubules (indicated by asterisks) are prevalent in *Nxf2*$^{-/Y}$ mice but not in wild type. Groups of Sertoli cells indicated by arrows are sloughed off the epithelium. (e, f) Histological analysis of testes from 9-month-old mice at high magnification (400×). Note the absence of germ cells in the *Nxf2*-deficient SCO tubule. Scale bars: 50 μm.

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Table 1

Testis weight and sperm production in *Nxf2*$^{+/Y}$ and *Nxf2*$^{-/Y}$ mice[a]

	Genotype			
	+/Y (n= 7[b])	**-/Y (n= 7[b])**	**Ratio of -/Y to +/Y**	**P value**
Body weight (g)	24.0±2.7	25.1±1.0	1.04	$p < 0.35$
Testicular weight (mg)	166±15	118±8	0.71	$p < 0.0001$*
Sperm/cauda (10^6)	6.95±2.11	3.65±0.43	0.52	$p < 0.0016$*

[a]Mice from the C57BL/6J background were used at 2 months of age.

[b]7 mice (n=7) for each genotype were used.

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

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Table 2

Motility of sperm from *Nxf2*$^{+/Y}$ and *Nxf2*$^{-/Y}$ mice[a]

Genotype	+/Y	-/Y
Motility (%)	56.3 ± 4.9	29.7 ± 1.5 *
VAP (μm/s)	126.8 ± 15.2	137.8 ± 27.0
VSL (μm/s)	75.5 ± 16.3	74.0 ± 21.2
VCL (μm/s)	257.3 ± 42.9	263.6 ± 96.4
ALH (μm)	14.6 ± 2.1	10.5 ± 5.7
BCF (Hz)	16.2 ± 0.8	14.3 ± 3.5
STR (%)	44.0 ± 3.0	23.3 ± 5.1 *
LIN (%)	56.7 ± 32.7	41.3 ± 13.9

[a] Values represent means ± standard deviations. Three adult mice of each genotype on the C57BL6/J inbred background were analyzed. Asterisks indicate statistically significant differences ($p<0.05$). Abbreviations: VAP, average path velocity; VSL, straight-line velocity; VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency; STR, straightness; LIN, linearity.