Nuclear export factor 3 is involved in regulating the expression of TGF-β3 in an mRNA export activity-independent manner in mouse Sertoli cells

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The NXF (nuclear export factor) family members are implicated in the transport of mRNA from the nucleus to the cytoplasm. Recently, some members of the NXF family have been reported to play divergent functional roles, such as post-transcriptional regulation, translational control, regulation of mRNA stability and trafficking. However, little is known about the roles of NXF3 in spermatogenesis. In the present study, we found that mouse NXF3, specifically expressed in principal cells in segment II of the caput epididymis, as well as Sertoli cells in the mouse testis, was required to mediate TGF-β (transforming growth factor β)-induced down-regulation of Tgfβ3 mRNA expression and protein secretion in Sertoli cells. In addition, NXF3 was also involved in TGF-β-induced transcriptional regulation of other genes associated with Sertoli cell maturation and the restructuring of the Sertoli cell BTB (blood–testis barrier), such as Gata1 (GATA-binding protein 1), Wt1 (Wilms’s tumour homologue 1), Cldh11 (claudin11) and Cdkn1a (cyclin-dependent kinase inhibitor 1A or p21Cip1). The transcriptional regulation of NXF3 was mediated through physical interaction with STRAP (serine/threonine kinase receptor-associated protein), where NXF3 inhibited the complex formation among Smad7, STRAP and activated type I TGF-β receptor. Taken together, our data provide mechanistic insights into the roles of NXF3 in TGF-β-mediated expression of Tgfb3 and other genes. NXF3 may be implicated in Sertoli cell maturation and the extensive restructuring of the Sertoli cell BTB.

Key words: mouse Sertoli cell, nuclear export factor 3 (NXF3), serine/threonine kinase receptor-associated protein (STRAP), Smad2/3 phosphorylation, transforming growth factor β3 secretion (TGF-β3 secretion), transforming growth factor β signalling pathway (TGF-β signalling pathway).

INTRODUCTION

Sertoli cells, known as the ‘nurse cells’ of the testis, secrete essential growth factors, such as TGF (transforming growth factor)-β3, trophic factors, regulatory proteins and nutritive factors, to the seminiferous tubule and create an appropriate environment necessary for germ cell development [1,2]. For example, TGF-β3 is implicated not only in spermatogenic cell differentiation [3], but also in regulating the extensive restructuring of the BTB (blood–testis barrier), which is conferred by adjacent Sertoli cells near the basement membrane [4,5]. BTB sequesters the more developed germ cells (i.e. pachytenic spermatocytes) from the systemic circulation by blocking the access of growth factors, nutrients and other biological substances from the seminiferous epithelium to the developing germ cells [6]. An example of BTB function is the fact that BTB restructuring allows the transit of preleptotene/leptotene spermatocytes from the basal to the apical compartment at stage VIII of the seminiferous epithelial cycle [7]. TGF-β3, which is located predominantly in stage V–VIII tubules and is also released by Sertoli cells [4,5], is needed to open up the BTB in late stage VIII to permit the migration of preleptotene and leptotene spermatocytes across the BTB [8]. Therefore an understanding of the factors regulating the synthesis and secretion of TGF-β3 will provide insights into the endocrine regulation of spermatogenesis and male fertility, or, conversely, identifying male contraceptive targets.

A recent study has shown that NXF3, a member of the nuclear export factor family, is specifically expressed in Sertoli cells in the testes of mice from puberty to adulthood [9]. The evolutionarily conserved NXF family is implicated in the transport of mRNA from the nucleus to the cytoplasm [10]. The yeast genome encodes a single NXF family protein (also called MEX67), but higher eukaryotes, such as Caenorhabditis elegans, Drosophila melanogaster and humans, generally encode more than two NXF family genes that share a similar domain organization with human NXF1 (also called TAP) in different tissues or in different phases of development. Studies suggest that NXF1 takes part in the nuclear export of mRNAs encoding housekeeping proteins, and the other NXF family members are essential for the nuclear export of developmental stage- and/or tissue-specific mRNAs [11]. In addition to mRNA transport, some members of the NXF family are reported to play more divergent functional roles. For example, human NXF1 also plays roles in translational control of an unspliced viral mRNA [12]; the non-essential C. elegans Ce-NXF2 post-transcriptionally regulates tra-2 mRNA which is required for female development [13], and mouse NXF2 plays a role in the regulation of mRNA stability and trafficking [14,15].

Abbreviations used: BTB, blood–testis barrier; Cy3, indocarbocyanine; DMEM, Dulbecco’s modified Eagle’s medium; dpp, days post-partum; ECL, enhanced chemiluminescence; EDL, efferent duct ligation; FISH, fluorescence in situ hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GST, glutathione transferase; HEK, human embryonic kidney; i.p., intraperitoneal; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; NXF, nuclear export factor; RBD, RNA-binding domain; RT, reverse transcription; SBE, Smad-binding element; siRNA, short interfering RNA; STRAP, serine/threonine kinase receptor-associated protein; TGF, transforming growth factor; TGF-β1, TGF-β receptor.

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NXF2 is highly expressed in the brain and testis [16], and its inactivation resulted in defects in spermatogenesis in mice [17]; however, Nxf3-null mutant mice appear to be grossly healthy and fertile [9]. Furthermore, Nxf2−/− Nxf3−/− double mutant males lack compound effects in the sperm output reduction compared with Nxf2−/− single mutant males. These results indicate that NXF3 function is likely to overlap with other proteins, and not with NXF2. However, NXF3 protein has not been assigned specific functions in Sertoli cells.

Since NXF3 is specifically expressed in Sertoli cells, of which the primary function is to synthesize and secrete proteins to create an appropriate environment for germ cell development [18], it is plausible to speculate that NXF3 may participate in the regulation of Sertoli cell protein synthesis or secretion. In the present study, the effects and mechanisms of NXF3 on TGF-β expression and secretion were characterized in mouse Sertoli cells for the first time.

EXPERIMENTAL

Animals, heat stress, CdCl2 treatment and unilateral EDL (efferent duct ligation)

ICR male mice were purchased from the Animal Center, University of Science and Technology of China (USTC, Hefei, China) and maintained under temperature (22°C)- and light cycle (14 h light/10 h dark)-controlled quarters. Mice were provided food and water ad libitum. This study received ethical approval from the institutional review boards of the USTC.

Male mice at the age of 60 days were anesthetized with sodium pentobarbitorne (50 mg/kg of body weight, i.p. injection), and the lower third of the body (hind legs, tail and scrotum) was submerged in a water bath of 43°C for 30 min. Control mice were anesthetized as above and left at room temperature (25°C).

After the heat treatment, the mice were dried and returned to their cages to recover from the effects of anaesthesia.

CdCl2 was prepared in 0.2% solution (in Milli-Q water), and was administered to groups of mice (n = 3 mice per time point) via i.p. (intraperitoneal) injection at 3 mg/kg of body weight, as described previously [19,20]. Thereafter, mice were killed at 1, 3, 6, 12, 16, 24, 48 and 72 h after CdCl2 treatment.

Adult mice were subjected to unilateral EDL under aseptic conditions, as described previously [21]. Briefly, mice were anesthetized as described above. After the epididymides were exposed through a low midline abdominal incision, a silk suture was tied tightly around the efferent ducts. Great care was taken to avoid damage to the regional blood vessels. The whole tissue was then returned to the scrotum, and the incision was closed with silk sutures. Control mice were killed immediately after ligation, and the others were killed at 3, 6 and 9 days after ligation. The tissues were fixed or lysed as described above.

Reagents

TGF-β1 and -β3 (R&D Systems) were reconstituted in acidified buffer [4 mM HCl (pH 5.7) and 1 mg/ml BSA] [22] to prepare 1 mg/ml stock solutions, which were added to media to yield a final concentration. For culture controls, media were treated with the acidified buffer alone in equal concentrations.

Sertoli cell isolation, cell culture and transfection

Primary Sertoli cell cultures were prepared from testes of 16-day-old mice, as described previously [23], with modifications. Briefly, testes were decapsulated, minced and digested in PBS containing 2 mg/ml collagenase (Sigma, Type IV) and 75 units/ml DNase I (Sigma) at room temperature for 5 min while being shaken softly. The digested tissues were centrifuged, and the pellets were suspended in PBS containing 2 mg/ml collagenase, 75 units/ml DNase I and 2 mg/ml hyaluronidase (Sigma, Type IV) at room temperature for 5 min with vigorous shaking. After the addition of DMEM (Dulbecco’s modified Eagle’s medium)/Ham’s F12 (Invitrogen) to dilute the digestion buffer, the suspension was filtered through a stainless steel filter (mesh 70) and then centrifuged for 5 min at 77 g. Thereafter, the supernatant was removed and the cells were washed twice with DMEM/Ham’s F12, before being cultured at 5 × 105 cells/cm² on Matrigel™-coated dishes (BD Biosciences) with DMEM/Ham’s F12 containing Hepes (15 mM), sodium bicarbonate (1.2 g/ml), bovine insulin (10 μg/ml), human transferrin (5 μg/ml), epidermal growth factor (2.5 ng/ml) and 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Life Technologies). The media were replaced daily. Sertoli cells were cultured at 35°C in a 5% CO2 atmosphere for 36 h, and then treated with 20 mM Tris/HCl (pH 7.4) for 3 min to lyse residual germ cells. Sertoli cells were harvested at the indicated time points. HEK (human embryonic kidney)-293T cells were cultured in DMEM supplemented with 10% FBS (fetal bovine serum) (Life Technologies) and 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C under a 5% CO2 atmosphere.

The siRNA (short interfering RNA) duplex targeting NXF3 and STRAP (serine/threonine kinase receptor-associated protein) were purchased from Santa Cruz Biotechnology and the negative control siRNAs were synthesized and purified by Shanghai GenePharma. Sertoli cells were transfected with siRNA duplex using Lipofectamine™ RNAiMAX (Invitrogen). HEK-293T cells were transfected using Lipofectamine™ 2000 (Invitrogen). The transfection procedure was performed following the manufacturer’s instructions.

Antibodies

Antisera were generated in two rabbits against the synthetic decapetide EDTISEDKFTQTDPRS, corresponding to the biologically active N-terminal peptide of mouse NXF3. Rabbit polyclonal anti-NXF3 antibody was produced by Abgent Biotechnology. Western blot analysis revealed that, in mouse testis, the anti-NXF3 antibody detected two closely positioned protein bands whose estimated molecular masses were 60 kDa. The other primary and secondary antibodies used in the present study are listed in Supplementary Tables S1 and S2 respectively at http://www.biochemj.org/bj/452/bj4520067add.htm.

Plasmid construction

The full-length cDNAs for mouse NXF3, TβR1 (TGF-β receptor 1) and STRAP were obtained by RT (reverse transcription)–PCR from total RNA of mouse testis using primers containing specific restriction sites. For the construction of expression vectors, Nxf3 cDNA was cloned into pXFLAG-myc-CMV-24 (Sigma) pEGFP-C1 (Clontech) or pGEX-5X-3 (GE Healthcare) vectors at EcoRI and SalI sites. Streap cDNA was cloned into the pXFLAG-myc-CMV-24 vector at BglII and SalI sites, and Tgfbr1 (TβR1) cDNA was cloned into the pEGFP-C1 vector at EcoRI and SalI sites. A series of truncated forms of NXF3 and STRAP were generated by PCR and subcloned into pGEX-5X-3 and pXFLAG-myc-CMV-24 expression vectors respectively. A constitutively active mutant of TβR1(T204D) was
overnight at 37°C in a buffer containing 1 pmol of oligo(dT) probes and incubated with a biotinylated secondary antibody (Abcam) for 2 h at room temperature. Immunosignals were visualized using streptavidin–biotinylated secondary antibody (Maixin Bio) as the chromogen. A negative control was included whereby the primary antibody was omitted.

Immunofluorescence analysis was performed as described by Liang et al. [24]. Nuclei were stained with Hoechst 33342 (Sigma). Fluorescent signals were examined using a Nikon Eclipse 80i epifluorescence microscope.

FISH (fluorescence in situ hybridization)

Cy3 (indocarbocyanine)-labelled oligo(dT) probes (red) were purchased from TaKaRa Bio and FISH was performed as described previously [24]. Briefly, slides with spermatogonia/Sertoli cells isolated from 16 dpp (days post-partum) mouse testis sections were fixed overnight at room temperature in 1% (w/v) paraformaldehyde and then pre-hybridized for 6 h at 37°C with 700 μl of pre-hybridization buffer [50% formamide, 5× saline sodium citrate (0.75 M NaCl/0.075 M sodium citrate), 5× Denhardt’s (0.1% Ficoll 400/0.1% polyvinylpyrrolidone/0.1% BSA), 200 μg/ml yeast RNA, 500 μg/ml salmon sperm DNA, 2% blocking reagents (Roche) and diethylpyrocarbonate-treated water]. Subsequently, slides were overlaid with 150 μl of hybridization buffer [pre-hybridization buffer containing 1 pmol of oligo(dT) probes] and incubated overnight at 37°C in a humidified chamber. After hybridization, rabbit anti-NXF2 antibody (1:50 dilution) or rabbit anti-NXF3 antibody (1:200 dilution) was used as primary antibody, and Alexa Fluor® 488-labelled anti-rabbit antibody (1:200 dilution) was used as secondary antibody, to detect NXF2 and NXF3 in situ samples. Hoechst was used to stain the nucleus. Fluorescent signals were examined using a Nikon Eclipse 80i epifluorescence microscope.

Western blotting

Tissues and cells were lysed in RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% SDS, 1% sodium deoxycholate and 1 mM EDTA) containing Complete™ EDTA-free protease inhibitor cocktail (Roche), 1 mM PMSF and phosphatase inhibitors (5 mM sodium orthovanadate). The nuclear and cytoplasmic extracts were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) according to the manufacturer’s instructions. Protein lysates were loaded on to SDS/PAGE gels, electroblotted on to Hybond™ ECL (enhanced chemiluminescence) nitrocellulose membrane (GE Healthcare), immunoblotted with antibodies and visualized using ECL (Kodak). Protein levels were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The primary and secondary antibodies used for immunoblotting are listed in Supplementary Tables S1 and S2 respectively.

GST (glutathione transferase) pull-down assay

GST–NXF3 full-length and GST–NXF3 fragment were expressed from the pGEX-5X-3 vector in Escherichia coli BL21(DE3)pLysS cells at 30°C and purified with glutathione–Sepharose 4B beads (GE Healthcare). The purified GST-fusion proteins were then incubated with testis lysates or cell lysates from HEK-293T cells transfected with the indicated plasmids. The GST precipitates were subjected to SDS/PAGE, followed by Coomassie Brilliant Blue staining or immunoblotting with anti-FLAG antibodies. Separated protein bands in SDS/PAGE were excised from the gel and processed for LC (liquid chromatography)–MS analysis.

IP (immunoprecipitation) assay

IP was performed as described previously [24] with some modifications. HEK-293T cells were transfected with the indicated plasmids using Lipofectamine™ 2000 and cultured for 36 h. Cells or 16 dpp mouse testes were lysed in TNE buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 1 mM PMSF, 1 mM activated sodium orthovanadate and 10 μg/ml protease inhibitor cocktail). The lysates were centrifuged, and the supernatants were pre-cleared with Protein G–Sepharose 4 Fast Flow (GE Healthcare) for 2 h at 4°C on an end-over-end rotator. The pre-cleared lysates were then immunoprecipitated with the anti-GFP (green fluorescent protein) or anti-NXF3 antibody overnight at 4°C on an end-over-end rotator. The immunoprecipitated proteins were resolved by SDS/PAGE.

RT–PCR and real-time PCR

Total RNA was extracted using the SV Total RNA Isolation System (Promega). RT–PCR was performed using a PrimeScript One-Step RT-PCR Kit Ver.2 (TaKaRa Bio), as described previously [24]. Real-time PCR was performed on the ABI Step One System (Applied Biosystems) using a SYBR Premix EX Taq II kit (TaKaRa Bio), according to the manufacturer’s protocols. All of the mRNA expression levels were normalized to GAPDH mRNA. Primer sequences for RT–PCR and real-time PCR are listed in Supplementary Tables S3 and S4 respectively at http://www.biochemj.org/bj/452/bj4520067add.htm.

Luciferase reporter assay

Luciferase reporter assays were performed as described by Lian et al. [25]. HEK-293T cells were transiently co-transfected with the p3TP-Lux reporter plasmid (a gift from Dr Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) or SBE (Smad-binding element)–Luc reporter plasmid (a gift from Dr Bart J.L. Eggen, Department of Neuroscience, University Medical Centre Groningen, Groningen, The Netherlands) and the expression vectors indicated. The total DNA concentration in each group was kept constant by supplementing with pEGFP-C1 empty vector DNA. Cells were harvested 48 h after transfection and assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega).

ELISA

To measure secreted TGF-β3, Sertoli cells were seeded in 12-well plates and treated with TGF-β1 (5 ng/ml) alone or together with SB435124 or SB203580 (10 μM), or were transfected with
genes such as role(s) in an mRNA export activity-independent manner in Sertoli cell localization in the cytoplasm of Sertoli cells and accumulated at one spot within the nucleus (Figures 1D and 1E, Supplementary Figure S1 at http://www.biochemj.org/bj/bj452/bj4520067add.htm). Unlike other members of the NXF family, such as NXF2 that co-localized with poly(A) RNAs in the spermatogonium nucleus, the NXF3 spots did not co-localize with the accumulated poly(A)+ RNAs in the nucleus (Figure 1E). This raises the possibility that NXF3 plays distinctive functional role(s) in an mRNA export activity-independent manner in Sertoli cells.

To understand the role of NXF3 during spermatogenesis, NXF3 protein levels in various mouse tissues and cellular localization in testsis were determined by Western blotting and immunohistochemistry respectively. Similar to previous studies [9], the NXF3 protein was detected only in the adult mouse testis among all of the tissues examined (Figure 1A), and expressed exclusively in Sertoli cells (Figure 1B) in a segment II epithelium (Figure 1F, panels i–iii). In addition, Western blot analysis of NXF3 expression in epididymides at different developmental stages showed that NXF3 was first present in the epididymides of 4-week-old mice (completion of the first wave of spermatogenesis), peaked at 5 weeks postparturition and was thereafter maintained (Figure 1G). The time of onset of NXF3 expression in epididymis is consistent with the entry of testicular fluid into the epididymis [29]. Furthermore, unilateral EDL, thereby preventing testicular fluid from entering the epididymis, revealed that NXF3 expression remained unaltered between the ipsilateral and contralateral testes (Figure 1H), but reduced on the ipsilateral epididymides (Figure 1I) without changing its localization (results not shown). These data suggest that NXF3 from the epididymis is regulated by factors from the testis.

RESULTS

NXF3 is specifically expressed in Sertoli cells of the mouse testis as well as principal cells of the proximal caput epididymis region (segment II)

To test further whether NXF3 negatively regulates TGF-β3 secretion, we specifically silenced NXF3 (si-NXF3) in Sertoli cells by using RNAi (RNA interference) (Figure 2E). Unexpectedly, knockdown of NXF3 in Sertoli cells did not up-regulate TGF-β3 production (Figures 2H and 2I). However, si-NXF3 (Figures 2H and 2I) and inhibition of TGF-β signalling by SB431542 (a specific inhibitor of TβR1 kinase) (Figures 2F and 2G) partially and totally abrogated TGF-β3 mRNA expression and protein secretion respectively. The NXF3 protein levels in various mouse tissues and cellular extracts or immunofluorescence staining showed a predominant localization in the normal mouse testes, and in CdCl2-, heat-treated testes. These data suggest that NXF3 from the epididymis is regulated by factors from the testis.

NXF3 mediates TGF-β-induced down-regulation of Tgfb3/TGF-β3 mRNA expression and protein secretion in an mRNA export activity-independent manner in Sertoli cells

To determine how NXF3 regulates TGF-β3 expression, we specifically silenced NXF3 (si-NXF3) in Sertoli cells by using RNAi (RNA interference) (Figure 2E). Unexpectedly, knockdown of NXF3 in Sertoli cells did not up-regulate TGF-β3 production (Figures 2H and 2I). However, si-NXF3 (Figures 2H and 2I) and inhibition of TGF-β signalling by SB431542 (a specific inhibitor of TβR1 kinase) (Figures 2F and 2G) partially and totally abrogated TGF-β3 mRNA expression and protein secretion (Figures 2F and 2G). These results indicate that NXF3 mediates TGF-β-induced regulation of TGF-β3 expression possibly through TGF-β/Smad signalling. Furthermore, these results also indicate that NXF3 mediates TGF-β-induced down-regulation of TGF-β3 expression in an mRNA export activity-independent manner, since knockdown of NXF3 has no effect on TGF-β3 expression in the absence of TGF-β1/β3 and enhances TGF-β3 expression in the presence of TGF-β1/β3 (Figures 2H and 2I).

NXF3 modulates TGF-β-induced transcription through physical interaction with STRAP

To determine how NXF3 regulates TGF-β-induced transcription, a p3TP-Lux reporter plasmid was used to assay for TGF-β transcriptional activity. The p3TP-Lux contains elements from the plasminogen activator inhibitor-1 promoter and drives the expression of a luciferase reporter gene in response to TGF-β1 [32]. We found that overexpression of NXF3 enhanced TGF-β-induced activation of a p3TP-Lux reporter in a dose-dependent manner (Figure 3A). To test whether the Smad pathway is involved in the enhancement of the p3TP-Lux activity by NXF3, HEK-293T cells were co-transfected with p3TP-Lux and GFP–NXF3, and then incubated with SB431542. The results showed that SB431542 decreased NXF3-mediated transcriptional activation of p3TP-Lux, but that SB203580 and U0126 (MEK1/2 [MAPK/ERK (extracellular-signal-regulated kinase) kinase 1/2] inhibitor) had no effects on NXF3-stimulated p3TP-Lux activity (Figure 3B). Similar to the effect of NXF3 on the p3TP-Lux reporter, overexpression of NXF3 in HEK-293T cells also caused a significant increase in TGF-β-induced activation of SBE–Luc (a Smad-specific reporter containing multimerized SBEs from the JunB gene [33]) in a dose-dependent manner (Figure 3C). Overexpression of NXF3 in HEK-293T cells (Figure 3D) and knockdown of NXF3 in Sertoli cells (Figure 3E) respectively showed that NXF3 mediates TGF-β-induced down-regulation of Tgfb3/TGF-β3 mRNA expression and protein secretion in an mRNA export activity-independent manner in Sertoli cells.

TGF-β3, one of growth factors produced by Sertoli cells, is involved in regulating BTB dynamics and spermatogenic cell differentiation [3–5]. CdCl2- or heat-induced BTB disruption was associated with a transient induction in testicular TGF-β2 and TGF-β3 [30,31]. In the present study, we examined the correlation between NXF3 and TGF-β expression in different developmental stages of mouse testes (Figures 2A and 2B), in CdCl2-treated (Figure 2C) and heat-stressed (Figure 2D) testes. A negative correlation was found between the levels of TGF-β3 and NXF3 in the normal mouse testes, and in CdCl2- or heat-treated testes. For example, in normal mouse testes, TGF-β3 expression peaked at 10 dpp and then gradually decreased afterwards, whereas NXF3 began to be expressed at 10 dpp (Figures 2A and 2B).

Statistical analysis

Experiments in the present study were repeated at least three times. Results are means ± S.E.M. Student’s t test was used to examine the differences among variables by using the SAS Software. A P value <0.05 was considered statistically significant.
NXF3 regulates TGF-β/Smad signalling in Sertoli cells

Figure 1 NXF3 expression in mouse testis and epididymis

(A) Western blot analysis of NXF3 protein expression in various mouse tissues. (B and C) Immunohistochemistry of NXF3 in the mouse testis at different developmental stages. (B) Representative images showing immunohistochemical localization of NXF3 in the adult (60 dpp) mouse testis. Pre-serum was used as a negative control for the primary antibody. Scale bars, 50 μm. (D) Subcellular localization of NXF3 in Sertoli cells. Western blot analysis of NXF3 in total, nuclear and cytoplasmic protein fractions of Sertoli cells isolated from 16 dpp mouse testis by NE-PER Nuclear and Cytoplasmic Extraction Reagents. (E) Co-localization of NXF3/NXF2 with poly(A) + RNA in the nucleus. A Cy3-labelled oligo(dT) probe (red) was used to detect mRNAs with a poly(A) tail in spermatogonia and Sertoli cells. After FISH with Cy3-labelled oligo(dT), NXF2 and NXF3 signals were revealed by immunofluorescence (green). Hoechst (blue) was used to stain the nucleus. Scale bars, 20 μm. (F) Immunohistochemical staining for NXF3 in the adult (60 dpp) mouse epididymis. The brown immunosignals for NXF3 were only observed in principal cells (arrow in panel iv) of segment II (panels ii and iii) of the caput epididymis (panel i). Scale bars, 500 μm (panel i), 100 μm (panels ii and iii) and 50 μm (panel iv). (G–I) Western blot analysis of NXF3 protein expression in the mouse epididymides at different developmental stages and in the brain (negative control) (G), testes (H) and epididymides (I) during the days after EDL. pc, principal cell; PS, primary spermatocyte; RS, round spermatid; SC, Sertoli cell; SPG, spermatogonia.
enhanced or partially inhibited TGF-β1-stimulated Smad-2 and -3 phosphorylation. Furthermore, TGF-β/Smad signalling regulated the expression of many important genes in Sertoli cells (Figure 3F) and si-NXF3 partially reversed the up-regulated (such as Cdkn1a) or down-regulated (such as Gata1, Wt1 and Cldn11) gene expression by TGF-β/Smad signalling (Figure 3G). These results indicate that NXF3 modulates TGF-β-induced Smad2/3-mediated transcription.

To elucidate the molecular mechanisms by which NXF3 regulates TGF-β-induced transcription, GST pull-down assays were performed using full-length NXF3 as bait to identify associated proteins in the mouse testes (results not shown). Several bands that bind specifically to GST–NXF3, but not to GST, were observed, and one band was identified as STRAP, a WD40 domain-containing protein that interacts with both TβR1 and TβRII and negatively regulates TGF-β signalling [34]. The NXF3–STRAP interaction was then verified in vitro and in vivo by Co-IP (co-immunoprecipitation) assays (Figure 3G). These results indicated that the association between NXF3 and STRAP was mediated through a less conserved region upstream of the RBD (RNA-binding domain) (between amino acids 1 and 21) [35] in NXF3 (Figure 4F) and the domain (amino acids 174–216) between the fourth and fifth WD40 repeat of STRAP (Figure 4G). In addition, mutation of the STRAP interaction domain in NXF3 attenuated NXF3-mediated p3TP-Lux luciferase activity (Figure 5A). Furthermore, knockdown of STRAP in Sertoli cells (Figure 5B) not only induced the down-regulation of Tgfb3/TGF-β3 mRNA expression and protein secretion, but also abrogated the effect of NXF3 on Tgfβ3/TGF-β3 mRNA expression and protein secretion in the presence of TGF-β1 (Figures 5C and 5D). These results suggest that NXF3-mediated activation of TGF-β signalling is partly dependent on its interaction with STRAP.

NXF3 destabilizes the complex among STRAP, Smad7 and TβR1(T204D)

To explore further the underlying mechanisms of NXF3 involved in TGF-β signalling via interactions with STRAP, we examined the effect of NXF3 on the association between Smad7 and the activated TβR1(T204D), because STRAP was known to inhibit...
NXF3 regulates TGF-β1/Smad signalling in Sertoli cells

Figure 3 NXF3 is involved in TGF-β-induced transcriptional regulation through the Smad pathway

(A–C) Luciferase activity derived from the co-transfected p3TP-Lux or SBE–Luc reporter construct. (A and C) HEK-293T cells were co-transfected with GFP, the p3TP-Lux reporter construct (or SBE–Luc reporter construct) and increasing amounts of plasmid expressing GFP–NXF3 for 24 h, and then treated with or without 1 ng/ml TGF-β1 or TGF-β3 for 24 h. (B) HEK-293T cells were transiently co-transfected with GFP, p3TP-Lux and GFP–NXF3-expressing plasmid for 24 h and then treated with 10 μM SB203580, U0126 or SB431542 for 24 h. All groups were co-transfected with pHRG-TK Renilla luciferase reporter vector to normalize for transfection efficiency differences. The cells were harvested and assayed for luciferase activities. Data obtained from untreated GFP-alone-transfected cells were set at 1.0. (D and E) Kinetics of Smad2/3 phosphorylation in HEK-293T cells and Sertoli cells. HEK-293T cells were transiently transfected with expression vectors encoding GFP–TβR1(T204D), Myc–Smad7, FLAG–STRAP and FLAG–NXF3. As shown in Figure 6(A), compared with control cells expressing GFP–TβR1(T204D), FLAG–STRAP and Myc–Smad7, the co-expression of NXF3 significantly inhibited the association between TβR1(T204D) and Smad7 (Figure 6A, top panel, fifth to eighth lanes from the left), indicating that NXF3 plays a role in preventing the formation of TβR1(T204D)–Smad7 complex. We then examined whether the co-expression of NXF3 affects the association between STRAP and Smad7 since STRAP stabilizes the association between TβR1(T204D) and Smad7 that is responsible for inhibiting TGF-β signalling [36]. As indicated in Figure 6(B), the interaction of Smad7 with STRAP was significantly decreased in the transfected cells expressing NXF3 compared with the control cells without NXF3 (fourth to seventh lanes from the left). Collectively, these results suggest that NXF3 inhibits the complex formation of TβR1–Smad7 for enhancing TGF-β signalling, via interaction with STRAP. The scheme shown in Figure 6(C) is a model for the mechanism of

TGF-β signalling by stabilizing the complex between TβR1 and Smad7 [36]. HEK-293T cells were transiently transfected with GFP–TβR1(T204D), Myc–Smad7, FLAG–STRAP and FLAG–NXF3. As shown in Figure 6(A), compared with control cells expressing GFP–TβR1(T204D), FLAG–STRAP and Myc–Smad7, the co-expression of NXF3 significantly inhibited the association between TβR1(T204D) and Smad7 (Figure 6A, top panel, fifth to eighth lanes from the left), indicating that NXF3 plays a role in preventing the formation of TβR1(T204D)–Smad7 complex. We then examined whether the co-expression of NXF3 affects the association between STRAP and Smad7 since STRAP stabilizes the association between TβR1(T204D) and Smad7 that is responsible for inhibiting TGF-β signalling [36]. As indicated in Figure 6(B), the interaction of Smad7 with STRAP was significantly decreased in the transfected cells expressing NXF3 compared with the control cells without NXF3 (fourth to seventh lanes from the left). Collectively, these results suggest that NXF3 inhibits the complex formation of TβR1–Smad7 for enhancing TGF-β signalling, via interaction with STRAP. The scheme shown in Figure 6(C) is a model for the mechanism of
**Figure 4  NXF3 interacts with STRAP specifically**

(A and B) IP analysis for the interaction between NXF3 and STRAP in vitro and in vivo. (A) HEK-293T cells were transfected with expression plasmids encoding GFP or GFP–NXF3, along with an expression vector encoding FLAG–STRAP. The cell lysates were first immunoprecipitated with anti-GFP antibody and then immunoblotted (WB) using anti-GFP and anti-FLAG antibodies. (B) Lysates from the 16 dpp mouse testes were immunoprecipitated with either rabbit pre-immune IgG (Control IgG) or rabbit anti-NXF3 antibody, and then immunoblotted with anti-STRAP antibody. One-tenth of cell extracts before IP was saved as input fraction. (C) Co-localization of STRAP and NXF3 in mouse testis and primary Sertoli cells. Adult mouse testis and primary Sertoli cells were immunostained using anti-NXF3 and anti-STRAP antibodies, followed by Alexa Fluor® 594-conjugated anti-mouse secondary antibody (for STRAP, in red) or Alexa Fluor® 488-conjugated anti-rabbit secondary antibody (for NXF3, in green) and then examined using fluorescence microscopy. Nuclei were visualized with Hoechst 33342. The yellow colour in merged images represents co-localization. Scale bars, 20 μm. (D) Schematic diagram of GST-fused wild-type and deletion mutants of NXF3. Residue numbers at domain boundaries are indicated. (E) Mapping the NXF3-interacting domain of STRAP by GST pull-down assay. Purified GST–NXF3 and its fragments were used to absorb FLAG–STRAP from the lysates of HEK-293T cells. The bound materials were then resolved by SDS/PAGE and immunoblotted with anti-FLAG antibody. (F) Schematic representation of wild-type STRAP and STRAP deletion mutants. Residue numbers at domain boundaries are indicated. (G) Mapping the STRAP-interacting domain of NXF3 by GST pull-down assay. Purified GST–NXF3 was used to absorb FLAG–STRAP and its fragments from the lysates of HEK-293T cells. The bound materials were resolved by SDS/PAGE and immunoblotted with anti-FLAG antibody.
NXF3 regulates TGF-β/Smad signalling in Sertoli cells

**Figure 5** The interaction between NXF3 and STRAP is required for the effect of NXF3 on TGF-β3 expression

(A) NXF3-mediated p3TP-Lux luciferase activity requires an interaction between NXF3 and STRAP. HEK-293T cells were transiently transfected with p3T P-Lux, GFP alone and increasing amounts of expressing vectors for GFP–NXF3 or GFP–NXF3 deletion mutant (NXF3 Δ2: residues 21–553), along with the expressing vectors for STRAP for 24 h and then treated with and without 1 ng/ml TGF-β3 for 24 h. The total DNA concentration in each group was kept constant by supplementing with GFP empty vector DNA. All groups were co-transfected with p HRG-TK Renilla luciferase reporter vector to normalize for transfection efficiency differences. Data obtained from untreated GFP alone-transfected cells were set at 1.0. (B) The efficacy of STRAP siRNA was evaluated by Western blotting after 48 h transfection with either siRNA negative control (si-NC) or STRAP siRNA (si-STRAP). (C and D) Analysis of Tgfb3/TGF-β3 mRNA expression and protein secretion in Sertoli cells. Sertoli cells were transfected with si-STRAP, alone or together with si-NXF3 or si-NC for 48 h followed by TGF-β3 or TGF-β1 (5 ng/ml) treatment. The medium was collected and subjected to ELISA for TGF-β3 after 24 h of treatment (D). The RNA was extracted from the Sertoli cells and subjected to real-time PCR after 12 h of treatment (C). Results are means ± S.E.M. for three independent experiments performed in triplicate. *P < 0.05; **P < 0.001.

NXF3 involved in TGF-β/Smad2/3 signalling in mouse Sertoli cells.

**DISCUSSION**

The evolutionarily conserved NXF family has been implicated in the nuclear export of bulk poly(A)+ RNAs [10,37,38]. Recently, several studies have indicated that some members play divergent functional roles in post-transcriptional regulation [13], translational control [12], the regulation of mRNA stability and trafficking [14,15]. In the present study, we found that NXF3 was also expressed specifically in the principal cells of the proximal caput epididymis region (segment II), as well as Sertoli cells in the mouse testis, and mediated TGF-β- induced down-regulation of Tgfb3/TGF-β3 mRNA expression and protein secretion in an mRNA export activity-independent manner in Sertoli cells. In addition, NXF3 was also involved in the transcriptional regulation of some other genes which are implicated in the regulation of Sertoli cell maturation and BTB restructuring. Furthermore, NXF3 was found to regulate transcriptional activity by physical interaction with STRAP. The NXF3–STRAP association destabilized the complex among Smad7, STRAP and activated TβR1. Notably, an NXF family member is demonstrated, for the first time, to participate in transcriptional regulation.

We have repeatedly found that NXF3 is exclusively expressed in the testicular Sertoli cells. Additionally, NXF3 is predominantly localized in the cytoplasm and accumulated at one spot within the nucleus in Sertoli cells (Figure 1E), HEK-293T cells transfected with GFP–NXF3 (Supplementary Figure S1, upper panels) and mouse testes (Supplementary Figure S1, lower panels). Real-time live cell imaging was performed with HEK-293T cells transfected with GFP–NXF3 and revealed that the spot moved rapidly along the nuclear envelope and, in some cases, into the nucleus, but made frequent contacts with the nuclear rim (Supplementary Figure S2...
Figure 6  NXF3 inhibits the association between Smad7 and activated TβR1

(A) Effect of NXF3 on the association of activated TβR1 and Smad7. HEK-293T cells were transfected with the indicated combinations of expression vectors for GFP alone, TβR1(T204D), Smad7, STRAP and increasing amounts of NXF3. The total DNA concentration in each group was kept constant by supplementing with FLAG and Myc empty vector DNA. Cell lysates were subjected to IP with anti-GFP antibody, and the presence of Smad7 in the immunoprecipitates was detected by immunoblotting with an anti-Myc antibody (top). To confirm the equivalent expression of Smad7, STRAP and TβR1(T204D), aliquots of total cell lysates were immunoblotted with anti-Myc, anti-FLAG and anti-GFP antibodies. (B) NXF3 inhibits the association between Smad7 and STRAP. HEK-293T cells were transfected with indicated combinations of FLAG, FLAG–STRAP, 6Myc–Smad7, and increasing amounts of GFP–NXF3. Cell lysates were immunoprecipitated with anti-FLAG antibody, and complex formation was determined by immunoblotting using an anti-FLAG antibody, as described in (A). (C) Schematic drawing illustrating the mechanism of NXF3 involved in TGF-β-induced down-regulation of TGF-β3 expression. The expression of TGF-β3 is inhibited by TGF-β/Smad2/3 signalling in Sertoli cells. The functional association between NXF3 and STRAP results in inhibiting the complex formation among TβR1, STRAP and Smad7, enhancing TGF-β/Smad2/3 signalling, ultimately leading to suppression of TGF-β3 secretion. mSC, mouse Sertoli cells.

at http://www.biochemj.org/bj/452/bj4520067add.htm). Unlike NXF1 [39]/NXF2 that co-localized with poly(A)+ RNAs in the nucleus, the NXF3 spot did not co-localize with the accumulated poly(A)+ RNAs. Combined with these observations, our results suggest that NXF3 might play different roles in Sertoli cells. In addition, NXF3 is found to also be specifically expressed in principal cells of caput epididymal segment II and the expression is developmentally regulated in the epididymis, which is affected by testicular factors. Sertoli cells, which undergo a differentiation and maturation process including dramatic morphological and functional changes around puberty, play a crucial role in supporting spermatogenesis [40]. The epididymis contributes to sperm transport, concentration, protection and storage as well as maturation [41]. Given that NXF3 expression coincides well with the differentiation of immature Sertoli cells in the mouse testis and the initial entry of testicular fluid containing the first wave of spermatooza into the epididymis, we speculate that NXF3 plays a role in facilitating spermatogenesis and epididymal sperm maturation. Furthermore, NXF3 function is likely to overlap with other protein(s), because Nxf3-null mice appear to be grossly healthy and fertile [9]. However, the precise function of NXF3 in the testis remains unknown.

It is well known that TGF-β3 and TNFα (tumour necrosis factor α) play a crucial role in regulating Sertoli cell BTB dynamics [8,30]. TGF-β3 predominantly localizes to stages V–VIII of the seminiferous epithelial cycle, becomes diminished in stages IX and X, and is virtually undetectable in stages XII–XIV [5]. The localization patterns allow TGF-β3 to transiently disrupt the BTB in late stage VIII and facilitate extensive restructuring at stages IX and X. In the present study, we observed that the NXF3 expression level is correlated negatively with TGF-β3 in the testes under normal and pathological conditions.
Furthermore, NXF3 is required to mediate TGF-β-induced down-regulation of Tgfb3/TGF-β3 mRNA expression and protein secretion in Sertoli cells. In addition to TGF-β3, NXF3 is also involved in TGF-β-induced down-regulation of some genes, such as Gata1, Wt1 and Cldn11, and up-regulation of Cdkn1a in Sertoli cells. GATA1 is a marker of mature Sertoli cells [42] and the cell cycle inhibitor p21Cip1 suppresses Sertoli cell proliferation [43]. Claudin11 is an important integral membrane protein of tight junctions constituting the BTB [44], and WT1 is involved in regulation of adherens junction-associated genes [45]. The developmental expression of NXF3 in the testis coincides well with the differentiation of immature Sertoli cells. It is therefore suggested that NXF3 may be involved in Sertoli cell differentiation by inhibiting cell proliferation. Additionally, these results also suggest that NXF3 may serve as a potential regulator of BTB dynamics by influencing TGF-β3 expression in the seminiferous epithelial cycle and down-regulating WT1 and Claudin11. Understanding the molecular mechanisms by which NXF3 regulates TGF-β-induced transcription will provide further insight into the precise function of NXF3 in spermatogenesis.

Regulation of specific gene transcription by endocrine signals usually involves the altered recruitment of transcriptional regulatory proteins to the promoter regions of target genes, or alterations of the activity of proteins already associated with the gene [46]. Protein–protein interactions could control the localization of proteins, their substrate-processing activity, and even their tagging for destruction or recycling [47,48]. In the present study, we identified STRAP as an interacting partner of NXF3 and the association between them contributed partly to NXF3-mediated activation of TGF-β signalling (resulting in Smad2/3 phosphorylation). In addition, we also found that NXF3 inhibited the complex formation among Smad7, STRAP and activated SmR1 by preventing the association between STRAP and Smad7. It is well known that protein–protein interactions can be mediated by prototypical protein-binding domains as well as disulfide linkages in the interacting proteins [49]. Although the functional motifs of STRAP are WD40 repeats that are generally involved in protein–protein interactions and the assembly of multienzyme complexes [50], the WD40 repeat domains appear to have no role in the association between NXF3 and STRAP. Similarly, part of a less conserved region upstream of the RBD in NXF family played a critical role in the association of NXF3 with STRAP. This may be the reason STRAP interacts specifically with NXF3, but not with NXF1 or NXF2.

In summary, NXF3, which is specifically expressed in principal cells of the caput epididymis segment II and Sertoli cells, is required for TGF-β-induced down-regulation of TGF-β3 secretion in Sertoli cells by interacting with STRAP. The physiological role of NXF3 in the testis reveals the potential application of NXF3 in the control of Sertoli cell BTB, or in the investigation of the roles of Sertoli cells and caput epididymis on sperm maturation by the generation of NXF3–Cre mice.

AUTHOR CONTRIBUTION

Yimeng Yin and Fei Sun designed the experiments. Yimeng Yin and Guishuan Wang carried out almost all experiments and interpreted results with the help of Fei Sun. Yimeng Yin wrote the paper with the help of Guishuan Wang and Fei Sun.

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REFERENCES

SUPPLEMENTARY ONLINE DATA

Nuclear export factor 3 is involved in regulating the expression of TGF-β3 in an mRNA export activity-independent manner in mouse Sertoli cells

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Department of Cell and Developmental Biology, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China, and Hefei National Laboratory for Physical Sciences at Microscale, Hefei, Anhui 230027, China

Immunofluorescence and image analysis

HEK-293T cells were transiently transfected with GFP–NXF3 and cultured for 24 h. Time-lapse recordings of transfected cells were performed at 37°C and captured in a GFP-configured channel every 3 min using a Leica AF6000LX imaging system and analysed using Leica application software.

![GFP-NXF3, Hoechst, Merge](image1.png)

**Figure S1** Immunofluorescence staining for NXF3 in HEK-293T cells (upper panels) and in mouse testis (45 dpp) (lower panels) transfected with plasmid expressing GFP–NXF3

A spot found in the nucleus of isolated Sertoli cells (Figure 1E of the main text) was also detected in transfected HEK-293T cells (upper panel; scale bars, 80 μm) and in the mouse testes (lower panels; scale bars, 20 μm).

![GFP-NXF3, time-lapse](image2.png)

**Figure S2** Time-lapse image of GFP–NXF3 in HEK-293T cells

A spot accumulated in the nucleus and moved along the nuclear rim. Scale bar, 10 μm.

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1 These authors contributed equally to this work.
2 To whom correspondence should be addressed (email feisun@ustc.edu.cn).
### Table S1  List of primary antibodies

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### Table S2  List of secondary antibodies

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### Table S3  List of primers for construction of expression vectors

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<td>Strap</td>
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### Table S4  List of primer pairs for real-time PCR

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