# The fragile X mental retardation protein interacts with a distinct mRNA nuclear export factor NXF2

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**ABSTRACT**

**Loss of fragile X mental retardation protein, FMRP, causes the fragile X syndrome. Highly expressed in the brain and testis, FMRP has been implicated in the transport and translation of specific mRNAs. Here we show that FMRP and the mRNA nuclear export factor NXF2 co-express in the mouse male germ cells and hippocampal neurons and that FMRP associates with NXF2 but not with its close relative NXF1. We thus hypothesize that FMRP and NXF2 may act in concert to promote the nucleocytoplasmic transport of specific mRNAs in male germ cells and neurons.**

**Keywords: fragile X; FMRP; NXF2; mRNA; transport; testis**

Fragile X syndrome, whose hallmark symptoms are mental retardation and macroorchidism, is caused by the silencing of the X-linked *FMR1* gene that leads to the loss of expression of the protein FMRP (for review, see Willemsen et al. 2004; Bardoni et al. 2006). While FMRP is widely expressed, it is particularly enriched in the neurons of the brain and in the germ cells of the testis (Devys et al. 1993; Bakker et al. 2000). FMRP has been shown to bind specific mRNAs and implicated in the transport of bound mRNAs from the nucleus to the cytoplasm, and from the cytoplasm to postsynaptic dendrites of neurons, where it regulates translation (for review, see Willemsen et al. 2004; Darnell et al. 2005; Bardoni et al. 2006). However, the detailed mechanism of how FMRP may function remains largely unclear.

mRNA nuclear export, in general, requires the ubiquitously expressed export receptor NXF1, which is recruited to the messenger ribonucleoprotein particles (mRNPs) via RNA-binding adapter proteins. After binding mRNPs, NXF1 interacts with nuclear pore components to promote export (for review, see Dimaano and Ullman 2004). Multiple RNA-binding proteins have been identified as adapter proteins in the mRNPs. As an essential mRNA export receptor, NXF1 is concentrated in the nucleus at steady state, but shuttles continuously between the nucleus and cytoplasm. The N-terminal region of NXF1 is capable of contacting adapter proteins and the C-terminal region binds both the essential cofactor p15/NXT1 as well as nuclear pore proteins (Fig. 1A). Metazoans encode additional NXF1 orthologs, including NXF2 (Herold et al. 2000; Sasaki et al. 2005; Tan et al. 2005). The overall domain organization of NXF2 follows a highly conserved modular architecture (Fig. 1A). Like NXF1, NXF2 interacts with p15 and with components of the nuclear pore complex (Herold et al. 2000), and is able to stimulate the nuclear export of reporter mRNAs in transient transfection assays (Sasaki et al. 2005; Tan et al. 2005). Intriguingly, NXF2 is present in both nucleus and cytoplasm of transfected cells at steady state (Tan et al. 2005), suggesting a possible role in the cytoplasm in addition to the nucleus. In the process of studying RNA-binding proteins in the mouse testis, we unexpectedly found that NXF2 and FMRP specifically interact with each other.

For histological and biochemical studies of NXF2 protein, we generated polyclonal antibodies. The mouse NXF2 bears a unique sequence containing five degenerate repeats in the C-terminal region of the LRR domain (Fig. 1A). A peptide sequence (PQDGKDLIVPTGN) derived from one of these repeats was used to generate polyclonal antibodies specific for the mouse NXF2. This antibody recognizes bands of about 100 kDa (Fig. 1B, left panel, lane 3) and 80 kDa (Fig. 1B, left panel, lane 4) from cells transfected with plasmids encoding a GFP-mNXF2 (Tan et al. 2005)

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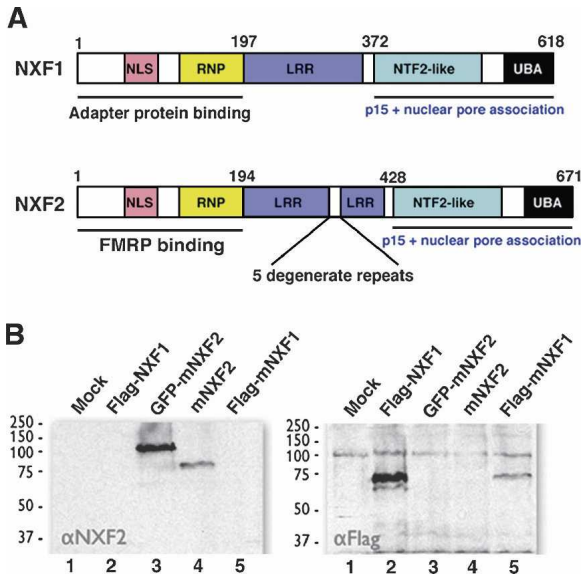


**FIGURE 1.** (*A*) Schematic representation of the structures of NXF1 and NXF2 (Herold et al. 2000; Tan et al. 2005). The numbers on *top* are in amino acids and the protein interaction domains are marked with the bars. NLS, nuclear localization signal; RNP, ribonucleoprotein motif; LRR, leucine-rich region. NTF2-like, nuclear transport factor 2 like; UBA, ubiquitin associated. (*B*) Antibody specificity. The *left* panel is a Western blot of protein samples extracted from transfected cells, using the NXF2 antibody. The *right* panel is a reprobing of the same blot with an anti-flag antibody (Sigma). Molecular weight markers in amino acids are on the *left*.

and an untagged mNXF2, respectively. However, it does not detect any proteins from cells that were mock transfected (Fig. 1B, left panel, lane 1), or transfected with plasmids encoding a flag epitope-tagged human (Fig. 1B, left panel, lane 2) (Huang et al. 2003) or mouse (lane 5) NXF1, despite the fact that these tagged proteins (size of 70 KDa) are expressed (Fig. 1B, right panel, lanes 2,5). To compare FMRP and NXF2 expression patterns in the testis, indirect immunofluorescence experiments on cryostatic sections of adult mouse testis were carried out using a well-characterized antibody specific for FMRP (Chemicon, AB2160), or the NXF2 antibody. FMRP is concentrated in the periphery of the seminiferous tubules where spermatogonia (Sg) and Sertoli cells (Se) reside (Fig. 2, panel a). At higher magnification (Fig. 2, panel b), we observe that FMRP is present in the cytoplasm of the spermatogonia, with no signal detected in Sertoli cells or in the maturing germ cells where FXR1P (a closely related FMRP family member) expression was reported to be pronounced (Bakker et al. 2000; Huot et al. 2001). Likewise, possible cross-reactivity of the FMRP antibody with another FMRP family member, FXR2P, was ruled out by our immunofluorescence and Western blot analyses (data not shown). The apparent exclusive spermatogonial FMRP staining is consistent with previously reported work (Devys et al. 1993; Bakker et al. 2000). Strikingly, the pattern of NXF2 mimics that of FMRP, with expression being exclusively restricted to the spermatogonia (Fig. 2, panels c,d). The NXF2 staining is predominantly at the nuclear rim or perinuclear in a punctate pattern (Fig. 2, panel d). This pattern resembles that seen in transient transfection experiments using epitope-tagged NXF2 fusion proteins (Herold et al. 2000; Tan et al. 2005). Further, FMRP and NXF2 co-localize at the perinuclear region in spermatogonia in our double staining immunofluorescence experiments (Fig. 2, panels e–g). Having found that in brain tissue, the NXF2 antibody works well in immunohistochemistry on paraffin sections, but not in immunofluorescence on frozen sections, we employed the former method for the brain studies. In this tissue, we observe strong staining of both NXF2 (Fig. 2, panel h) and FMRP (Fig. 2, panel i) in hippocampal neurons, with FMRP being predominantly cytoplasmic and NXF2 being both nuclear and cytoplasmic.

The particular enrichment of FMRP and NXF2 in both spermatogonia and hippocampal neurons raised the possibility that these proteins may interact with one another in cells of these two tissues. To test this, we performed immunoprecipitation (IP) and Western blot experiments. FMRP-containing protein complexes were isolated from

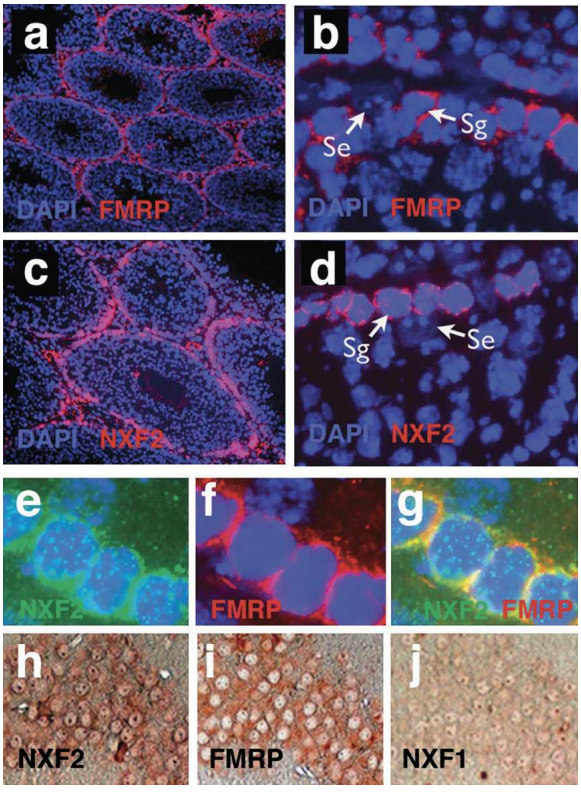


**FIGURE 2.** (*a–g*) Fluorescent images of cryostatic sections of mouse testes. Panels *a–f* show merged images between DAPI and the indicated protein staining., and panel *g* is a merged image between *e* and *f*. (*h–j*) Immunohistochemistry of adult mouse brain paraffin sections using antibodies specific for NXF2 (*h*), FMRP (*i*) and NXF1 (*j*), respectively.

freshly prepared mouse testicular or brain lysates by IP using anti-FMRP. As a negative control, an unrelated antibody, anti-MS2, was used in parallel experiments. Given that both FMRP and NXF2 are RNA-binding proteins, IP experiments were carried out in the presence of an excess amount of RNase A to disrupt possible RNA-bridged interactions. Resulting IP complexes, supernatants, and inputs were subjected to SDS-PAGE, followed by Western blotting using the indicated antibodies (Fig. 3A, B). The IP efficiency of FMRP is about 25% (Fig. 3A, cf. lanes 3 and 1 in panel I), and the control antibody did not bring down detectable levels of FMRP (Fig. 3A, lane 4 in panel I). Strikingly, while NXF2 is present in the FMRP-containing complex (Fig. 3A, lane 3 in panel II), three other RNA-binding proteins, NXF1, DAZL (Reijo et al. 2000) and 9G8, are not (Fig. 3A, cf. panel II and panels III, IV, and V in lane 3). 9G8 is an mRNA splicing factor shown to act as an export adapter for NXF1 (Huang et al. 2003). Given that about 10% of NXF2 in the total lysate is in complex with FMRP (Fig. 3A, cf. lanes 3 and 1 in panel II), and that about 25% of FMRP is brought down with the FMRP antibody (Fig. 3A, cf. lanes 3 and 1 in panel I), we estimate that at least 30% of NXF2 is in complex with FMRP in the testis. This correlates well with the immunofluorescence results where FMRP and NXF2 only partially colocalize in the spermatogonia (Fig. 2, panel g). In the brain, similar results were seen (Fig. 3B). Although NXF1 is expressed in spermatogonia (data not shown) and hippocampal neurons (Fig. 2, panel j), it is, however, not present in the FMRP-containing complexes (Fig. 3A,B). That the FMRP/NXF2 association is specific and stable is strongly supported by the fact that nonspecific reassociation of FMRP and NXF2 with NXF1, 9G8 and DAZL during IP incubation did not occur, although these proteins were present in the lysates at comparable levels as indicated in the supernatants and inputs (Fig. 3A,B). To support this further, we performed in vitro pull-down assays using recombinant proteins. As shown in Figure 3C, NXF2 is preferentially selected by GST-FMRP (Fig. 3C, lane 2) (but not the control GST alone, Fig. 3C, lane 3) from a mixture that contains both NXF2 and NXF1 (Fig. 3C, lane 1). Using the same strategy, we were able to map the FMRP-interacting domain to the N-terminal 194 amino acids of NXF2 Figs. (3D, 1A).

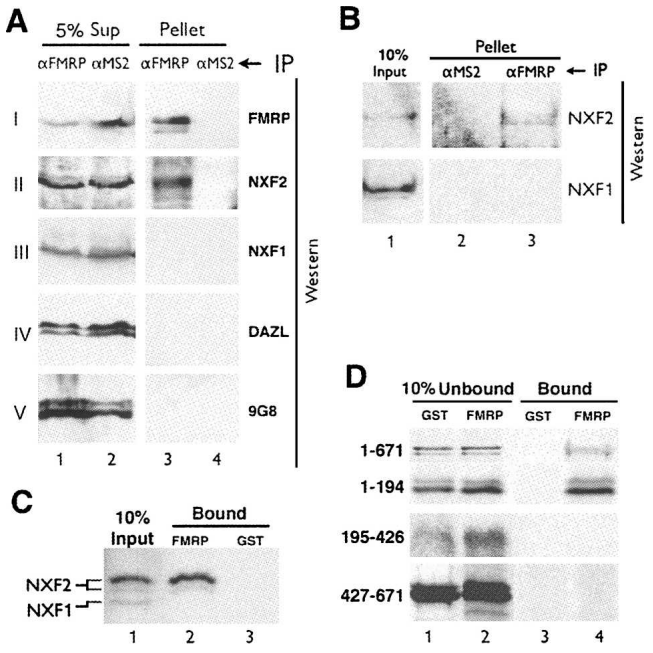


**FIGURE 3.** FMRP and NXF2 interact with each other biochemically. The antibody specific for FMRP (αFMRP) or an antibody against an unrelated protein (αMS2) was used to isolate FMRP-containing protein complexes from adult mouse testicular (*A*) or brain (*B*) lysates in the IP experiments. Purified protein complexes (Pellet), supernatants (Sup) or inputs (Input) were resolved by SDS-PAGE. Indicated antibodies (marked on the *right*) were used to probe the membranes in Western blot analyses. (*C*) In vitro pull-down assays. A mixture of $^{35}$S-labeled full-length NXF2 (appears as a doublet) and NXF1 proteins (lane *1*) was incubated with GST-FMRP (lane *2*) or GST alone (lane *3*) pre-bound on glutathione beads as previously described (Huang et al. 2003). Input and proteins selected by the beads were resolved by SDS-PAGE and labeled proteins visualized by autoradiography. (*D*) Interaction domain mapping. $^{35}$S-labeled full-length as well as various domains of NXF2 were incubated with GST alone (lane *3*) or with GST-FMRP (lane *4*) pre-bound on glutathione beads. Unbound and bound fractions were resolved by SDS-PAGE and labeled proteins visualized by autoradiography. The full-length (1–671), N-terminal (1–194), middle (195–426), and C-terminal regions (427–671) of NXF2 are marked on the *left* in amino acids.

It has long been suspected that FMRP may play a role in the export of its bound mRNAs from the nucleus to the cytoplasm (for review, see Willemsen et al. 2004). However, no report so far has clearly demonstrated that FMRP indeed exports mRNAs, nor the pathway(s) it may take to export them. Here, we provide evidence important and necessary to establish the role of FMRP in mRNA nuclear export, since FMRP interacts with a bona fide mRNA nuclear export factor. Our findings also suggest that one mode of action of FMRP may be to serve as an export adapter, since FMRP interacts with the same domain of NXF2 that has been implicated in binding to export adapters, as is in the case for NXF1. Further, our results point to a novel mechanism by which NXF2 may export mRNAs—by interacting with a distinct class of adapters such as FMRP. The observation that 9G8, a known export adapter for NXF1 (Huang et al. 2003), and which is also expressed in spermatogonia (data not shown), is not complexed with FMRP, while NXF2 is, supports the notion that NXF2 and NXF1 may use different sets of export adapters. Finally, the apparent highly coordinated expression of FMRP and NXF2 and specific interactions between the two in the same cell population suggest that an NXF2-mediated pathway may be coupled to the subsequent translational control of a specific subset of mRNAs by FMRP, a process that is essential for the function of neurons and the development and differentiation of germ cells.

It has been proposed that FMRP may export its mRNA targets through interaction with CRM1, although it has never been demonstrated that FMRP actually interacts with CRM1 (Eberhart et al. 1996; Fridell et al. 1996; Tamanini et al. 1999). Nor is it clear whether CRM1 indeed participates in the nuclear export of cellular mRNAs (for review, see Cullen 2003). Taken together with our findings, we envision several possibilities. CRM1 may be responsible for the nuclear exit of FMRP (for yet unknown functions) when FMRP is not bound to mRNAs, while NXF2 is required for FMRP-mediated mRNA export. Alternatively, either CRM1 or NXF2 may be sufficient for FMRP-mediated mRNA export. Finally, both CRM1 and NXF2 may be required for FMRP-mediated mRNA export. These models can be tested once true in vivo FMRP mRNA targets have been identified.

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