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# Nuclear RNA export factor 7 is localized in processing bodies and neuronal RNA granules through interactions with shuttling hnRNPs

**Jun Katahira[1,2,*], Takashi Miki[1], Keizo Takano[1], Mitsuji Maruhashi[3], Masanori Uchikawa[3], Taro Tachibana[4] and Yoshihiro Yoneda[1,2]**

[1]Biomolecular Networks Laboratories, Biomolecular Dynamics Laboratory, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, [2]Department of Cell Biology and Neuroscience, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, [3]Developmental Biology Laboratory, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871 and [4]Department of Bioengineering, Graduate School of Engineering, Osaka City University, Osaka 558-8585, Japan

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## ABSTRACT

**The nuclear RNA export factor (NXF) family proteins have been implicated in various aspects of post-transcriptional gene expression. This study shows that mouse NXF7 exhibits heterologous localization, i.e. NXF7 associates with translating ribosomes, stress granules (SGs) and processing bodies (P-bodies), the latter two of which are believed to be cytoplasmic sites of storage, degradation and/or sorting of mRNAs. By yeast two-hybrid screening, a series of heterogeneous nuclear ribonucleoproteins (hnRNPs) were identified as possible binding partners for NXF7. Among them, hnRNP A3, which is believed to be involved in translational control and/or cytoplasmic localization of certain mRNAs, formed a stable complex with NXF7 *in vitro*. Although hnRNP A3 was not associated with translating ribosomes, it was co-localized with NXF7 in P-bodies. After exposing to oxidative stress, NXF7 trans-localized to SGs, whereas hnRNP A3 did not. In differentiated neuroblastoma Neuro2a cells, NXF7 was co-localized with hnRNP A3 in cell body and neurites. The amino terminal half of NXF7, which was required for stable complex formation with hnRNP A3, coincided with the region required for localization in both P-bodies and neuronal RNA granules. These findings suggest that NXF7 plays a role in sorting, transport and/or storage of mRNAs through interactions with hnRNP A3.**

## INTRODUCTION

Shuttling transporters are essential for gene expression, since RNA transcription and protein translation occur at two cellular locations in eukaryotes. The nucleus is the site of transcription of mRNAs, while translation of mRNAs into protein occurs in the cytoplasm. Nuclear RNA eXport Factor 1 (NXF1), originally named Tap, is a non-importin-β type shuttling mRNA transporter (1–4). Tap/NXF1 is a member of the NXF protein family, which is evolutionarily conserved across species from the budding yeast *Saccharomyces cerevisiae* to humans (1,5–12). In metazoa, the gene family is structurally and functionally divergent and some members do not participate in mRNA export from the nucleus to the cytoplasm. In humans and mice, at least four different NXF family gene products are expressed in different tissues. Among them, the Tap/NXF1 and NXF2 proteins of both human and mouse origin exhibit nuclear mRNA export activity, while others, including human and mouse NXF3, human NXF5, and mouse NXF7 do not (5–7,10,11). In neuronal cells, both mouse NXF7 and human NXF5, as well as mouse NXF2, show prominent cytoplasmic localization (7,12,13). Such unique localization distinguishes theses factors from other family members. It has been proposed that mouse NXF2 and NXF7 are components of cytoplasmic mRNA granules in neuronal cells and possess additional cytoplasmic functions via interactions with microtubule-associating proteins such as cytoplasmic motor proteins and MAP1B (11–13).

During or soon after transcription, mRNAs undergo various maturation steps including capping, splicing, and 3′-end formation in the nucleus. Throughout these processes, mRNAs are associated with various proteins, thus forming messenger ribonucleoproteins particles

*To whom correspondence should be addressed. Tel: +81 6 6879 4606; Fax: +81 6 6879 4609; Email: katahira@anat3.med.osaka-u.ac.jp
Correspondence may also be addressed to Yoshihiro Yoneda. Tel: +81 6 6879 4605, Fax: +81 6 6879 4609, Email: yyoneda@anat3.med.osaka-u.ac.jp

(mRNPs) (14,15). The most abundant components of mRNPs are the heterogeneous nuclear ribonucleoproteins (hnRNPs), which consist of more than 20 different proteins (16). In addition, a series of mRNA-binding proteins, including Aly/REF, Y14, magoh, Upf3 and so on, bind mRNAs during splicing reactions (17–20). Subsequently, on mature mRNAs, these proteins are recognized by Tap/NXF1. The bound mRNPs are then transported into the cytoplasm through nuclear pore complexes (NPCs) via the affinity of Tap/NXF1 for FG-repeat containing nucleoporins (14,21–26). It has also been shown that a subset of proteins containing serine-arginine rich (SR)-domain bind mRNAs, in this case most likely independent of splicing. These proteins are recognized by Tap/NXF1 and, therefore, also play an important role in nucleo-cytoplasmic transport of mRNAs (27,28). It appears that these proteins act as adaptor molecules that tag fully matured mRNAs, thus exporting only functional mRNAs out of the nucleus (14). After transport to the cytoplasm, peripheral components of mRNPs, such as Aly/REF, dissociate from mRNAs; whereas, the core components of exon-junction complex (EJC) and certain hnRNPs may remain bound, contributing to downstream events (21,29,30). For example, EJC components including the Y14-magoh heterodimer, as well as non-EJC components such as hnRNP A/B family proteins, MARTA1/KSRP and their orthologues, are involved in cytoplasmic mRNA localization in various organisms (31–38). In addition, Upf3 triggers degradation of aberrant mRNAs containing premature stop codons (39).

This study demonstrates that NXF7 associated with translating ribosomes, processing bodies (P-bodies) and stress granules (SGs), the latter two of which are proposed to be the sites of storage, degradation and/or sorting of translationally repressed mRNAs (40–43). In addition, NXF7 interacted with a series of shuttling hnRNPs, including hnRNP A3. We show that the amino-terminal region together with the leucine-rich repeat (LRR) domain of NXF7 is responsible for the abilities to bind hnRNP A3 and to be targeted to P-bodies and cytoplasmic processes in cultured neuronal cells. These data indicate that NXF7 may recognize mRNAs through interaction with hnRNPs and may participate in cytoplasmic mRNA storage and/or localization in P-bodies.

## EXPERIMENTAL PROCEDURES

### Plasmid construction

A cDNA encoding full-length NXF7 was isolated from pEGFP-NXF7 (10) and subcloned into pGBKT7 (Clontech) to obtain a bait plasmid (pGBKT7-NXF7) for yeast two-hybrid screening.

A mammalian expression vector comprised of full-length NXF7 with a carboxyl-terminal GFP-tag was constructed by inserting the NXF7 cDNA into the pEBO-GFP vector (21), which had been linearlized by Xho I and Nru I digestion.

Mammalian expression vectors for fusion proteins consisting of CFP and various NXF7 domains were constructed by inserting the corresponding cDNA fragments, synthesized by PCR using full-length NXF7 as the template, into the pECFP-C1 vector (Clontech).

Bacterial expression vectors for mouse KSRP and hnRNP E1 were constructed by inserting full-length cDNAs for the corresponding proteins, which had been isolated by RT-PCR from a mouse cDNA library, into the pET-$NH_6$ vector (13). The resulting plasmids, designated pET-$NH_6$-KSRP and pET-$NH_6$–hnRNP E1, respectively, encode a 6xhistidine tag, KSRP and hnRNP E1, controlled by the phage T7 promoter.

Baculovirus expression vectors for mouse hnRNP A2/B1 and hnRNP A3 were constructed by inserting the corresponding cDNAs, which also had been isolated by PCR, along with GST ORF, isolated from the pGEX6P3 vector, into the pFASTBac1 vector (Invitrogen). Baculovirus expression vectors for full-length and an amino-terminal fragment (aa 99–374) of NXF7 were constructed in the same way. Isolation of Bacmid DNAs was performed by transforming DH10Bac competent cells (Invitrogen) according to the manufacturer's protocol.

A bacterial expression vector for an amino-terminal fragment of mouse Tap (aa 96–371) was obtained by inserting the corresponding cDNA fragment into the pGEX-6P3 vector. The resulting expression vector was designated pGEX-6P3-TapRBD.

A cDNA encoding human Dcp1a (GenBank accession NM_018403) was cloned by PCR from a human liver cDNA library. The amplified cDNA was subcloned into the pmRFP-C1 vector to obtain a mammalian expression vector, pmRFP-Dcp1a.

A cDNA encoding amino acids (aa) 1–125 of NXF7 was amplified by PCR and subcloned into the pET21d vector (Novagen). The resulting plasmid was designated pET21d-NXF7N and was used to express antigen for monoclonal antibody production.

### Antibodies

Mouse monoclonal and rabbit polyclonal antibodies against MARTA1/KSRP were generous gifts from Drs Douglas Black (UCLA) and Monika Rehbein (University of Hamburg), respectively (44,45). Rabbit polyclonal antibody against Dcp1a was kindly provided by Dr Jens Lykke-Andersen (University of Colorado) (46). Rabbit polyclonal antibodies against hnRNP A3 and hnRNP A2/B1 were kind gifts from Dr Ross Smith (University of Queensland) (47). The following antibodies, mouse monoclonal anti-FLAG (Sigma), mouse monoclonal anti-β-actin (Sigma), mouse monoclonal anti-pentaHis (Qiagen), rabbit anti-GFP (Molecular Probes) and mouse monoclonal anti-G3BP (BD Transduction Laboratories), were commercially acquired. Alexa-conjugated secondary antibodies were purchased from Molecular Probes. A rat monoclonal antibody was raised against the amino-terminal fragment (aa 1–125) of NXF7 using a published method (48). The specificity of the anti-NXF7 antibody was examined by Western blot using total cell extracts prepared from undifferentiated and differentiated mouse embryonic stem (ES) cells (see supplementary Figure S1). The specificities of

anti-Dcp1a and anti-hnRNP A3 antibodies were tested by Western blot using total cell extracts prepared from HeLa and Neuro2a cell lines (see supplementary Figure S2).

### Yeast two-hybrid screening

The yeast two-hybrid screening strain AH109 harboring the pGBKT7-NXF7 bait plasmid was transformed with a mouse 7-day embryo cDNA library (Clontech) as previously described (13). In total $1.2 \times 10^6$ transformants were screened for growth on SDC (-leu, -trp, -his, -ade) and for *MEL1* gene expression according to the manufacture's protocol. Finally, 20 clones, which fulfilled the requirement for specific interaction with NXF7, were analyzed by DNA restriction patterns and the cDNA inserts were sequenced.

### Cell culture, transfection and establishment of stable cell line

L929, HeLa, and Neuro2a cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (DMEM-10% FBS) at 37°C in 5% $CO_2$ atmosphere. Transfection was performed using the Effectene Transfection Reagent Kit according to the manufacturer's protocol (QIAGEN). Differentiation of Neuro2a cells was induced as previously reported (49). An L929 cell line stably expressing FLAG-tagged NXF7 was established as reported previously (21). The stable cell line was maintained in DMEM-10% FBS containing 400 μg/ml of hygromycin B. Sf9 cells were grown in Sf-900II medium (Invitrogen) supplemented with 10% fetal bovine serum. R1 ES cells (a kind gift from Dr J. Takeda, Osaka University) were cultured in DMEM supplemented with 20% FCS and 1000 units/ml of LIF (ESGRO, Chemicon) in the presence of mouse embryonic fibroblasts (Invitrogen) as feeder cells. Formation of embryoid bodies was performed as reported previously (50). For immunofluorescence analysis, cystic embryoid bodies (CEBs) of 10 days after suspension culture were transferred to poly-L-lysine-coated glass bottom dishes and further cultured for 24 h before fixation.

### Protein expression and purification

Cultures of *E. coli* strain BL21(DE3), harboring pET-$NH_6$-KSRP and pET-$NH_6$–hnRNP C1, were grown in LB medium containing 100 μg/ml ampicillin. Protein expression was induced by adding 1 mM IPTG for 20 h at 20°C. After induction, the cells were harvested, resuspended in buffer A [20 mM Tris–HCl (pH 7.5)/250 mM NaCl] containing 20 mM imidazol and 5 mg/ml lysozyme, and lysed by sonication. Soluble fractions were subjected to Ni-NTA agarose column chromatography. After extensive washing with buffer A, bound proteins were eluted using imidazole (220 mM in buffer A). After passing through a PD-10 desalting column that had been pre-equilibrated with 20 mM Tris–HCl (pH 7.5) buffer, 6xhistidine-tagged KSRP underwent additional purification using Mono Q FPLC column chromatography.

Cultures of *E. coli* strain BL21(DE3), harboring pGEX-6P3 or pGEX-6P3-TapRBD, were grown as described earlier. Protein expression was induced by adding 1 mM IPTG for 20 h at 30°C. The cells were harvested, resuspended in buffer A containing 1 mM DTT and 5 mg/ml lysozyme, and lysed by sonication. The lysates were subjected to centrifugation and the soluble supernatants were purified using glutathione Sepharose column chromatography. After washing with buffer A, the bound proteins were eluted with 100 mM Tris–HCl (pH 8.8) containing 20 mM glutathione. Purified proteins were passed through PD-10 desalting columns that had been pre-equilibrated with 20 mM Tris-HCl (pH 7.4)/150 mM NaCl/1 mM DTT.

The recombinant Bacmid plasmids encoding GST-NXF7, GST-hnRNP A2/B1 and GST-hnRNP A3 were transfected into Sf9 cells using the Effectene Reagent (QIAGEN). Recombinant Baculoviruses were harvested and amplified according to the manufacturer's protocol. For large-scale expression, 200 ml cultures of $2 \times 10^6$ cells/ml were infected with each recombinant Baculovirus at MOI 10. The infected cells were cultured for 72 h at 28°C and harvested by centrifugation. The cells were resuspended in lysis buffer [20 mM Tris–HCl (pH 7.4)/150 mM NaCl/1 mM DTT/0.5% Triton X-100] and disrupted by brief sonication. Both GST-hnRNP A2/B1 and GST–hnRNP A3 were almost fully solubilized under these conditions. Each protein-containing lysate was subjected to glutathione Sepharose column chromatography. After extensive washing with lysis buffer, the bound proteins were eluted by treatment with PreScission protease. After passing through PD-10 columns pre-equilibrated with 20 mM Tris–HCl (pH 7.0)/1 mM DTT, eluted proteins underwent additional purification using Mono Q FPLC chromatography. GST-NXF7 and its derivative were not solubilized under these conditions. After sonication, the remaining insoluble materials were pelleted down and resuspended in 20 mM Tris–HCl (pH 7.4)/250 mM KCl/5 mM DTT. After incubation for 20 h at 4°C with mild agitation, the GST-fusion proteins in the supernatants were purified by glutathione Sepharose column chromatography as above except that elution was done by 100 mM Tris–HCl (pH 8.8) buffer containing 20 mM glutathione. Excess glutathione was removed using a PD-10 desalting column.

### Miscellaneous

RNA isolation and indirect immunofluorescence assays were performed as previously reported (10,13). Pull-down assays using glutathione Sepharose beads were done as described previously (3,23). Fractionation of cytoplasmic lysates using 20–50% (w/v) linear sucrose gradients was performed according to the method of Feng *et al.* (51) using a Gradient Master and a Piston Gradient Fractionator (Biocomp). Disruption of translating ribosomes was achieved by treating the cells with puromycin (1 mM) for 5 h, while degradation of RNAs was induced by addition of RNase A to cytoplasmic lysates at the concentration of 100 μg/ml.

## RESULTS

### Shuttling hnRNPs interacted with NXF7

Previously, it has been shown that NXF7 does not exhibit mRNA nuclear export activity and is exclusively localized in the cytoplasm (10,12). In accordance with this observation, NXF7 did not interact with Aly/REF, an adaptor molecule required for nuclear mRNA export (21,26), in the yeast two-hybrid assay (data not shown). To gain further insight into the cytoplasmic function of NXF7, a yeast two-hybrid screening was performed. Since expression of NXF7 was observed early in embryonic development, especially in extra-embryonic tissues (see supplementary Figure S1), a mouse 7-day embryo cDNA library was used for screening. Of the $1.2 \times 10^6$ independent clones screened, 20 positive candidates were obtained. Among the candidate clones, several hnRNPs known to be involved in cytoplasmic mRNA localization and/or translational control were obtained. Those include hnRNP A2/B1, hnRNP A3, MARTA1/KSRP and hnRNP E1/PCBP1.

To test whether NXF7 interacts directly with these hnRNPs, pull-down assays using recombinant proteins were performed. As shown in Figure 1A, NXF7 bound hnRNP A3 very efficiently and Coomassie stainable amount of hnRNP A3 was readily pulled down from diluted samples. MARTA1/KSRP (Figure 1C) and hnRNP A2/B1 (Figure 1A and B) also bound NXF7, although with much lower affinity, as these proteins were detectable only by Western blot. Binding of NXF7 and hnRNP E1/PCBP1 could not be reproduced under the *in vitro* assay condition employed in this study (data not shown). Thus, the interaction of NXF7 and hnRNP E1/PCBP1 was not further investigated.

The N-terminal region of Tap/NXF1 is required for interaction with various RNA-binding proteins, such as Aly/REF, etc., which act as adaptor molecules for nuclear export of mRNAs (14,21,25,26). To examine whether the corresponding region of NXF7 binds hnRNP A3, pull-down assays were performed using a truncated mutant. As shown in Figure 1A, a region including a portion of the N- and LRR-domains (aa 99–374 of NXF7) bound hnRNP A3 and hnRNP A2/B1, although the interactions were significantly weakened compared with full-length NXF7. Since the N-terminal region of Tap/NXF1 (aa 96–371), corresponding to the minimal hnRNP A3-binding domain of NXF7, did not exhibit any affinity for hnRNP A3 or hnRNP A2/B1 (Figure 1A and B), it was concluded that hnRNP A2/B1- and hnRNP A3-bindings were specific for NXF7. Binding of MARTA1/KSRP to the truncated NXF7 mutant was not detectable (Figure 1C), and therefore, it is conceivable that the mode of the binding is somewhat different.

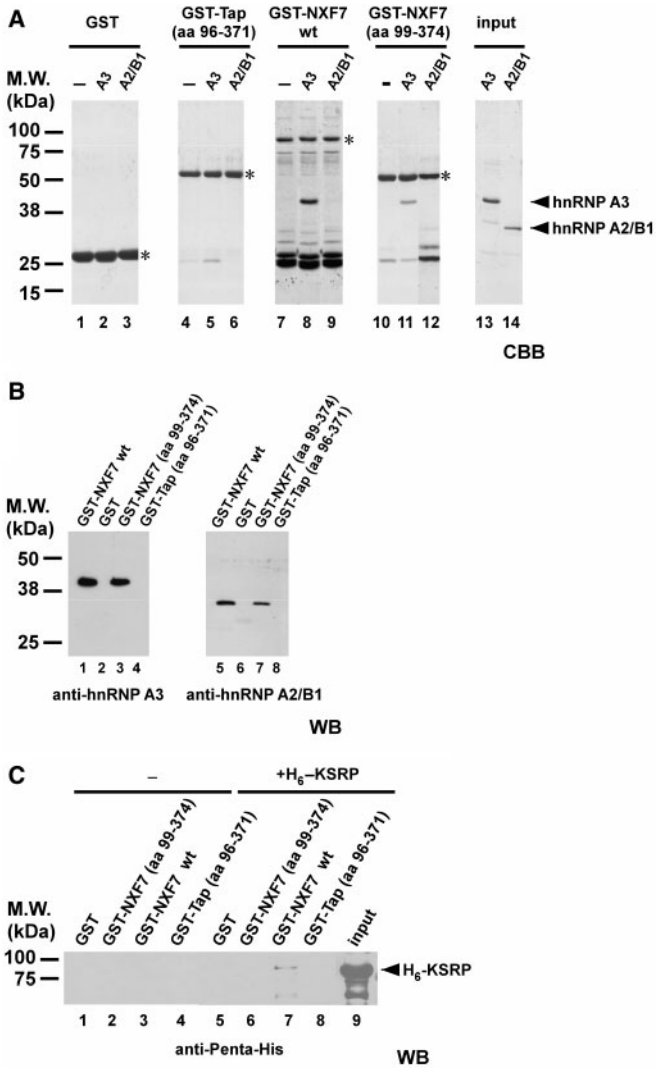


**Figure 1.** NXF7 binds a series of shuttling hnRNPs. (**A**) GST (lanes 1 to 3), GST-Tap (aa 96–371; lanes 4 to 6), GST-NXF7 (full length; lanes 7 to 9), and GST-NXF7 (aa 99–374; lanes 10 to 12), each pre-adsorbed to glutathione Sepharose beads, were incubated for 2 h at 4°C with either buffer (lanes 1, 4, 7, 11), hnRNP A3 (lanes 2, 5, 8, 11) or hnRNP A2/B1 (lanes 3, 6, 9, 12). After washing extensively, bound proteins were eluted by boiling in SDS sample buffer and were then loaded on SDS-12% polyacrylamide gels. In lanes 13 and 14, ~5% of input was loaded. Proteins were visualized by staining the gel with Coomassie brilliant blue. Migration positions of molecular weight markers are indicated on the left in kilo Daltons and those of hnRNP A3 and hnRNP A2/B1 are on the right side of the panel, indicated by arrowheads. Asterisks indicate the positions of GST and GST fusion proteins. (**B**) Same as in A, but proteins were detected by Western blot using the indicated antibodies. (**C**) Same as in A, but 6xHis-tagged KSRP was subjected to binding reactions. Proteins were detected by Western blot using anti-pentaHis antibody.

### NXF7 associated with polyribosomes

Since NXF7 shows prominent cytoplasmic localization, the role of NXF7 and hnRNPs in the cytoplasm was examined. Recently, exogenously expressed NXF7 was shown to co-localize with ribosomal RNAs, as a component of RNA granules, in cytoplasmic processes of neuronal cells (12). Therefore, to test the possibility that NXF7 is directly involved together with hnRNPs in mRNA translation, NXF7 association with ribosomes was examined. The subcellular distribution of NXF7 was analyzed by fractionating cytoplasmic extracts prepared from L929 cells stably expressing the NXF7-FLAG fusion protein, using sucrose gradient centrifugation. As shown in Figure 2A, NXF7 was recovered in the

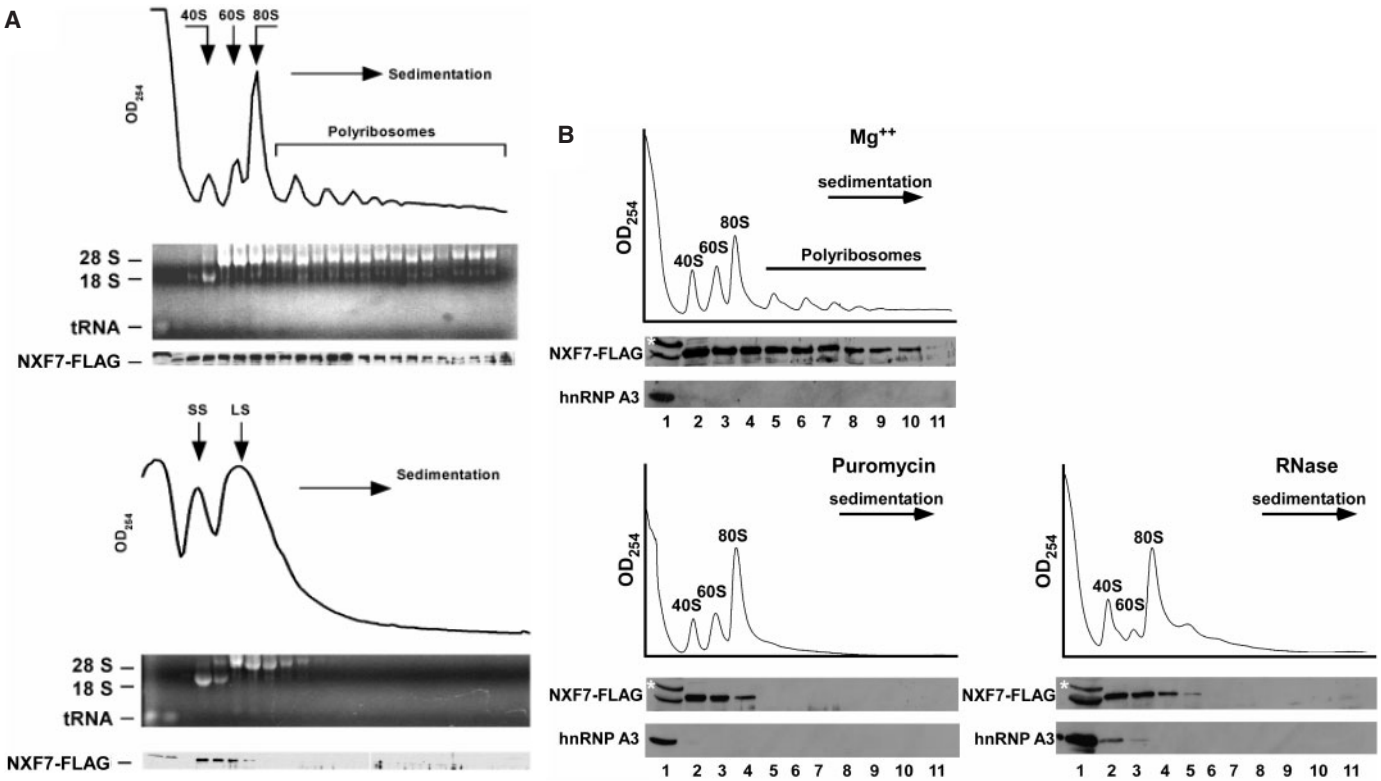


**Figure 2.** NXF7 is localized in polyribosomes containing fractions. (**A**) *Upper panel*: Cytoplasmic lysate of a mouse L929 cell line stably expressing FLAG-tagged NXF7 was prepared using $Mg^{++}$-containing buffer and was fractionated over a linear 20–50% sucrose gradient, which also contained $Mg^{++}$. The ribosome profile ($OD_{254}$ nm trace) is depicted. Arrows indicate the positions of 40, 60 and 80 S ribosomal subunits in the gradient, while a bracket illustrates positions of polyribosomes. Total RNA and protein were isolated from each fraction. Distribution of tRNAs and rRNAs was examined by denaturing agarose gel electrophoresis followed by ethidium bromide staining of the gel. FLAG-tagged NXF7 fusion protein was detected by Western blot with an anti-FLAG antibody. *Lower panel*: Cytoplasmic lysate of a mouse L929 cell line stably expressing FLAG-tagged NXF7 was prepared using an EDTA-containing buffer. Fractionation was performed as above, but the gradient contained 50 mM EDTA. The positions of small and large ribosome subunits are shown by arrows in the $OD_{254}$ nm trace. Distribution of RNAs and FLAG-tagged NXF7 was examined as above. (**B**) *Upper panel*: Cytoplasmic lysate of a mouse L929 cell line stably expressing FLAG-tagged NXF7 was prepared and fractionated under $Mg^{++}$-containing condition as in *A*. *Lower left panel*: Cytoplasmic lysate was prepared after the cells were treated with 1 mM puromycin for 5 h. *Lower right panel*: Cytoplasmic lysate was prepared in the presence of RNase A (100 μg/ml). In both experiments, fractionation was performed under $Mg^{++}$-containing condition. The ribosome profiles ($OD_{254}$ nm trace) are depicted. The positions of 40, 60 and 80 S ribosomal subunits and polyribosomes in the gradients are indicated in each panel. FLAG-tagged NXF7 and hnRNP A3 in the gradients were detected by Western blot using anti-FLAG and anti-hnRNP A3 antibodies, respectively. A non-specific band that migrates slower than NXF7-FLAG is indicated by asterisks.

polyribosome-containing heavy fractions. Addition of EDTA in extraction buffer and gradient media causes dissociation of ribosomes into the subunits. Under the ribosome dissociating condition, NXF7 was recovered in the upper fractions (Figure 2B), indicating that NXF7 was associated with ribosomes. When the cells were treated with puromycin, a peptide chain elongation inhibitor that causes loss of translating polyribosomes, NXF7 was recovered in the upper fractions (Figure 2B lower left panel). This observation excludes a possibility that NXF7 is involved in $Mg^{++}$-dependent large RNPs that are not associated with polyribosomes. It was also found that association of NXF7 to polyribosomes was RNA-dependent (Figure 2B lower right panel). Similar behavior of NXF7 was confirmed in another cell line (e.g. human 293F) stably expressing GST-tagged NXF7 (data not shown), as well as in cystic embryoid bodies (CEBs) derived from mouse embryonic stem (ES) cells, which express NXF7 endogenously (see supplementary Figure S1). In contrast to NXF7, hnRNP A3 was not detected in polyribosome-containing fractions of these cell lines (see Figure 2B upper panel, for CEBs see supplementary Figure S1). These results indicate that NXF7 associated with translating ribosomes independently of hnRNP A3, and that the NXF7-hnRNP A3 complex was not directly involved in mRNA translation.

### NXF7 was localized in both P-bodies and SGs

During the course of this study, it was observed that, in transfected HeLa cells, especially in those expressing low levels of the fusion protein, NXF7-GFP accumulated in the cytoplasm both as dot-like structures and as diffuse signals. The dot-like cytoplasmic structures were reminiscent of P-bodies, the site of translational repression and mRNA degradation. To examine whether the dot-like structures were in fact P-bodies, HeLa cells expressing NXF7-GFP were immunostained with an antibody against Dcp1a, a protein marker for P-bodies.

The NXF7-GFP fusion protein was expressed as a single band with a molecular weight approximately equal to the expected value (Figure 3A). As shown in Figure 3B, almost all of the NXF7-GFP containing dots co-localized with Dcp1a. In addition, this co-localization was recapitulated by exogenously expressed Dcp1a as an mRFP fusion protein (Figure 3C). These results indicate that NXF7 was targeted to P-bodies.

To examine which region of NXF7 was required for P-body localization, various fragments of NXF7, used as CFP fusion proteins (Figure 4A), together with mRFP-Dcp1a were co-expressed and localization patterns were observed. Although degradation bands were observed for several truncation mutants, fusion proteins, of the sizes expected, were expressed (Figure 4B). As shown in Figure 3C, the N-terminal half, consisting of the N- and LRR-domains (aa 1–374) showed the same pattern, as did the full-length protein; whereas, the C-terminal half (aa 375–620) did not. A mutant lacking the N-terminal 90 amino acids and the C-terminal half (aa 91–374) did not show the dot-like P-body localization pattern. Further truncation (aa 211–374) completely abolished P-body localization (Figure 4C). Since the N-fragment alone (aa 1–210) did not exhibit the P-body localization (Figure 4C), it was concluded that the N-and LRR-domains, arranged in *cis*, were required for P-body targeting (Figure 4A).

In cells expressing higher levels of fusion protein, NXF7-GFP formed cytoplasmic aggregates that were much larger than P-bodies, as reported previously (11). NXF7-containing aggregates were positive for both G3BP (Figure 3D, upper panels) and TIA1 (data not shown), both of which are well-established protein markers for SGs, indicating that NXF7 spontaneously induced the formation of SGs upon overexpression. Furthermore, arsenite treatment caused NXF-GFP relocation to SGs, even in cells expressing low levels of fusion protein (Figure 3D, lower panels). The truncation mutants lacking M- and C-domains were targeted to SGs. However, the region of the N-terminal required for SG localization was different from that needed for P-body localization, as the N-terminal 90 amino acids appeared to be dispensable (Figure 4A and E).

### hnRNP A3 co-localized with NXF7 in P-bodies

Proteins belonging to the hnRNP A/B family shuttle continuously between the nucleus and the cytoplasm. Thus, the role of the observed interactions with hnRNP A/B family molecules in the localization of NXF7 in P-bodies was examined. When intracellular localization of hnRNP A3 in HeLa cells was examined by indirect immunofluorescence using a monospecific antibody (for specificity see Supplementary Figure S2), cytoplasmic dot-like signals and strong nuclear staining were observed. NXF7-GFP co-localized with the hnRNP A3-containing foci (Figure 5A). The hnRNP A3-containing cytoplasmic foci were observed in other cell lines such as MDCK, Neuro2a and 293 cells (data not shown, see also below). To the contrary, hnRNP A2/B1, which binds weakly to NXF7, did not show such cytoplasmic signals

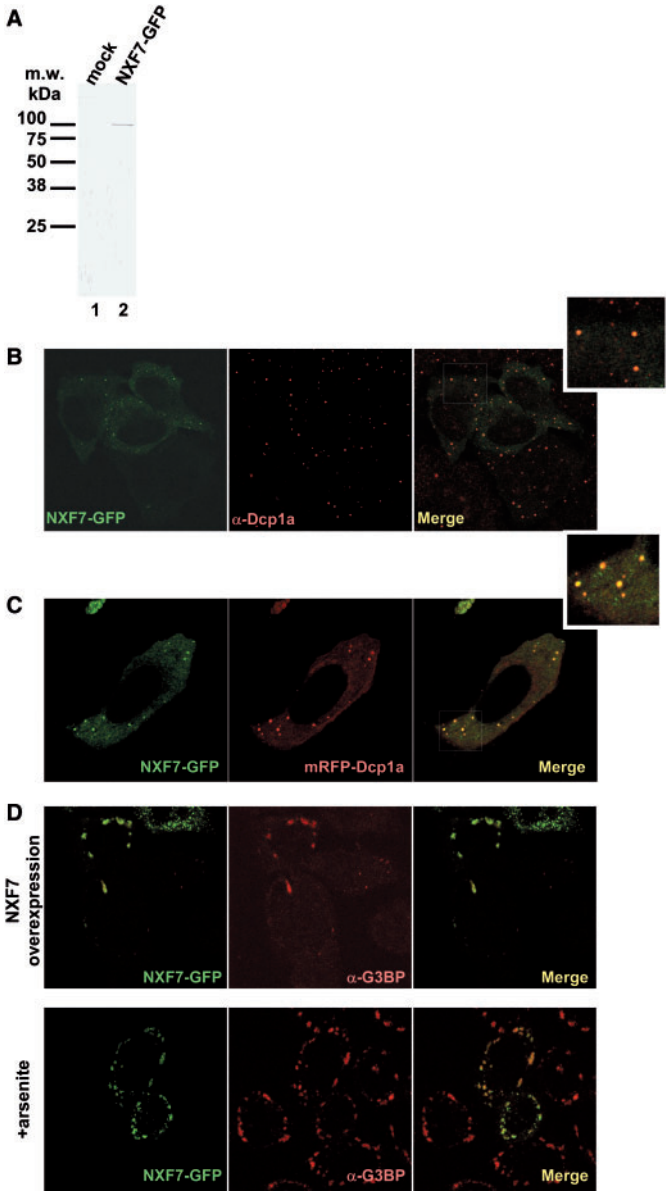


**Figure 3.** NXF7 is localized in P-bodies and SGs. (**A**) HeLa cells were transiently transfected with a vector harboring NXF7-GFP fusion protein (lane 2). Untransfected HeLa cells were used as negative control (lane 1). At 48 h after transfection, total cell extracts were prepared and subjected to Western blot using anti-GFP antibody. Positions of molecular weight markers are indicated on the left in kDa. (**B**) HeLa cells were transiently transfected with a vector harboring NXF7-GFP fusion protein. The cells were fixed 20 h after transfection and localization of Dcp1a was detected by immunofluorescence using an anti-Dcp1a antibody followed by Alexa568-labeled anti-rabbit IgG. The upper right panel is a magnified view of the area indicated by the white box in the merged image. (**C**) HeLa cells were transfected with vectors harboring NXF7-GFP and mRFP-Dcp1a fusion proteins. At 20 h after transfection, the cells were fixed and observed by epifluorescent microscopy. The upper right panel is a magnified view of the area indicated by the white box in the merged image. (**D**) *Upper panels*: HeLa cells expressing NXF7-GFP were fixed and subjected to immunofluorescence using an anti-G3BP antibody followed by Alexa568-labeled anti-mouse IgG. Note that in highly expressing cells, aggregated NXF7-GFP contained G3BP. *Lower panels*: HeLa cells expressing NXF7-GFP were exposed to oxidative stress for 1 h, fixed, and subjected to immunofluorescence as above. Note that even in the cells expressing low amounts of the fusion protein, NXF7 relocalized to SGs.

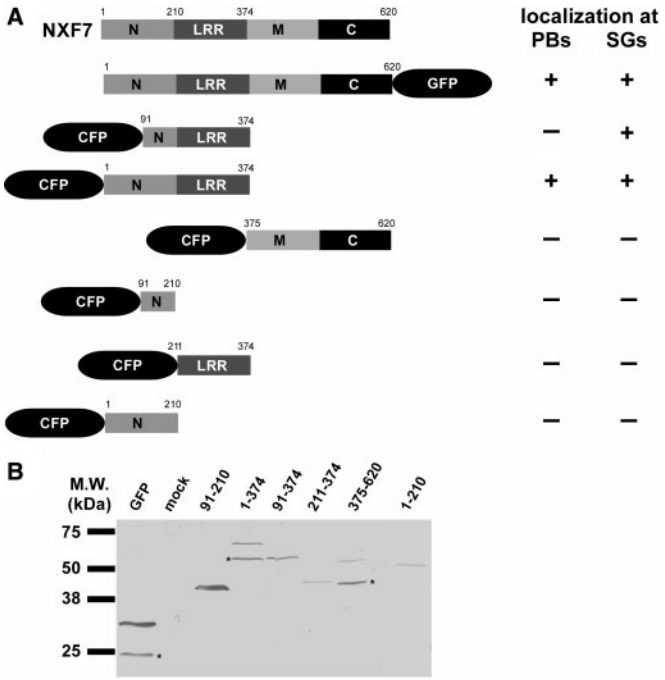


**Figure 4.** Identification of domains required for P-body and SG localization. (**A**) A schematic representation of the mutants used. The names of each domain are based on our previous reports (10). The numbers above each rectangle indicate amino acid positions. + and − signs indicate the presence or absence of mutants in P-bodies and SGs. (**B**) Total cell extracts prepared from HeLa cells transiently expressing each mutant were separated by SDS–PAGE and subjected to Western blot using a rabbit anti-GFP antibody. The positions of molecular weight markers are indicated on the left side in kDa. Asterisks indicate the positions of degradation products. (**C**) HeLa cells co-expressing mRFP-Dcp1a and the CFP-tagged mutants were observed by epifluorescent microscopy. (**D**) Co-localization of CFP-NXF7(1–374) and mRFP-Dcp1a in transfected HeLa cells was observed by confocal microscopy. Insets are magnified view of the areas indicated by the white boxes. (**E**) HeLa cells expressing CFP-tagged mutants were cultured for 1 h in the presence of 0.5 mM arsenite before fixation. Localization of G3BP was detected by immunofluorescence using mouse anti-G3BP antibody followed by Alexa568-labeled anti-mouse IgG. The cells were observed by confocal microscopy.

(data not shown). To determine if the hnRNP A3-containing cytoplasmic foci were P-bodies, mRFP-Dcp1a-expressing cells were stained with the anti-hnRNP A3 antibody. As shown in Figure 5A, the mRFP-Dcp1a signals almost completely overlapped those of hnRNP A3 in the cytoplasm. When immunostaining with anti-hnRNP A3 was performed in mRFP-Dcp1a and NXF7-GFP double positive cells, all three signals overlapped (Figure 5B). Endogenous NXF7 in CEB showed similar localization pattern (i.e. diffuse as well as dot-like cytoplasmic signals). In many cases, NXF7-containing foci were also positive for Dcp1a (Figure 5C) and hnRNP A3 (Figure 5D). Thus, one simple interpretation of these results is that NXF7 was localized in P-bodies as a complex with hnRNP A3.

It was recently reported that hnRNP A1 is recruited to SGs in response to oxidative, as well as osmotic, stress (52). In contrast to hnRNP A1, oxidative stress did not cause hnRNP A3 accumulation in SGs (Supplementary Figure S3). Even when NXF7-GFP was over-expressed, re-localization of hnRNP A3 to arsenite-induced SGs was hardly observed (Figure 5E). Although enlargement of the Dcp1a-containing foci was observed in the present study, as was reported previously (53), Dcp1a did not re-localize to arsenite-induced SGs (Figure 5E). On the other hand, localization of hnRNP A3- and Dcp1a-containing foci (i.e. P-bodies) to close proximity to NXF7-containing SGs or complete fusion was observed (Figure 5E) as previously reported (53).

### hnRNP A3 was co-localized with NXF7 in neuronal processes

A recent report indicated that NXF7 is a component of neuronal RNA granules (12). In addition, P-bodies in somatic cells are similar to RNA granules in neuronal cells in terms of composition and function (40,41,43,54,55). Therefore, co-localization of hnRNP A3 and NXF7 in RNA granules in neurites was examined. NXF7-GFP was transiently expressed in the mouse neuroblastoma cell line, Neuro2a. Upon exposure to differentiation condition, a fraction of the cells extended cytoplasmic processes. As reported, NXF7-GFP was localized in cell bodies, as well as in the distal cytoplasmic processes. As shown previously, the NXF7-containing granules in neuronal cells were positive for both poly(A)$^+$ RNAs (see Supplementary Figure S4) and co-transfected mRFP-Staufen (J. K. and T. M., unpublished data). Moreover, endogenous hnRNP A3 was often co-localized with NXF7-GFP at both sites (Figure 6A) and NXF7-GFP-containing granules also contained Dcp1a (Figure 6B). Consistent with *in vitro* binding data, the N + LRR (aa 1–374), but not the M + C (aa 375–620), domain of NXF7 showed co-localization with hnRNP A3, as did wild-type protein (Figure 6C and D). The shorter fragment showing weaker binding to hnRNP A3 in the pull-down assays (aa 91–374), did not show the punctate localization at any location (Figure 6E). These results indicate that the granules in the cell bodies and the cytoplasmic processes of neuronal cells are a kind of P-body, suggesting NXF7 involvement in translational control and/or subcellular localization of hnRNP A3-bound target mRNAs.

## DISCUSSION

It has been suggested that NXF7, a member of the NXF mRNA exporter family, plays a role in cytoplasmic mRNA metabolism. It already has been shown that NXF7 is localized exclusively in the cytoplasm of non-neuronal cells where it forms aggregates of unknown nature (10,11). By examining heterologous localization patterns, it was found that NXF7 localized in translating ribosomes, P-bodies, neuronal mRNA granules and SGs under various conditions. In addition, a series of hnRNPs that were able to bind NXF7 were identified by yeast two-hybrid screening.

Since a fraction of NXF7 was found to associate with polyribosomes, the possibility that NXF7 plays a direct role in protein translation was examined. Direct tethering of an MS2 phage coat-NXF7 fusion protein did not alter the expression level of a luciferase reporter gene containing MS2-binding sites at the 3′-untranslated region (UTR)

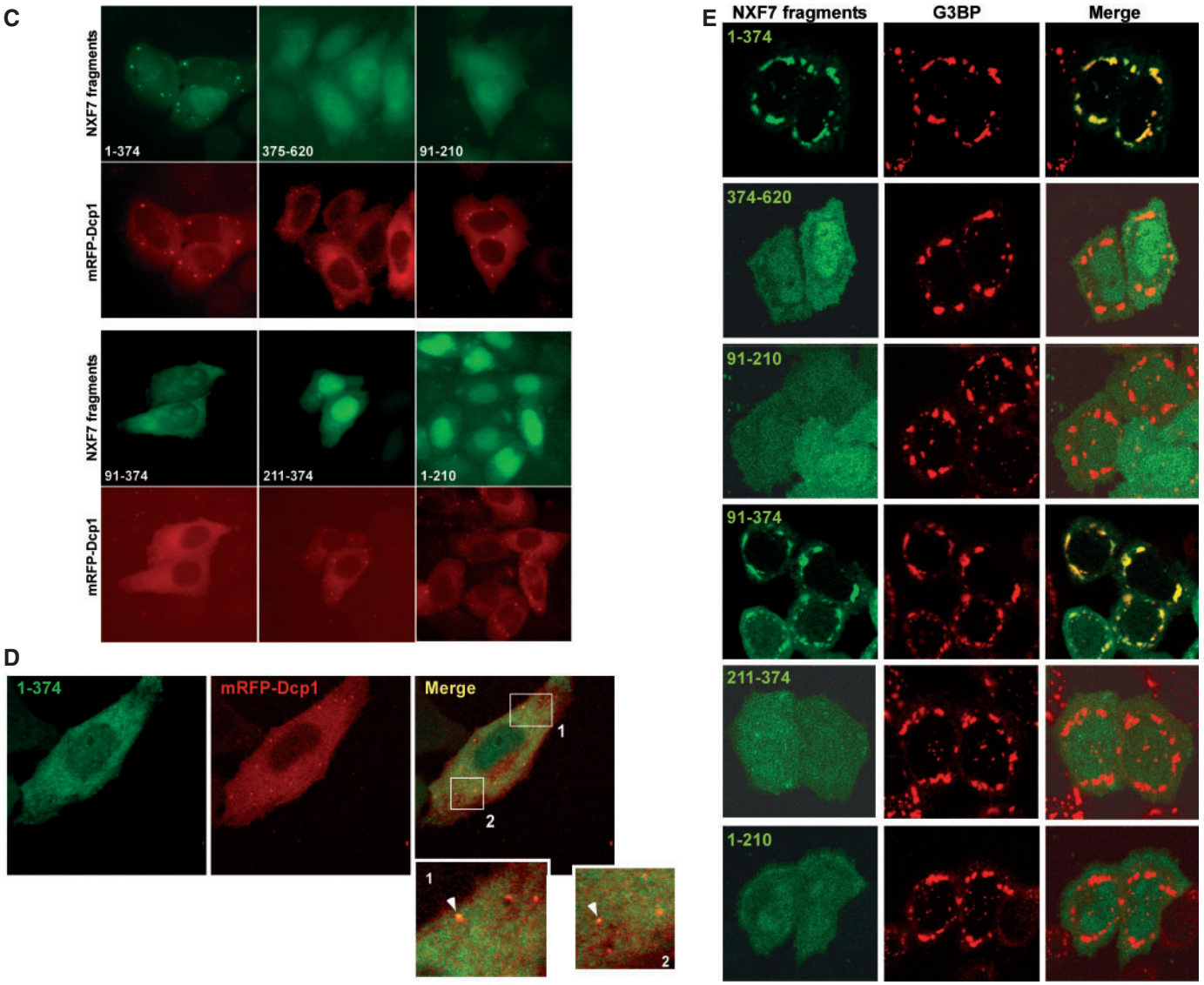


**Figure 4.** Continued.

(see Supplementary Figure S5). In addition, there was no effect on bulk translation, as judged from ribosome profiles, in different cell lines stably expressing NXF7 (J.K. unpublished observation). These findings suggest that NXF7 was not directly involved in translation process.

Among the binding partners of NXF7 identified in this study, the focus was on hnRNP A3, because hnRNP A3 may be involved in translational control and/or cytoplasmic localization of certain mRNAs in mammalian cells (47). Since hnRNP A3 was never co-fractionated with NXF7 in polyribosomes-containing fractions, it was concluded that the complex, as well as NXF7 alone, does not play a direct role in translation. In contrast, a fraction of hnRNP A3 was co-localized with NXF7 in P-bodies and in cytoplasmic processes of neuronal cells, as reported previously (47). Since the mutant truncated at the N-terminus (aa 91–374) of NXF7 exhibited weaker binding to hnRNP A3 and did not localize in P-bodies, the simplest interpretation for our observations is that the interaction with hnRNP A3 was required for targeting of NXF7 to P-bodies.

It has been proposed that translational repression and degradation of mRNA occur in P-bodies. On the other hand, it has been postulated that the cytoplasmic fates of different mRNAs are defined during their biogenesis in the nucleus. Specific sequences and/or structures within mRNAs, such as 'ZIP-code' in the 3′-UTRs and EJCs, function as primary determinants of mRNA fate (36,56–58). Nucleo-cytoplasmic shuttling mRNA binding proteins that recognize these determinants play key roles in downstream events. Indeed, a subset of EJC components, such as magoh/Y14, and non-EJC components, including hnRNP A/B family proteins from various species (i.e. Hrp48 and squid from fruit fly and hnRNP A2/B1 from mammals), play key roles in translational control and localization of bound mRNAs in the cytoplasm (31–37,59). In addition, other shuttling hnRNPs such as hnRNP I from *Xenopus* and

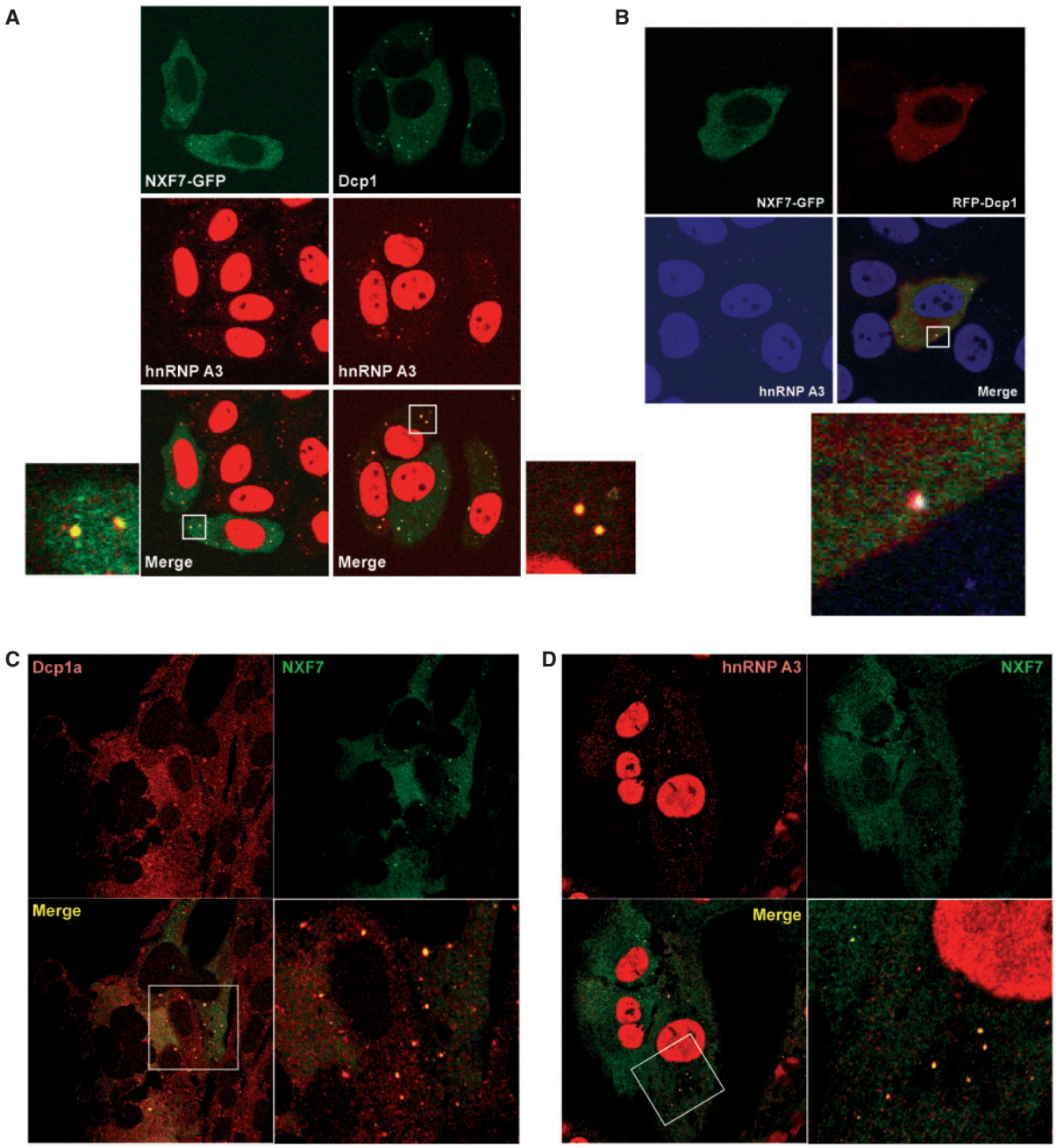


**Figure 5.** NXF7 is co-localized with hnRNP A3 in P-bodies. (**A**) HeLa cells expressing NXF7-GFP (left panels) or GFP-Dcp1a were fixed and subjected to immunofluorescence using rabbit anti-hnRNP A3 antibody. The lower left and right panels are magnified images of the areas indicated by white boxes. Localization was observed by confocal microscopy. (**B**) HeLa cells expressing NXF7-GFP and mRFP-Dcp1a were fixed and subjected to immunofluorescence as in A. Visualization of localization of hnRNP A3 was done using Alexa647-labeled anti-rabbit IgG. The lowest panel shows a magnified view of the area indicated by a white box in the merged image. (**C**) CEB-derived cells cultured on glass bottom dishes were fixed and immunostained with anti-Dcp1a and anti-NXF7 antibodies followed by Alexa568-labeled anti-rabbit and Alexa488-labeled anti-rat secondary antibodies. The cells were observed by confocal microscopy. The lower right panel shows a magnified view of the area indicated by the white box in the merged image. (**D**) Same as in C, but the cells were immunostained with anti-hnRNP A3 and anti-NXF7 antibodies. The cells were observed by confocal microscopy. The lower right panel shows a magnified view of the area indicated by the white box in the merged image. (**E**) HeLa cells expressing NXF7-GFP were cultured in the presence of 0.5 mM arsenite for 1 h. The cells were fixed and immunostained with anti-Dcp1a (upper panels) and anti-hnRNP A3 (lower panels) antibodies followed by Alexa568-labeled anti-rabbit IgG. Localization of each protein was detected by confocal microscopy.

Vera/ZBP/KSRP from *Xenopus*, chicken and mammals also are implicated in translational control and localization of specific mRNAs in the cytoplasm (35,37,38,60–65). It remains unclear, however, how the localized mRNAs are sorted and separated from the pool of translating mRNAs and then are packed within cytoplasmic granules, such as P-bodies and neuronal mRNA granules. Based on the results of the present study, a plausible mechanism is

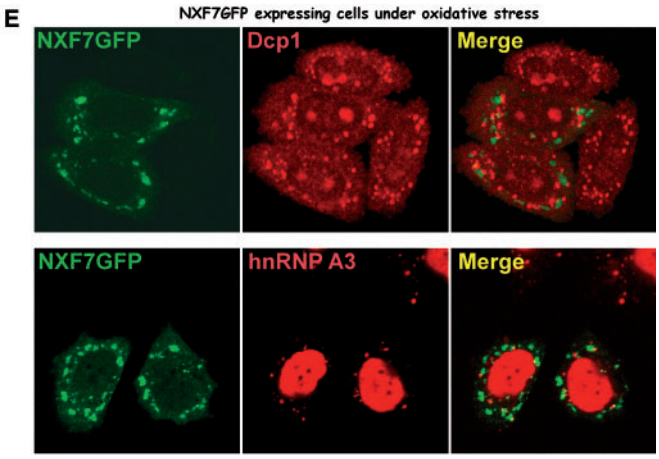


**Figure 5.** Continued.

that attachment of NXF7 to translating ribosomes allows for co-translational scanning of newly exported mRNAs and that NXF7 sorts mRNA through binding to hnRNP A3. Once target mRNAs bound to hnRNP A3 are picked up, NXF7 may stabilize the mRNAs in a conformation that suppresses translation, as proposed for *oskar* mRNA in Drosophila (33). This 'scanning model' resembles 'Pioneer round of translation', a model which was first proposed for nonsense-mediated mRNA decay (66,67). Generally, proteins involved in cytoplasmic mRNA localization and translational control show heterologous localization like NXF7. Such proteins, including FMRP and Staufen, also are localized in translating ribosomes and SGs (51,68–72) and some of them are localized in P-bodies (40,41). Thus, it is conceivable that these proteins are recruited on target mRNAs by a similar co-translational scanning mechanism.

Localization of NXF7 in SGs was observed in both arsenite-treated and NXF7-overexpressing cells. It is believed that storage or sorting of translationally dormant mRNAs occurs in SGs in higher eukaryotes (41,53). However, re-localization of hnRNP A3 in SGs was not observed and spontaneous formation of SGs did not occur in cells expressing endogenous NXF7 (e.g. CEB, see Figure 5) or stable cell lines exogenously expressing low level of NXF7 (J. K. unpublished data). Therefore, it is

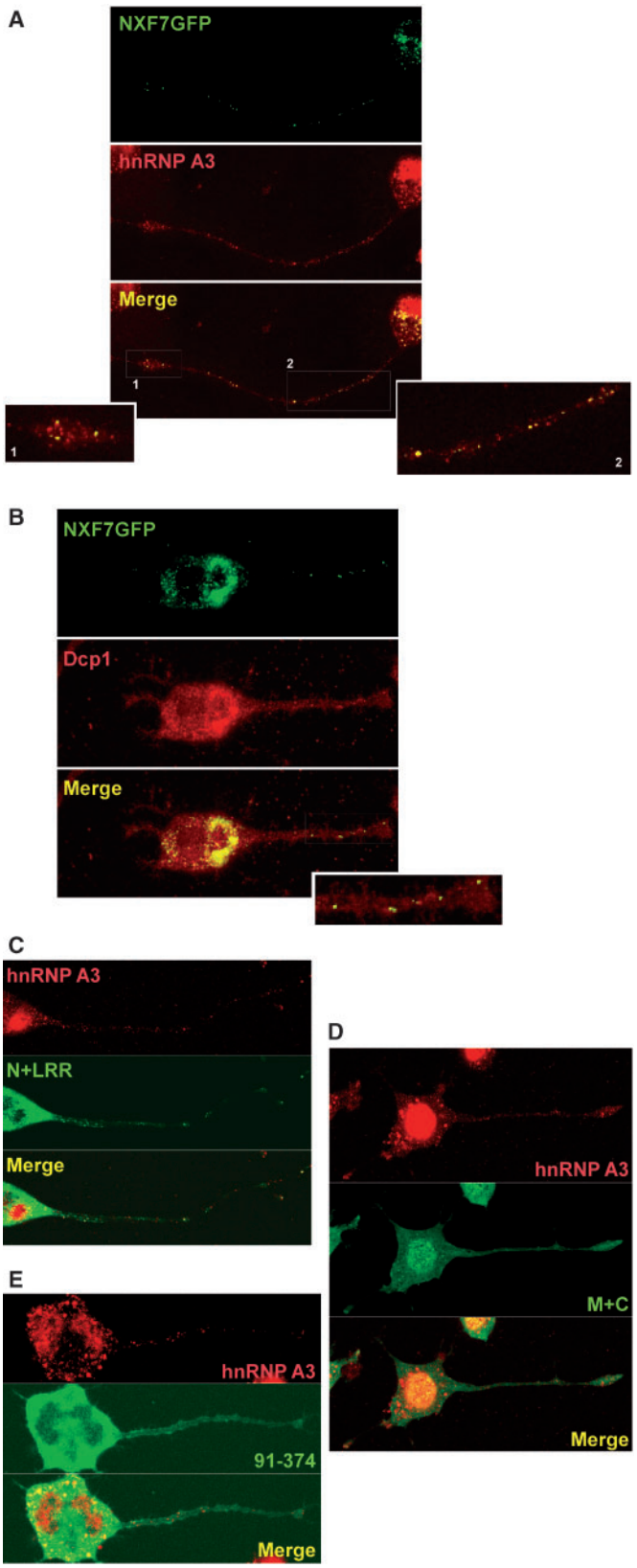


**Figure 6.** Co-localization of NXF7 and hnRNP A3 in distal sites of neurites. (**A**) Neuro2a cells expressing NXF7-GFP were fixed and subjected to immunofluorescence using anti-hnRNP A3 antibody. Localization was observed by confocal microscopy. Insets are magnified view of the areas indicated by the white boxes. (**B**) Same as in A, but immunofluorescence was performed using anti-Dcp1a antibody. Inset is a magnified view of the area indicated by the white box. (**C**) Neuro2a cells expressing a CFP-fusion protein containing the N + LRR domain (aa 1–374) of NXF7 was subjected to immunofluorescence using anti-hnRNP A3 antibody as in A. (**D**) Neuro2a cells expressing a CFP-fusion protein containing the M + C domain (aa 375–620) of NXF7 was subjected to immunofluorescence using anti-hnRNP A3 antibody as in A. (**E**) Neuro2a cells expressing a CFP-fusion protein containing the minimal hnRNP A3-binding domain (aa 91–374 of NXF7) was subjected to immunofluorescence using anti-hnRNP A3 antibody as in A.

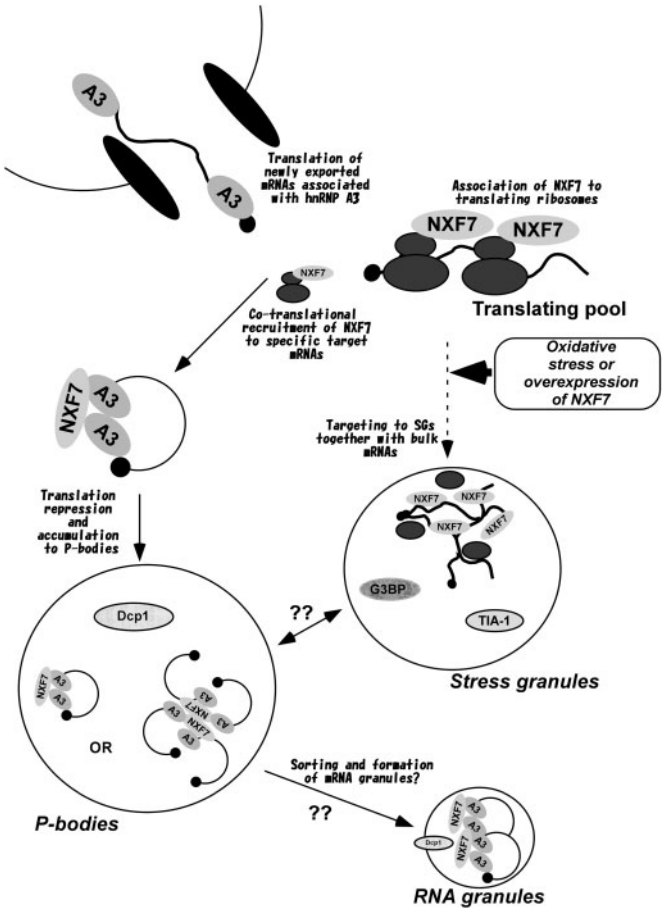


**Figure 7.** A model for the possible function of NXF7. See 'Discussion' section for details.

possible that NXF7 may be non-specifically captured within SGs because of its affinity for both ribosomes and mRNAs and that over-production of NXF7 by transient transfection may disturb bulk translation by binding to mRNAs and ribosomes non-specifically, inducing the formation of SGs. The *cis* arrangement of RNP motifs and the LRR domain was required for binding of Tap/NXF1 to the viral CTE RNA (73) as well as to non-specific RNA sequences (3,5). The observation that targeting of NXF7 to SGs required the same domain organization, which exhibited non-specific interaction with RNAs as does Tap/NXF1 (J. K. unpublished data), but not the full binding activity to hnRNP A3, is consistent with this explanation.

As reported in this and previous (10) studies, the expression of NXF7 appears to be restricted in a spacio-temporal manner, although hnRNP A3 is expressed ubiquitously (47). Tight regulation of NXF7 expression may be important to avoid inappropriate formation of SGs and to efficiently regulate cytoplasmic mRNA transport. Furthermore, it is highly probable that NXF7 plays non-essential roles in mRNA localization and/or storage, by modulating the functions of hnRNP A3, including the return of target mRNAs from storage sites to the translation pool. Such non-essential functions of NXF7 may allow for tighter regulation such as is required in specific tissues or during development. To validate these or other possibilities and to fully understand the functional role of NXF7, identification of endogenous target mRNAs is essential.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Conflict of interest statement*. None declared.

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