In eukaryotes, the nuclear export of mRNA is mediated by nuclear export factor 1 (NXF1) receptors. Metazoans encode additional NXF1-related proteins of unknown function, which share homology and domain organization with NXF1. Some mammalian NXF1-related genes are expressed preferentially in the brain and are thought to participate in neuronal mRNA metabolism. To address the roles of NXF1-related factors, we studied the two mouse NXF1 homologues, mNXF2 and mNXF7. In neuronal cells, mNXF2, but not mNXF7, exhibited mRNA export activity similar to that of Tip-associated protein/NXF1. Surprisingly, mNXF7 incorporated into mobile particles in the neurites that contained poly(A) and ribosomal RNA and colocalized with Staufen1-containing transport granules, indicating a role in neuronal mRNA trafficking. Yeast two-hybrid interaction, coimmunoprecipitation, and in vitro binding studies showed that NXF proteins bound to brain-specific microtubule-associated proteins (MAP) such as MAP1B and the WD repeat protein Unrip. Both in vitro and in vivo, MAP1B also bound to NXF export cofactor U2AF as well as to Staufen1 and Unrip. These findings revealed a network of interactions likely coupling the export and cytoplasmic trafficking of mRNA. We propose a model in which MAP1B tethers the NXF-associated mRNA to microtubules and facilitates their translocation along dendrites while Unrip provides a scaffold for the assembly of these transport intermediates.

The proteins of the NXF1 family share homology and domain structure and are conserved from yeast to humans. The best studied family members are the metazoan TAP/NXF1 and their Saccharomyces cerevisiae orthologue Mex67p, which are essential for general mRNA export from the nucleus and act as direct export receptors by linking their mRNA cargo to the nuclear pore complex. TAP/NXF1 is added to the export-ready messenger ribonucleoproteins (mRNP) in a manner that is coupled to splicing via the interactions of its N-terminal region with cofactors such as exonic junction complex components (1, 2), including Y14/MAGO1 (3, 4), as well as with shuttling SR (serine/arginine-rich) splicing factors such as U2AF (5), 9G8, SRp20, and ASF/SF2 (6–8) (for recent reviews see Refs. 9–12).

In mouse, there are two genes encoding such additional factors, mNXF2 and mNXF7 (20, 21). Like their human analogues, both mNXF2 and mNXF7 mRNAs are preferentially expressed in the brain (20). Here, we show that mNXF2 has properties similar to those of TAP/NXF1. In contrast, mNXF7 has properties of a cytoplasmic RNA transport factor. We further show that TAP/NXF1, mNXF2, and mNXF7 bind to the light chain (L chain) of brain-specific microtubule-associated protein MAP1B, which interacts with cytoplasmic microtubules and actin filaments and participates in the development and function of the nervous system (22–26). Supporting a role after export, we found that mNXF7 colocalizes with Staufen1 (Stau1) protein, which is a marker of neuronal RNA transport granules (27–35), as well as with poly(A) and ribosomal RNA.

Another NXF-interacting factor identified in this study is Unrip/STRAP, which belongs to a family of WD-repeat proteins (for review, see Ref. 36). Human Unrip participates in the inhibition of transforming growth factor β signaling by direct binding to its receptor (37–39). Unrip also associates with an RNA-binding protein, Unr, which has been implicated in trans-

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lution regulation and mRNA turnover (40, 41). Because WD-repeat proteins are thought to act as scaffolds for the assembly of multiprotein complexes, it is possible that Unrip may facilitate the recruitment of soluble factors onto the NXF-containing mRNP complexes or that it may act by anchoring such complexes to specific subcellular locations.

MATERIALS AND METHODS

Cell Culture, Fractionation, and Immunofluorescence—Transfections in human 293 or HeLa-derived HAT cells, luciferase and GFP measurements, DM128 export assays, and chloramphenicol acetyltransferase (CAT) activity measurements were performed as described previously (42). Mouse neuroblastoma Neuro2a (N2a) cells (ATCC number CCL-131) were transfected using calcium phosphate or the LT1 (Mirus, Madison, WI) protocol. Primary chicken forebrain neurons were isolated, cultured, and transfected by electroporation as described previously (43, 44). Live cell treatments with colchicine (1 μg/ml), taxol (20 μM), and cytochalasin B (10 μM) were performed for 30–60 min at room temperature. The cytoplasmic and nuclear extracts of 293 cells were prepared as described previously (5). For indirect immunofluorescence, cells were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized with 0.2% Triton X-100/PBS for 10 min. If cells were permeable in permeabilization, they were reattached with 0.004% dimethyl sulfoxide in PBS followed by a 2-min fixation in 3.7% formaldehyde/PBS at room temperature. The Alexa 590-, Alexa 488-, or Alexa 320-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes, Eugene, OR) were used as secondary antibodies.

In Situ Hybridization—Cells were grown and transfected in 35-mm glass-bottomed polylsine-coated plates (MatTek, Ashland, MA) fixed in 3.7% formaldehyde in PBS for 10 min and stored in 70% ethanol at 4 °C. After rehydration in PBS, cells were permeabilized in 0.2% Triton X-100/PBS, equilibrated in 2 × SSC/40% formamide for 5 min, and hybridized overnight at 37 °C with 5′-biotinylated oligodeoxyribonucleotide probes. Hybridization mixtures contained 1 μg/ml probe in 2 × SSC, 40% formamide, 1 × Denhardt’s solution, 10% dextran sulfate, and 100 μg/ml denatured herring sperm DNA. Cells were washed twice with 2 × SSC, once with 0.2% SSC at room temperature, incubated in blocking solution (DAKO, Carpinteria, CA), and stained with Alexa 594-conjugated streptavidin.

Recombinant DNA and Proteins—mNXF2 (GenBank™ accession numbers AY017476 and AF490577) and mNXF7 (GenBank™ accession numbers AY266683 and AY260550) are described elsewhere (21). The mammalian expression plasmids for the tagged, HA- and GFP-tagged mNXF2 and mNXF7 and for GFP-tagged Unrip were constructed as described previously (42). The plasmids expressing GFP-TAP/NXF1 and its mutants, GFP-U2AF35 and GFP-U2AF65, were described previously (5, 42). GFP-mNXF7 plasmid was constructed by inserting the mNXF7 coding region in pCEP4 (BD Clontech, Palo Alto, CA). YFP and HA-Stau1 expression plasmids were a generous gift from M. Kiebler. The fluorescently tagged mNXF7 expression plasmid contains a region encoding the light chain of MAP1B that was inserted into p2FLAG-CMV-14 (Sigma). The expression plasmids for the Ref and p15-1 proteins were provided by E. Izaurralde. For expression in Escherichia coli, MAP1B L chain and its portions were inserted in the pGEX-2T plasmid in-frame with GST, and the proteins were purified following the standard protocols. The plasmid pRed2-Dmio expresses a fluorescently tagged mitochondrial marker protein (BD Clontech).

Microscopy—For microscopy, cells were grown in 35-mm glass-bottomed plates. The wide-field epifluorescence images were captured using an inverted microscope (Axiovert 135TV) with PlanApo X40 or X100 objective, equipped with an Axiocam MRm charge-coupled device camera, appropriate filter sets, and Axiosvision software (Carl Zeiss, Thornwood, NY). Confocal microscopy was performed as described previously (42, 45). For some live cell experiments, plates were maintained at 37 °C in a closed chamber. The dual color experiments were performed using appropriate controls to exclude leakage between the channels. Some images of N2a neurites and 293 processes were acquired with parameters that maximized the pixel intensity while maintaining signal linearity in these compartments. Under these conditions, the cell body fluorescence intensity was saturated. Image refinement, digital deconvolution, and colocalization were performed using the AutoDeblur and Imaris software (Bitplane, Saint Paul, MN).

In Vitro Protein Binding Assays—Reticulocyte-produced proteins were synthesized and metabolically labeled in a coupled transcription/translation system (TNT T7 Quick, Promega, Madison, WI), using T7 promoter-containing PCR fragments as templates and were adjusted with unprogrammed extract to equal molar concentrations. Equimolar amounts of these proteins were used in binding reactions that contained 1–2 μg of E. coli–produced GST-tagged proteins that were immobilized on glutathione-Sepharose beads (Amersham Biosciences). The binding was performed in 200 μl of RBB buffer (15 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, and 0.2% Triton X-100) supplemented with 200 or 400 mM NaCl. Following incubation for 15 min at room temperature, the beads were pelleted and washed three times with binding buffer. Bound proteins were eluted by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE, and detected using a phosphorimaging device.

Immunoprecipitations—Complexes of epitope-tagged proteins were immunopurified from transiently transfected 293 cells. Typically, about 3 × 106 cells were extracted with 200 μl of RBB-200 buffer (15 mM HEPES, pH 7.9, 50 mM KCl, 200 mM NaCl, 0.1 mM EDTA, and 0.2% Triton X-100), which was supplemented with RNase and protease inhibitors for 10 min at 4 °C. The extracts were cleared by centrifugation at 10,000 × g for 15 min at 4 °C and, in some experiments, were further fractionated by gel filtration on Chromaspin-200 columns (BD Clontech) to enrich for high molecular mass complexes. Immunoprecipitations were performed at 4 °C for 10 min in 200 μl of RBB-200 buffer, using covalently attached antibodies (anti-HA and anti-GFP agarose, VectorLabs, Burlingame, CA) or anti-FLAG M2-agarose (Sigma). The precipitates were analyzed on immunoblots using horseradish peroxidase-conjugated epitope tag antibodies.

RESULTS

mNXF2 but Not mNXF7 Exhibits mRNA Export Activity—In cultured cells, exogenous TAP/NXF1 stimulates the expression of DM128 mRNA, a reporter transcript that contains an intron-encoded CAT reporter gene embedded within a portion of HIV-1 env (46, 47), and is normally retained in the nucleus. The DM128 mRNA also contains a HIV-1 Rev-responsive element element that, in the presence of the HIV-1 Rev protein, leads to strong activation of CAT expression. Although TAP/NXF1 alone leads to a small stimulation, coexpression of its cofactor p15 augments this effect (48). We have shown previously that the exogenous TAP/NXF1-p15 as well as the export cofactor of TAP/NXF1, U2AF65, acts by increasing the nuclear export of cat mRNA (5), and therefore CAT activation provides a measure of nuclear export stimulation in this reporter system. We examined the activity of mNXF2 and mNXF7 in this assay and compared it with that of TAP/NXF1.

Mouse neuronal N2a cells were cotransfected with the pDM128 plasmid and vectors encoding untagged TAP/NXF1, mNXF2, or mNXF7 in the presence or in the absence of the p15-1 expression plasmid. The cloning and characterization of mouse NXF cDNAs are described in detail elsewhere (21). All transfections contained a GFP expression plasmid as an internal reference. As positive control, pDM128 was cotransfected with the rev expression plasmid. We found that coexpression of Rev activated the DM128 mRNA expression 16-fold (Fig. 1), as expected (46, 47), confirming the validity of our assay conditions. In the absence of p15-1, TAP/NXF1 activated the DM128 expression about 2-fold, whereas co-transfection of TAP/NXF1 and p15-1 led to an at least 20-fold activation, in agreement with previous data (48). Similarly, we found that mNXF2 alone led to 7-fold activation, and
cotransfection of p15-1 resulted in 14-fold effect. In contrast, mNXF7 was inactive both in the absence and in the presence of p15-1 (Fig. 1). Neither of the coexpressed proteins had significant effects on GFP expression (Fig. 1), and similar results were obtained when using mouse PA317 or human 293 cells (21). Because mNXF7 was localized exclusively to the cytoplasm of N2a cells (see Fig. 2A), the lack of export activity could be due to its absence from the nucleus. The lack of an effect by mNXF7 is likely not because of its lower levels but rather its intrinsic properties, because mNXF7 and NXF1 are always expressed to higher levels compared with mNXF2 (see e.g. Fig. 5A). Together, our results show that the mouse mNXF2 is an active export receptor, whereas mNXF7 is inactive in this assay. However, we cannot exclude the possibility that mNXF7 has a more specialized export role that was not revealed using the DM128 reporter mRNA and N2a cells.

**mNXF7 Colocalizes with Stau1 in RNA Transport Granules**—Subcellular localization of mouse NXF factors was studied using GFP- or HA-tagged proteins that were transiently expressed in neuronal N2a cells. GFP-TAP/NXF1 localized to the nucleoplasm and nuclear membrane, whereas GFP-mNXF2 was found both in the cytoplasm and nucleus and was most prominent at the nuclear membrane (Fig. 2A). Hence, the localization of these proteins was that of typical NXF factors, in agreement with their export activity in N2a cells (Fig. 1). In contrast, GFP-mNXF7 was found uniquely in small cytoplasmic foci, both in the cell body and in the neurites, and it was excluded from the nucleus (Fig. 2A and B). The observed localization did not depend on the tag because the HA-tagged mNXF7 was localized similarly (Fig. 2A) and was not sensitive to actinomycin D or leptomycin B treatments (data not shown).

To assess the motility of mNXF7-containing particles in neurites, we used time-lapse microscopy of live N2a cells coexpressing GFP-mNXF7 and, as internal control, a mitochondria-tagged DsRed2-Mito protein. Fig. 2B shows that mNXF7 and mitochondria were segregated into discrete, non-colocalizing particles and were present abundantly in neurites. The major-

**Fig. 1.** mNXF2 but not mNXF7 stimulates the expression of DM128 CAT mRNA in neuronal N2a cells. N2a cells were transfected with 1 mg of pDM128 and 1 mg of GFP expression plasmid pFRED253Nae in the absence (none) or in the presence of 0.5 mg of untagged NXF expression plasmids. Some transfections contained 0.2 mg of human p15-1 or 0.2 mg of the HIV-1 Rev expression plasmid BsRev, as indicated. CAT activity values (black bars) are plotted as percent chloramphenicol conversion, and relative GFP fluorescence values are given. Similar results were obtained in several independent experiments.

**Fig. 2.** Subcellular localization of mNXF2 and mNXF7. GFP- or HA-tagged NXF proteins (A) or GFP-mNXF7 and DsRed2-mito (B) were transiently expressed in N2a cells and visualized by confocal (A-C) and wide-field (D and E) microscopy. A, localization of NXF factors within the cell body. GFP-tagged proteins (green) were detected in live cells, and HA-mNXF7 (blue) was detected in fixed cells using indirect immunofluorescence with HA antibody. Raw images are shown, representing midsections through the nuclei. Bar, 20 μm. C, images of live cells coexpressing GFP-mNXF7 and DsRed2-mito. Right, merge of GFP-mNXF7 and DsRed2 (mitochondria) signals. Left, differential interference contrast (DIC) image of the same field. Bar, 20 μm. C, time-lapse images of GFP-mNXF7 in N2a neurites. Arrows indicate the particles undergoing directional movement. Top row, fields shown in B by rectangles, elapsed time is shown in minutes, and bar is 20 μm. Bottom row, time is in seconds, and bar is 4 μm. D, live primary chicken forebrain neurons expressing GFP-mNXF7. Cells were electroporated with GFP-mNXF7 expression plasmid immediately after isolation, and the GFP and bright field images (top two panels) were captured at day 2. Bottom panel, time-lapse images of GFP-mNXF7 in the field indicated by rectangles in top panels. Bar, 10 μm. E, in situ colchicine treatments of N2a cells expressing GFP-mNXF7. Time-lapse GFP images of the same neurite were acquired over a period of 30 s, before and after treatment. Data are presented as pseudo-three-dimensional rendering, where the elapsed time is plotted on the vertical axis.
ity of mNXF7 particles oscillated along neurites with amplitudes of several micrometers (see also Fig. 2E), and the movements of mNXF7 granules were distinct from those of mitochondria at all times (data not shown). Some particles showed sustained unidirectional translocations with velocities ranging from 1 to 40 μm/min (Fig. 2C). Similarly, when expressed in primary chicken forebrain neurons, GFP-mNXF7 formed mobile particles in the neurites, and a fraction of them moved unidirectionally with velocities of ~20 μm/min (Fig. 2D). In both cell types, both anterograde and retrograde translocations were observed.

To probe the involvement of cytoskeletal components in mNXF7 particle motility, we studied the effects of colchicine, taxol, and cytochalasin B on cells expressing GFP-mNXF7. In both N2a (Fig. 2E) and primary chicken forebrain neurons (not shown), a short-time colchicine treatment completely abolished the motility, indicating involvement of microtubules. In contrast, taxol and cytochalasin B had no effect even after prolonged incubation (data not shown), suggesting that the motility was supported by native or stabilized microtubules but did not require the integrity of actin filaments.

Overall, the properties of mNXF7 particles were comparable with those of neuronal RNA trafficking intermediates termed RNA transport granules (28, 34, 49). We therefore probed the colocalization of mNXF7 with Staufen1 protein, a marker of such intermediates. Fig. 3A shows that in the neurites of live N2a cells coexpressing CFP-mNXF7 and YFP-Stau1, the Stau1 protein was found in granules, as expected, and exhibited extensive colocalization with mNXF7. A similar degree of colocalization was observed in fixed cells for YFP-Stau1 and HA-mNXF7 as well as for HA-Stau1 and GFP-mNXF7 (data not shown).

We next studied the mNXF7-Staufen1 colocalization in non-neuronal 293 cells, which frequently form elongated cytoplasmic processes. By using the same approach as in Fig. 3A, we found that both proteins were incorporated in granules and showed colocalization within the processes (Fig. 3B), suggesting that this property is not restricted to cells of neuronal.
origin. This is in agreement with a study showing that Staufen1 complexes purified from 293 cells are similar to those detected in neurons (33) and is consistent with the view that the RNA granule trafficking revealed in neurons/oligodendrocytes can be part of a general pathway that is active in a variety of systems (50). Interestingly, coimmunoprecipitation (as performed for Fig. 5) of the epitope-tagged mNXF7 and Stau1 proteins expressed in 293 cells did not reveal any significant association (data not shown), and thus the two proteins may belong to distinct RNP complexes. In addition, because only a minor fraction of Stau1 is present in the processes and co-localizes with mNXF7 (Fig. 3B), the possible binding of this fraction to mNXF7 could be difficult to detect by coimmunoprecipitation.

We next examined the presence of RNA in mNXF7 granules by ethidium bromide (EtBr) staining of digitonin-permeabilized, fixed N2a cells expressing GFP-mNXF7. Fig. 4A shows that GFP-mNXF7 granules were stained with EtBr, and the signal disappeared after RNase treatment in situ. The GFP-mNXF7 signal was also reduced about 2.5-fold, suggesting that RNA is required for the integrity of the granules (Fig. 4A). The RNase sensitivity of GFP-mNXF7-labeled granules was also observed when cells were not permeabilized prior to fixation (not shown). We concluded that GFP-mNXF7 can recruit RNA and associates with the granules in an RNA-dependent manner.

Because the neuronal RNA transport granules contain mRNPs packed with ribosomes (34), we examined the presence...
of ribosomal RNA and poly(A) in mNXF7-containing particles in N2a cells using in situ hybridization with 28 S rRNA or oligo(dT) probes (Fig. 4, B and C). As control, we used a probe for the U1 small nuclear ribonucleoprotein (snRN), which is not expected to undergo dendritic transport. In the cell body, each probe produced a distinct staining pattern that was typical for the respective target RNA (poly(A), cytoplasm, nucleoplasm, excluded from the nucleoli; 28 S rRNA, cytoplasm and nucleoli; U1 snRN, nucleoplasm, splicing factor compartment, excluded from the nucleoli), confirming the specificity of detection (data not shown). In the neurites, 28 S rRNA (Fig. 4B) and poly(A) RNA (Fig. 4C) were found abundantly in granules and showed a high degree of colocalization with mNXF7, albeit the RNA/mNXF7 signal ratio varied between individual granules (Fig. 4, B and C). Under the same conditions, we observed only a background U1 snRN signal in the neurites, and similar results were obtained using different probes for the same RNAs (data not shown), further validating the specificity. We concluded that in the neurites of N2a cells mNXF7 colocalized non-randomly and specifically with poly(A) and 28 S ribosomal RNA. In agreement with the colocalization data, we found that, similarly to TAP/NXF1 (5), mNXF7 can incorporate into general mRNP complexes (data not shown).

To facilitate detection, MAP1B L chain was tagged with the 3XFLAG epitope, and NXF factors were tagged with GFP. After immunoprecipitation with an antibody targeted to MAP1B L chain (anti-FLAG), the precipitates were analyzed on Western blots. Detection of MAP1B L chain (Fig. 5A, IP, α-FLAG) confirmed uniform, efficient precipitation. Detection of GFP-containing proteins on the same blots revealed that TAP/NXF1 and mNXF2 efficiently coprecipitated with MAP1B L chain, validating the yeast two-hybrid interaction. In addition, we also found interaction with mNXF7 but not with the GFP protein alone (Fig. 5A, IP, α-GFP). Similar results were obtained when using antibodies targeted to NXFs (anti-GFP) for immunoprecipitation followed by Western blot detection of MAP1B L chain (data not shown). Immunoprecipitations were carried out under conditions that favored destabilization of microtubules; however, we observed that microtubule stabilization (EGTA, warm extracts) did not affect the association significantly (not shown).

Because MAP1B L chain is known to localize to the cytoplasm, its association with NXF factors may take place in that compartment. However, under the conditions of our study, MAP1B L chain was not excluded from the nucleus of 293 cells, as revealed by indirect immunofluorescence using FLAG antibodies (Fig. 5B), in agreement with the previous data showing nuclear accumulation of this protein (51). In addition, MAP1B L chain and TAP/NXF1 were found in the cytoplasm in response to overexpression of their binding partners such as U2AF35.2 Therefore, MAP1B L chain has the potential to interact with TAP/NXF1 and mNXF2 in the nucleus.

We next probed the association between Unrip and NXFs using coimmunoprecipitation. The NXF proteins (HA-tagged) and GFP-Unrip were coexpressed as described above, and immunoprecipitations were performed using GFP antibodies. Western blot analysis with HA antibodies revealed that mNXF7, but not mNXF2 or TAP/NXF1, coprecipitated with Unrip (Fig. 5C). Because GFP-Unrip is enriched in the cytoplasm of 293 cells (Fig. 5D), it is likely that its preferential association with mNXF7 reflects the exclusively cytoplasmic localization of this particular NXF protein (Fig. 2A) rather than an intrinsic inability of other NXFs to interact. In summary, these results demonstrate that NXF factors can form stable complexes with MAP1B and Unrip in vivo.

**MAP1B Binds Stau1, Unrip, and NXF1 Export Cofactors Ref**

MAP1B binds Stau1, Unrip, and NXF1 Export Cofactors Ref and U2AF35—We further studied the binding of MAP1B L chain to Stau1 and Unrip as well as to the NXF1 export cofactors Ref1-II, Ref2-II, MAGOH, UAP56, and U2AF35 in vitro.

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1. I. Tretyakova, A. S. Zolotukhin, W. Tan, J. Bear, F. Propst, G. Ruthel, and B. K. Felber, unpublished data.
For comparison, we used the proteins representing amino acids 1–400 of TAP/NXF1, mNXF2, and mNXF7. Pull-downs were performed using immobilized recombinant GST-MAP1B L chain and reticulocyte-produced, metabolically labeled partners (Fig. 6A, full-length LC). As expected, TAP/NXF1, mNXF2, and mNXF7 bound efficiently to MAP1B L chain (Fig. 6A), confirming the validity of the assay and corroborating our yeast two-hybrid (Table I) and immunoprecipitation (Fig. 5A) data. Using this approach, we observed a strong binding of Ref1-II and Ref2-II as well as U2AF35, which have been proposed to facilitate the addition of TAP/NXF1 to its export substrate, whereas other export cofactors such as Y14, MAGOH, and UAP56 did not bind detectably. Interestingly, Stau1 and Unrip also bound to MAP1B L chain (Fig. 6A, full-length LC).

In the yeast two-hybrid screen, NXFs interacted with both the MAP1B and MAP1A light chains, which share strong homology within a region spanning 120 amino acids at their C termini and likely contained a shared NXF binding site. We therefore performed the pull-downs under the same conditions, using a GST-tagged MAP1B peptide representing this C-terminal homology region (Fig. 6A, C terminus). Interestingly, the NXFs bound to this region efficiently, but the binding of the Ref proteins and U2AF35 was significantly reduced, whereas the binding of MAGOH, Stau1, and Unrip did not bind detectably. Interestingly, Stau1 and Unrip also bound to MAP1B L chain (Fig. 6A, full-length LC).

Fig. 5. Association of NXF factors with MAP1B L chain and Unrip in vitro. All indicated proteins were transiently expressed in 293 cells. A, FLAG-tagged MAP1B L chain and GFP-NXFs were coexpressed, and MAP1B L chain-containing complexes were precipitated under native conditions using FLAG antibodies. The immunoprecipitates (IP, αFLAG) and 1:100 aliquots of input extracts (Load) were analyzed on Western blots using GFP and FLAG antibodies. Positions of GFP-NXF factors (black arrowheads) and GFP protein (gray arrowheads) are indicated. B, FLAG-tagged MAP1B L chain was detected with FLAG antibodies using indirect immunofluorescence and confocal microscopy. C, GFP- Unrip and HA-tagged NXFs were coexpressed, and NXFs were analyzed in immunoprecipitated Unrip-containing complexes (IP, αGFP) and 1:100 aliquots of input extracts (Load). D, Unrip-GFP was detected by confocal microscopy as in Fig. 2A.

In the yeast two-hybrid screen, NXFs interacted with both the MAP1B and MAP1A light chains, which share strong homology within a region spanning 120 amino acids at their C termini and likely contained a shared NXF binding site. We therefore performed the pull-downs under the same conditions, using a GST-tagged MAP1B peptide representing this C-terminal homology region (Fig. 6A, C terminus). Interestingly, the NXFs bound to this region efficiently, but the binding of the Ref proteins and U2AF35 was significantly reduced, whereas the binding of MAGOH, Stau1, and Unrip was either unaffected or slightly increased (Fig. 6A). These data narrowed down the NXF-binding determinant to the C-terminal MAP1A-MAP1B homology region and indicated that NXFs and some of their cofactors use distinct binding sites within MAP1B L chain and therefore may be able to bind concurrently. We note that this C-terminal homology region also binds the MAP1B heavy chain (24) as well as β-actin (23), whereas the N-terminal portion of MAP1B L chain contains a microtubule binding site (23). It is therefore possible that the binding partners identified here may compete for the above described binding sites, leading to the regulation of different aspects of the function of MAP1B L chain.

We next sought to verify these interactions by coimmunoprecipitation of GFP- or YFP-tagged candidate binders with FLAG-tagged MAP1B L chain. Fig. 6B shows that TAP/NXF1 coprecipitated with MAP1B L chain efficiently, as expected. We found that Unrip, Stau1, U2AF35, and the large subunit of U2AF (U2AF65) showed efficient association that was proportional to their overall expression levels, whereas Ref2-II was undetectable in the precipitates (Fig. 6B, compare IP with Load). It is possible that Ref binds to MAP1B L chain in insoluble complexes or that its binding is weak and/or transient and therefore difficult to detect. Together, these results demonstrated that in addition to its binding to NXF proteins, MAP1B L chain interacts both in vivo and in vitro with the NXF-binding factors, Unrip and U2AF, as well as with the Stau1 protein.

**DISCUSSION**

A general model of cytoplasmic mRNA trafficking has been proposed in which the transcripts are packaged into transport complexes in the nucleus and then exported to the cytoplasm, where they are tethered to microtubules and are transported to the sites of translation (50, 52). In particular, some mRNAs are translocated over extremely long distances in neurons and
oligodendrocytes, and the number of mRNAs undergoing dendritic transport was estimated at 5% of neuronal transcripts. It was proposed that such mRNAs are delivered to their correct locales (e.g., postsynaptic compartment) in a translationally silent form, allowing subsequent activation leading to regulated local expression. Supporting this model, some dendritically localized mRNAs were shown to undergo local translation after synaptic activity (for recent reviews, see Refs. 53–55).

In neurons and oligodendrocytes, such complexes appear microscopically as RNA-containing particles (termed RNA transport granules), which are able to translocate along neurites in a microtubule-dependent manner (28, 34, 49, 56, 57). Their biochemical correlates are large ribonucleoprotein complexes that are enriched in microtubule- and RNA-binding Stau1 protein and translation factors and contain densely packed clusters of ribosomes while remaining translationally inactive (29, 34, 58–60). Besides Staufen1, a variety of factors were implicated in the cytoplasmic mRNA translocation (for reviews, see Refs. 54, 55, and 61). Some of them act on specific transcripts and are segregated into separate populations of granules (62), suggesting a high degree of specialization among such intermediates.

Here, we show that mNXF7 colocalizes with Staufen1, poly(A), and 28 S RNA within mobile cytoplasmic granules both in neurites and in the extended cytoplasmic processes of non-neuronal cells. However, mNXF7 does not coprecipitate with Staufen1 in vivo, and therefore it may define a distinct subset of transport intermediates. The trafficking of Staufen1-containing granules relies on microtubules (28), and Staufen1 binds to tubulin directly via a region similar to the microtubule-binding domain of MAP1B, suggesting that Staufen1 plays an active role in transport by tethering the mRNP to the cytoskeleton (32). Extending the analogy between Staufen1 and mNXF7, we show here that mNXF7 (and other NXFs) binds directly to MAP1B, which may facilitate its association with microtubules.

There is growing evidence of links between cytoplasmic localization and nuclear export/splicing of mRNA. As an example, the export and nonsense-mediated decay cofactors of NXF1 such as Y14/MAGO play a role in mRNA localization and are linked to proteins implicated in cytoplasmic transport (Stau1-binding protein Barentsz) and nonsense-mediated decay (eIF4AIII) (63–65). In this report, we demonstrate that besides the NXFs, MAP1B also binds to the TAP/NXF1 export cofactors Ref and U2AF as well as to Staufen1. Of particular interest is the ability of a WD-40 repeat protein Unrip to bind both mNXF7 and MAP1B. Considering its predicted properties, Unrip may serve as a scaffold for the assembly of mNXF7 and MAP1B with the export and trafficking cofactors. In agreement with our findings, a recent report has identified Ref and Unrip as components of kinesin-containing neuronal ribonucleoprotein complexes that have properties of mRNA trafficking intermediates (35).

These data led us to hypothesize that cytoplasmic factors such as mNXF7, Unrip, Staufen1, and MAP1B L chain can assemble with mRNA transport intermediates in the nucleus. In support of this idea, MAP1B L chain (51), Staufen1 (66), and Unrip (this study)
can enter the nucleus. Although mNXF7 is cytoplasmic at steady state, our mutational analysis revealed the presence of active nuclear localization and nuclear pore complex association determinants (21), suggesting that this protein can also enter the nucleus. While this paper was under review, Sasaki et al. (67) confirmed the cytoplasmic localization of mNXF7 and showed that this protein, unlike mNXF2, fails to bind to p15 and to the phenylalanine-glycine repeat sequences of nucleoporins. These data suggest that mNXF7 is unable to act as an export receptor and further support its role in the cytoplasm.

Although mNXF7, unlike a typical NXF, apparently has a dedicated role in the cytoplasm, there is evidence that TAP/NXF1 and mNXF2 can also participate at post-export steps. First, TAP/NXF1 remains associated with general mRNP in the cytoplasm (5), associates with the rapidly translated mRNP population, and activates translation of constitutive transport factor (Ref), Stau1 (via MAP1B L chain), and Unrip (via NXF and microtubules for trafficking via MAP1B L chain and Stau1 (via MAP1B L chain), and Unrip (via NXF and MAP1B L chain). The export-ready complexes (Fig. 7, steps I and III) translocate to the cytoplasm and are tethered to microtubules for trafficking via MAP1B L chain and Stau1 (via MAP1B L chain). Because mNXF2 and mNXF7 (20) as well as MAP1B (70) are expressed at the highest levels in the developing brain, it is possible that the above links between nuclear export and cytoplasmic trafficking are relevant for neuronal development. We speculate that these NXFs act as part of a developmental expression program by controlling a subset of neuronal transcripts. Identification of transcripts that are subject to such regulation will be central for understanding the biological roles of brain-specific NXF family proteins.

Acknowledgments—We thank P. Aplan, G. Dreyfuss, M. Green, E. Izaurralde, M. Hurt, and A. Krichevsky for discussions and help in some experiments, and T. Jones for editorial assistance. We also thank our sum-
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