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Mammalian pre-mRNA 3′ End Processing Factor CF I_m68 Functions in mRNA Export

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Export of mRNA from the nucleus is linked to proper processing and packaging into ribonucleoprotein complexes. Although several observations indicate a coupling between mRNA 3′ end formation and export, it is not known how these two processes are mechanistically connected. Here, we show that a subunit of the mammalian pre-mRNA 3′ end processing complex, CF I_m68, stimulates mRNA export. CF I_m68 shuttles between the nucleus and the cytoplasm in a transcription-dependent manner and interacts with the mRNA export receptor NXF1/TAP. Consistent with the idea that CF I_m68 may act as a novel adaptor for NXF1/TAP, we show that CF I_m68 promotes the export of a reporter mRNA as well as of endogenous mRNAs, whereas silencing by RNAi results in the accumulation of mRNAs in the nucleus. Moreover, CF I_m68 associates with 80S ribosomes but not polysomes, suggesting that it is part of the mRNP that is remodeled in the cytoplasm during the initial stages of translation. These results reveal a novel function for the pre-mRNA 3′ end processing factor CF I_m68 in mRNA export.

INTRODUCTION

The removal of introns by splicing as well as cleavage and polyadenylation at the 3′ end of RNA polymerase II primary transcripts (pre-mRNAs) are usually required before they can be exported from the nucleus as mature mRNAs (Erkmann and Kutay, 2004). This observation has suggested that transport factors interact with the RNA during pre-mRNA processing. Indeed, recent discoveries have lent support to this hypothesis. The splicing reaction deposits on the mRNA a specific subset of proteins called the exon junction complex (EJC, for review see Tange *et al.*, 2004). REF, a component of the EJC, facilitates mRNA export by interacting with the mRNA export factor NXF1 (also called TAP, for review, see Reed and Hurt, 2002). NXF1 was originally identified as the export receptor for type D retroviral RNAs that associate with NXF1 through a sequence-specific interaction with the constitutive transport element (CTE). However, NXF1 recruitment on cellular mRNAs requires adaptor proteins such as Aly/REF (hereafter named REF). In yeast Mex67 (the homolog of NXF1) is recruited by Yra1 (homolog of REF), which is also essential for the export of poly(A) RNA in *Saccharomyces cerevisiae*. In contrast in metazoans, REF is dispensable for bulk mRNA export. This raises the possibility that multiple and partially redundant adaptor proteins may be responsible for the recruitment of NXF1. Indeed, spliceosomal proteins, including U2AF35 (Zolotukhin *et al.*, 2002) and some members of the SR family of splicing factors, were shown to interact with NXF1 and act as adaptors for NXF1-dependent export of poly(A) mRNAs (Huang and Steitz, 2001; Huang *et al.*, 2003; Lai and Tarn, 2004; Hargous *et al.*, 2006; Tintaru *et al.*, 2007).

Several observations have linked 3′ end cleavage and polyadenylation to mRNA export (for review see Zhao *et al.*, 1999). For example, RNA polymerase II reporter transcripts lacking a polyadenylation signal are retained in the nucleus of yeast cells. Positioning a transcribed poly(A) tract at the end of an mRNA by ribozyme cleavage does not result in efficient nuclear export, indicating that the 3′ end processing reaction itself, and not simply the presence of the poly(A) tail, is required for nuclear export (Huang and Carmichael, 1996). Moreover, the NS1A protein of influenza A virus specifically inhibits export of cellular but not viral mRNAs by targeting two essential components of the pre-mRNA 3′ end processing machinery (Nemeroff *et al.*, 1998). Although three yeast polyadenylation factors, Hrp1/Nab4, Nab2, and Pab1 (Kessler *et al.*, 1997; Hector *et al.*, 2002; Brune *et al.*, 2005), and the mammalian nuclear poly(A) tail–binding protein (PABPN1, Calado *et al.*, 2000), are nucleocytoplasmic shuttling proteins, to date there is no evidence for a direct role of 3′ end processing factors in mRNA export.

The mature 3′ ends of most eukaryotic mRNAs are generated by endonucleolytic cleavage of the primary transcript followed by the addition of a poly(A) tail to the upstream cleavage product (for reviews see Zhao *et al.*, 1999; Gilmartin, 2005). Cleavage Factor I_m (CF I_m) is a component of the 3′ end processing complex that participates in the cleavage reaction. CFI_m is a heterodimer composed of a small subunit of 25 kDa and a large subunit of 59, 68, or 72 kDa (Rüegsegger *et al.*, 1998). The 25- and 68-kDa subunits have been

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shown to be sufficient to reconstitute CFI_m activity for poly(A) site cleavage in vitro upon addition to partially purified 3′ processing factors (Rüegsegger *et al.*, 1998). Both subunits contact the RNA substrate, as demonstrated by UV cross-linking studies (Rügsegger *et al.*, 1996). The structure of the CFI_m 68-kDa subunit is strikingly similar to that of the SR family of proteins, which plays an essential role in basal and regulated pre-mRNA splicing (reviewed by Graveley, 2000). The 68-kDa protein possesses an N-terminal RNP-type RNA recognition motif (RRM) and an RS-like C-terminal region enriched in RS/D/E dipeptides that is reminiscent of the RS domain of SR proteins. The RS-like domain of CF I_m 68-kDa subunit is sufficient for the localization in nuclear speckles (and in the nucleoplasm) and mediates the interaction in vitro with a subset of shuttling SR proteins (Dettwiler *et al.*, 2004; Cardinale *et al.*, 2007).

Here, we demonstrate that CF I_m68 is a nucleocytoplasmic shuttling protein that can stimulate mRNA export. First, we show that CF I_m68 is associated with components of the EJC. Second, we establish that CF I_m68 interacts with the mRNA export factor NXF1. Finally, we demonstrate that CF I_m68 is directly involved in mRNA export. Although CF I_m68 can stimulate export when tethered to a luciferase reporter mRNA and its overexpression results in an increase of endogenous mRNAs in the cytoplasmic fraction, RNAi-mediated silencing leads to the retention of polyadenylated transcripts in the nucleus. Furthermore, we show by sucrose gradient centrifugation that CF I_m68 cosediments with the 80S ribosome particle. CF I_m68 is a well-established component of the pre-mRNA 3′ end processing complex. Our data showing that CF I_m68 is part of the mature mRNP particles and contributes to their export to the cytoplasm highlight a novel function for CF I_m68 and provide a link between pre-mRNA 3′ end formation and mRNA export.

MATERIALS AND METHODS

Oligonucleotides, Plasmids, and Antibodies

Sequences for real-time RT-PCR probes and description of plasmids and antibodies are presented in Supplemental Material.

Cell Culture and Heterokaryon Assay

HeLa and HEK293 cells were cultured in DMEM supplemented with 10% FBS and transfected using Effectene Transfection Reagent (Qiagen, Chatsworth, CA) or Escort V (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Drug treatments were carried out as follows: actinomycin D treatment (5 μg/ml, Sigma-Aldrich) for 2 h or leptomycin B (LMB; 25 μg/ml, Sigma-Aldrich) for 1 h in DMEM, followed by fixation and fluorescence microscopy.

For heterokaryon nucleocytoplasmic shuttling assays, HeLa cells were transiently transfected with different vectors as described. Twenty-four hours after transfection, an equal number of HeLa cells was seeded onto the same coverslip as NIH3T3 cells. The cells were incubated for 3 h in the presence of 50 μg/ml cycloheximide and for 30 min in the presence of 100 μg/ml cycloheximide before fusion. Cell fusions were done as described (Pinol-Roma and Dreyfuss, 1992). Heterokaryons were incubated further for 2 h in media containing 100 μg/ml cycloheximide before fixation.

Immunoprecipitations

After transfection, HEK293 cells were washed and harvested. For the immunoprecipitations of Flag-hUpf3b complexes, 8 × 100-mm dishes of transfected cells were used for each assay. The pellets were quickly frozen in liquid nitrogen, thawed, and resuspended in lysis buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP40, and 1× protease inhibitor cocktail [PIC]; Roche, Indianapolis, IN) and then incubated on ice for 1 h with occasional shaking. For the coimmunoprecipitation of hemagglutinin (HA)-CF I_m68 with either Flag-CF I_m25 or Flag-NXF1, cells were lysed in high-salt lysis buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% NP40). The samples were then centrifuged at 15,000 rcf for 15 min at 4°C, and the supernatants were collected. RNase A digestion (200 mg/ml, Roche) was performed for 30 min at 30°C.

Flag-tagged proteins were immunoprecipitated from the precleared, RNase A–treated cell lysate with M2 anti-Flag agarose (Sigma-Aldrich) at 4°C for 2 h on a rotator wheel in IP150 buffer (10 mM $MgCl_2$, 10% NP40, 150 mM NaCl). Then samples were centrifuged ten times at 600 × *g* for 10 min at 4°C and washed five times with 500 μl of IP150 or IP250 buffer. Precipitated proteins were eluted either with SDS sample buffer or M2 peptide and analyzed by Western blotting. Detection was performed with an ECL detection kit (Amersham, Piscataway, NJ).

GST-Fusion Protein Purification and GST-Pulldown Assays

To study protein–protein interactions in vitro, GST fusion proteins were expressed in *Escherichia coli* BL21(DE3)LysS or BL21(DE3) RIPL transformed with pGEX-derived plasmids encoding glutathione *S*-transferase (GST; negative control) or GST-fusions with CFI_m25, NXF1, or NXF1–202. After purification over glutathione Sepharose 4B beads (GE Healthcare, Waukesha, WI), GST-CFI_m25 and GST-NXF1 were further purified by gel filtration over a Superdex 75 column (GE Healthcare).

GST pulldown assays with hexahistidine-tagged CF I_m68 were performed as follows. The purified GST fusions were coupled to glutathione Sepharose 4B beads, by using equimolar amounts of either GST-CF I_m25 or GST-NXF1 (1.5 and 3 μg, respectively) and 3 μg of GST as negative control. The beads and proteins were incubated in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) supplemented with 0.1% NP40 (PBS/NP40) at 4°C for 1.5 h with gentle agitation on a wheel, followed by the addition of 400 ng histidine-tagged CF I_m68 and incubation for 1.5 h at 4°C. Subsequently, the beads were washed with PBS/NP40, and the bound material was analyzed by SDS-PAGE and subsequent Western blotting. Because input one-fifth of the added recombinant CF I_m68 was loaded on the gel, His-CF I_m68 was detected by incubation of the blot with a mouse monoclonal anti-His antibody (HIS1, Sigma-Aldrich) and a species-specific horseradish peroxidase–coupled antibody (Promega, Madison, WI) and developed by the enhanced chemiluminescence method (ECL-Plus, GE Healthcare). To visualize the bound recombinant GST-fusions, the membrane was stained with Coomassie Brilliant Blue R-250.

GST pulldown assays with in vitro–translated proteins were performed as follows. The purified GST-fusions were coupled to glutathione Sepharose 4B beads (GE Healthcare) in PBS/NP40 for 1 h and subsequently incubated with RNAse treated [^{35}S]methionine-labeled proteins obtained by coupled in vitro transcription/translation in rabbit reticulocyte lysate (TNT T7 kit, Promega). For nonradioactive in vitro translation, methionine was added to a final concentration of 20–30 μM. The samples were incubated in PBS supplemented with 0.1% NP40 at 4°C for 2 h with gentle agitation. Subsequently beads were washed with PBS/NP40 (NP40 concentration increased to 1%), and input and the bound fraction were analyzed by SDS-PAGE and detected on a storm 820 Phosphorimager (Amersham).

Tethered mRNA Export Assay

Assays were carried out as described in Hargous *et al.* (2006) and Tintaru *et al.* (2007), with the exception that *renilla* luciferase rather than ß-galactosidase was used for the normalization of transfection efficiency. For each transfection, 700 ng of each of the plasmids encoding the MS2-protein, 50 ng of luc-RRE firefly construct, and 5 ng of pRL-TK, a thymidine kinase renilla luciferase control vector, were cotransfected in 24-well plates. Detection of luciferase activity was performed with the Dual-luciferase Reporter Assay System (Promega) according to the manufacturer's instruction. Luminescence measurements were performed by using a Berthold luminometer. Four independent sets of transfections were carried out in triplicate with two different plasmids preparations. The average normalized luciferase activity in all the experiments was calculated and expressed as percentage of the activity measured for REF.

For analysis of the tethering experiments on the RNA-level, 1.4×10^6 HeLa cells were transfected with 10 μg MS2 fusion plasmid and 500 ng of pLuc-SalRRE-6MS2 using Dreamfect (OZ Biosciences, Marseilles, France). The cells were harvested 48 h after transfection. Nuclei were isolated as described below and RNA was prepared by using an "Absolutely RNA RT-PCR Miniprep Kit" (Stratagene, La Jolla, CA). RNA, 1 μg, was reverse-transcribed with random hexamers and StrataScript 6.0 reverse transcriptase (Stratagene) according to the manufacturer's protocol. Real-time RT-PCR was performed as described below.

Fluorescent In Situ Hybridization

For the visualization of the luciferase reporter RNA, the fluorescent in situ hybridization (FISH) probes were 390 nt biotinylated antisense RNA molecules transcribed in vitro from pRRE-Luc linearized with EcoRV with the BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, New York, NY). HeLa cells were transiently transfected with pLUCRRE6MS2 reporter alone or cotransfected with pCNMS2CFI_m68GFP, pCNMS2GFP, pCNMS2TAP, pCNMS2REF, or pCNMS2REF-RRM. After 30 h, the cells were fixed and FISH was performed according to standard protocols. Briefly, cells were incubated in prehybridization buffer (2× SSC, 20% formamide, 0.2% BSA, 1 μg/μl tRNA) for 30 min at 37°C and then in hybridization solution (2× SSC, 20% formamide, 0.2% BSA, 10% dextran sulfate, 1 μg/μl tRNA) in the

presence of the biotinylated RNA probe (50 ng/slide) for 3 h at 37°C. Stringent washes were performed in order to wash out unlabeled probe (twice with 2× SSC + 20% formamide, twice with 2× SSC, once with 1× SSC for 15 min at 45°C, once with 0.5× SSC for 15 min at 45°C, once with 0.5× SSC + 0.3% NP40 for 2 min at 72°C, once with 2× SSC + 0.1% NP40 for 1 min at room temperature (RT), and once with 2× SSC for 10 min at RT). After a preincubation wash with 4× SSC + 0.1% Triton X-100 for 5 min at RT, the FISH probe was revealed with 3 μg/ml streptavidin-APC conjugate (BD Biosciences, San Diego, CA) diluted in 4× SSC, 1% BSA for 1 h at RT. Finally, cells were stained with DAPI and mounted with FluorSave reagent (Calbiochem, La Jolla, CA). Images were collected with a TCS SP2 AOBS confocal microscope (Leica Microsystems, Exton, PA) and by using LSC software. Twelve-bit images were acquired by using the same setting parameters for all the samples (gain, offset); for each field, five different *xy* sections along the *z*-axis were acquired. FISH quantification was carried out with the same LCS software. Measurements of the FISH probe were obtained for the nuclear fluorescence (Sn), the total cell fluorescence (Sc), the area of the nucleus (An), and area of the cell (Ac). The cytoplasmic (C′) amount of mRNA was calculated as follows: $C' = 1 - [(An\ Sn)/(Ac\ Sc)]$. C/N ratios were calculated as $C/N = C'/(1 - C')$ (for reference see Valencia *et al.*, 2008).

For the visualization of poly(A)+ RNA, HeLa cells were grown in six-well plates on coverslips and transfected with the different plasmids, as indicated. Seven days past transfection, the cells were washed two times in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) before they were fixed for 10 min at RT in 3.7% paraformaldehyde (PFA). After a wash in PBS for 5 min, the cells were permeabilized for 10 min with 100% methanol, followed by a 10-min incubation in 70% ethanol, and a 5-min incubation in 1 M Tris, pH 8.0. The cells were then blocked for 30 min at 40°C in prewarmed prehybridization solution (2 x SSC, 20% formamide, 0.2% BSA, 1 μg/μl tRNA in DEPC-H_2O). Prewarmed hybridization solution (2× SSC, 20% formamide, 10% dextran sulfate, 0.2% BSA, 1 μg/μl tRNA, 1 ng/μl Cy3-labeled oligo-$(dT)_{50}$ in DEPC-H_2O) was added to the coverslips and incubated at 37°C for 2 h. Next, the cells were washed twice with prewarmed 2× SSC and 20% formamide in PBS, twice with prewarmed 2× SSC in PBS for 5 min each at 42°C and once in 1× SSC in PBS for 5 min at RT. Before the coverslips were mounted (Mowiol 4–88, Calbiochem, containing 0.25 μg DAPI/ml), they were washed three times for 5 min in PBS.

RNA Interference

HeLa cells stably transfected with pcDNA3-ß-globin cDNA were transfected either with two siRNAs against CF I_m68 or NXF1 (Silencer Select Pre-Designed & Validated siRNA, Applied Biosystems, Foster City, CA) at 25 nM concentration each or a nontargeting siRNA control at 50 nM concentration, by using Lullaby reagent (OZ Biosciences) according to the manufacturer's instructions. After splitting, cells were subjected to a second round of transfection with 50% of the amount of siRNAs indicated above. Cells depleted from CF I_m68 were harvested 4 d after the first transfection for analysis, whereas cells depleted from NXF1 were harvested 48 h after transfection.

Real-Time PCR Analysis

For the analysis of the nuclear and cytoplasmic distribution of mRNAs, HeLa cells were cotransfected with pSUPuro constructs or pcDNA3-HA–derived plasmids containing a puromycin selection marker by using Dreamfect (Oz Biosciences). Culturing the cells in the presence of 1.5 μg/ml puromycin eliminated untransfected cells. Three days after transfection cells were harvested.

Nuclear-cytoplasmic fractionations of HeLa cells were performed as described elsewhere (Carneiro and Schibler, 1984), except that RNase A treatment was omitted. From each fraction, 20 μl aliquots were taken for Western blot analysis. Subsequently RNA was isolated by using TRI-Reagent (Ambion, Applied Biosystems, Inc., Foster City, CA), and DNA contamination was removed by using Turbo DNA-free (Ambion). Total RNA, 2 μg, from each fraction was reverse transcribed with Stratascript reverse transcriptase (Stratagene) according to the manufacturer's instruction. cDNA corresponding to 40 ng RNA was amplified with specific primers and TaqMan probes in an ABI SDS7000 Sequence Detection System (Applied Biosystems). The relative mRNA levels of the cytoplasmic and nuclear fractions were normalized to 18S rRNA levels. The RT-PCR for X-ist and MIC 2a was performed in 50 μl 1× Fast Start Master Mix (Roche) supplemented with 400 nM f.c. of each primer pair, and 38 cycles were performed.

Polysome Profile

Sucrose gradient fractionation was performed as described in Sanford *et al.* (2004). Briefly, HEK293 cells were cotransfected with plasmids expressing HA-CF I_m68 and T7-ASF. After 24 h cells were washed and collected by short centrifugation at 4,000 rpm at 4°C and resuspended cold lysis buffer (100 mM NaCl, 10 mM $MgCl_2$, 30 mM Tris HCl, pH 7.5, 1 mM DTT, 0.05% Triton X-100) containing 100 μg/ml cycloheximide, RNase, and protease inhibitors. After 5 min on ice, the extract was centrifuged for 4 min at 12,000 × *g* at 4°C to pellet nuclei and debris. For negative controls, extracts were treated before sucrose gradient analysis either with 30 mM EDTA for 10 min at 4°C on a rotator wheel, followed by the addition of 2 μg RNase A (Roche) and further incubation for 30 min at 30°C, or with 20 mM puromycin for 1 h at 4°C on a rotator wheel. Supernatants were resolved on 15–50% sucrose gradients prepared in (100 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris HCl, pH 7.5). The gradient was centrifuged at 4°C at 38,000 rpm in a Beckman SW41 rotor for 3 h. After centrifugation, fractions were collected from the bottom, precipitated with TCA, and analyzed by immunoblotting.

Online Supplemental Materials are as follows: Supplemental File 1: legends to Supplemental Figures S1–S8; Supplemental File 2: Supplemental methods; Supplemental Figure S1: CF I_m is imported into the nucleus as a heterodimer; Supplemental Figure S2: Nucleocytoplasmic shuttling is an active process; Supplemental Figure S3: Western blot analysis of HEK293 cell extracts used in the mRNA export assay; Supplemental Figure S4: Western blot assessing the purity of the nuclear fractions used in the experiments shown in Figure 6D and 7E; Supplemental Figure S5: NXF1 depletion increases nuclear levels of ß-globin mRNA; Supplemental Figure S6: sucrose gradient fractionation of puromycin-treated extracts; Supplemental Figure S7: color version of Figure 1; and Supplemental Figure S8: color version of Figure 2.

RESULTS

CF I_m Large Subunits Are Nucleocytoplasmic Shuttling Proteins

We reported previously that the 68-kDa subunit of CF I_m interacts with a subset of SR proteins that were shown to shuttle continuously between the nucleus and the cytoplasm (Dettwiler *et al.*, 2004). In addition, the 25-kDa subunit interacts with PABPN1, which is a nucleocytoplasmic shuttling protein (Calado *et al.*, 2000). On the basis of these observations we wanted to test whether also the CF I_m subunits shuttle between the nucleus and the cytoplasm. We analyzed their migration in an interspecies heterokaryon fusion assay. HeLa cells expressing HA-tagged CF I_m68 were fused to mouse NIH3T3 cells in the presence of cycloheximide to produce heterokaryons. Shuttling of HA-CF I_m68 would lead to its equilibration into the nuclei of fused NIH3T3 cells. As a control, HeLa cells were cotransfected with a plasmid expressing GFP-hnRNP A1, a well-known shuttling protein, or GFP-hnRNP C, a protein that is always restricted to the nucleus (Pinol-Roma *et al.*, 1988). In a representative heterokaryon, 2 h after fusion, HA-CF I_m68 and GFP-hnRNP A1 were present both in the two HeLa cell nuclei and in the mouse cell nucleus (Figure 1A, top row). In contrast, GFP–hnRNP C was restricted, as expected, to the HeLa cell nucleus (Figure 1A, bottom row). Quantitative analysis showed that NIH3T3 nuclei were positive for HA-CF I_m68 in all of the 51 heterokaryons examined. Thus, although CF I_m68 is nuclear at steady state, it is continuously traversing the nuclear envelope. A similar assay was performed with HeLa cells expressing either GFP-CF I_m59 or GFP-CF I_m25. As shown in Figure 1B, although CF I_m59 can efficiently migrate into the mouse nucleus (Figure 1B, top row), shuttling of CF I_m25 is less efficient (Figure 1B, bottom row). A possible explanation for the observed, inefficient migration of CF I_m25 into the mouse nucleus could be that this subunit is not able to shuttle on its own and may be imported into the nucleus only in association with one of the endogenous larger polypeptides. Therefore, if GFP-CF I_m25 is overexpressed but the endogenous large subunits are limiting, this may result in poor nuclear import of GFP-CF I_m25. Cotransfection experiments with CFP-CF I_m68 and YFP-CF I_m25 constructs in which both ORFs are expressed under the same strong promoter (so that the two CF I_m subunits should be present in the cell in almost equimolar amounts and could therefore efficiently dimerize) demonstrated that, under these conditions, the CF I_m 25-kDa subunit is imported into nucleus more efficiently, in agreement with the explanation proposed above (Supplemental Figure S1).

To identify the region involved in the export of CF I_m68, we analyzed the shuttling behavior of various domain deletion mutants fused to GFP (68ΔN, 68ΔRS, 68RS, and

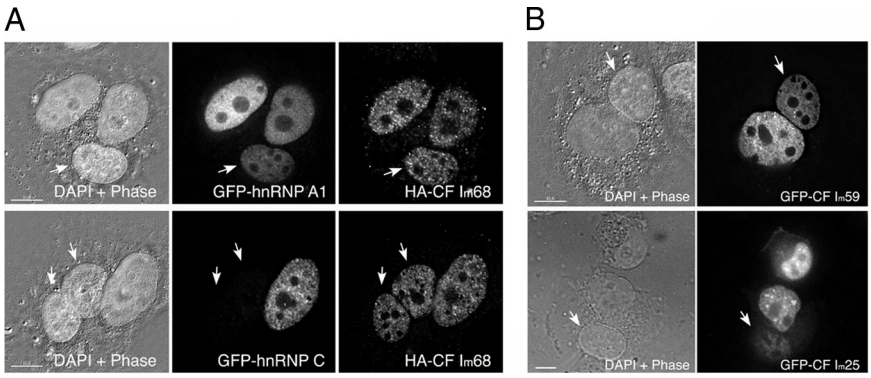

Figure 1. The two large CF I_m subunits migrate between nuclei in interspecies heterokaryons. (A) CF I_m68 is a shuttling protein. Left, merge of DAPI staining of HeLa and NIH3T3 nuclei (indicated by broken arrows) and phase-contrast microscopy view. Middle, localization of GFP–hnRNP A1 or GFP-hnRNP C. Right, localization of HA-CF I_m68. (B) CF I_m59 but not the 25-kDa subunit shuttles between the nucleus and the cytoplasm. Representative heterokaryons of HeLa cells, transfected with either GFP-CF I_m59 or GFP-CF I_m25. Broken arrows, the mouse nuclei. A full-color version of this figure is available as Supplementary Figure S7.

68RRM/RS, depicted in Supplemental Figure S2; Dettwiler *et al.*, 2004) at 37°C or, in addition, at 4°C to check for passive diffusion. The rationale for this assay is that, at 4°C, both receptor-mediated nuclear import and export are blocked, whereas passive diffusion continues to occur. Temperature-shift experiments were performed as described in Michael *et al.* (1995). As shown in Supplemental Figure S2, all the mutants were able to shuttle at 37°C but were restricted to the HeLa cell nucleus at 4°C.

Nuclear Export of CF I_m68 is CRM1-independent and Requires Active Transcription

The most common mechanism for the nuclear export of proteins in eukaryotic cells is based on CRM1-dependent systems. CRM1-mediated export is specifically blocked in the presence of the fungal metabolite LMB, which inhibits the formation of the ternary complex between CRM1, Ran-GTP, and the cargo protein. To check whether CRM1 is responsible for CF I_m68 nuclear export, heterokaryon assays were thus performed the presence of LMB. HeLa cells treated in this way were transfected with plasmids expressing HA-CF I_m68 in combination with GFP-HMGB1, a protein that requires CRM1 for export (Figure 2A). Treatment with LMB specifically restricted HMGB1 to the HeLa cell nucleus. while CF I_m68 could still be efficiently exported thus indicating that its export does not require CRM1.

Previous studies have shown that CF I_m is an RNA-binding factor (Rüegsegger *et al.*, 1996). CF I_m68 contacts the RNA mainly via the charged C-terminal domain, whereas the RRM is primarily involved in protein interactions with the small subunit (Dettwiler *et al.*, 2004). Therefore, it is possible that CF I_m68 is leaving the nucleus by "piggy-backing" on RNA molecules being exported by other factors, either in association with the 25-kDa subunit or because of a direct interaction via the C-terminal domain. To determine whether shuttling of CF I_m68 depends on mRNA synthesis, we performed heterokaryon assays in the presence of actinomycin D. As shown in Figure 2B, GFP-CF I_m68 shuttling is blocked in presence of the transcription inhibitor, whereas relocalization of the protein in cap-like structures around the nucleoli of the human cell can be observed, as previously described (Cardinale *et al.*, 2007; Figure 2B). Although we cannot formally rule out the possibility that actinomycin D could affect the synthesis of a short-lived protein required for export, the most likely conclusion of these experiments is that CF I_m68 shuttling is dependent on mRNA synthesis and possibly export. Therefore, on the basis of this observation, we began to investigate a possible involvement of CF I_m68 in mRNA export.

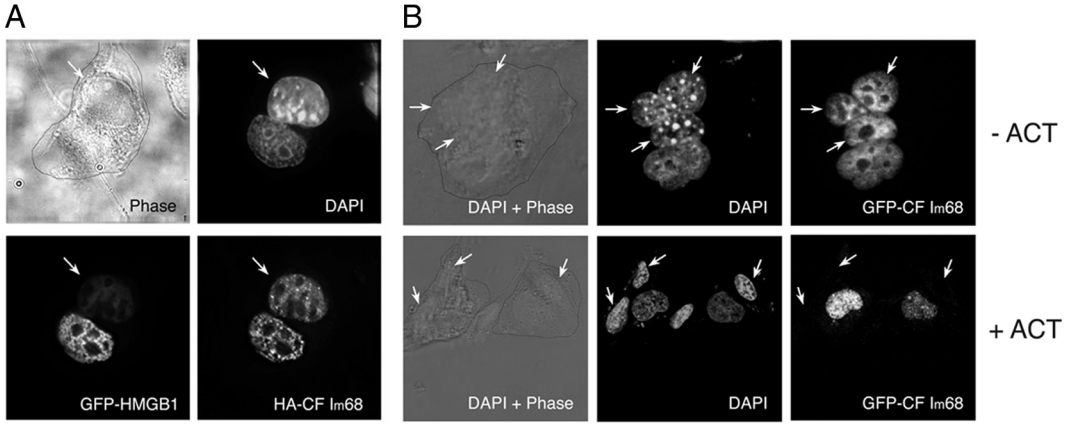

Figure 2. Export of CF I_m68 depends on mRNA traffic. (A) Inhibition of the CRM1-mediated nuclear export pathway does not affect CF I_m68 shuttling activity. HeLa cells were cotransfected with expression constructs for HA-CF I_m68 and GFP-HMGB1. Two hours before fusion with NHI3T3 cells and throughout the experiment, cells were incubated in the absence (not shown) or in the presence of LMB as described in *Materials and Methods.* HA-CF I_m68 was detected by immunofluorescence. (B) HeLa cells transfected with GFP-CF I_m68 and mouse NIH3T3 cells were treated with actinomycin D and then fused as described in *Materials and Methods.* Left, merge of DAPI staining of HeLa and NIH3T3 nuclei (indicated by broken arrows) and phase-contrast microscopy view. A full-color version of this figure is available as Supplementary Figure S8.

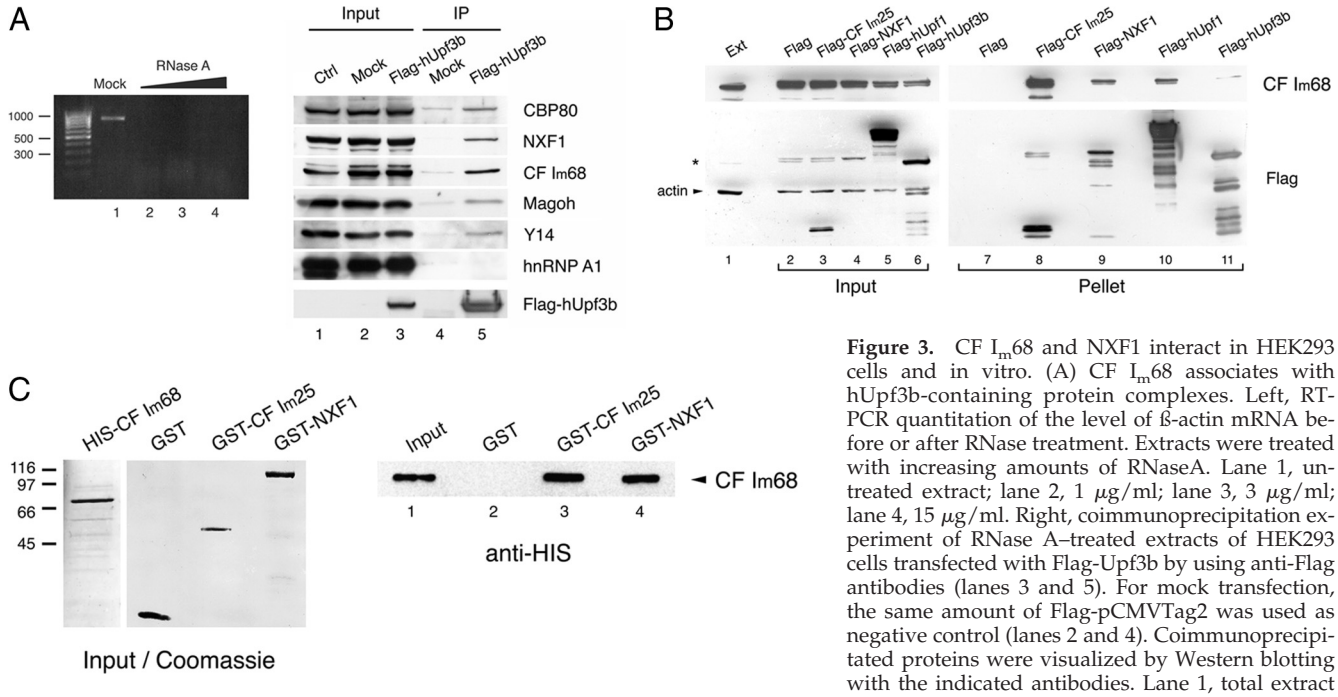

Figure 3. CF I_m68 and NXF1 interact in HEK293 cells and in vitro. (A) CF I_m68 associates with hUpf3b-containing protein complexes. Left, RT-PCR quantitation of the level of ß-actin mRNA before or after RNase treatment. Extracts were treated with increasing amounts of RNaseA. Lane 1, untreated extract; lane 2, 1 μg/ml; lane 3, 3 μg/ml; lane 4, 15 μg/ml. Right, coimmunoprecipitation experiment of RNase A–treated extracts of HEK293 cells transfected with Flag-Upf3b by using anti-Flag antibodies (lanes 3 and 5). For mock transfection, the same amount of Flag-pCMVTag2 was used as negative control (lanes 2 and 4). Coimmunoprecipitated proteins were visualized by Western blotting with the indicated antibodies. Lane 1, total extract from untransfected cells. (B) CF I_m68 interacts with NXF1 in HEK293 cell extract. Coimmunoprecipitations with anti-Flag antibody of RNase A–treated extracts of HEK293 cells transfected with Flag-tagged CF I_m25 (positive control, lanes 3 and 8), NXF1 (lanes 4 and 9), hUpf1 (lanes 5 and 10), or hUpf3b (lanes 6 and 11), and HA-tagged CF I_m68, as indicated. For mock transfections (lanes 2 and 7), the same amount of Flag-pCMVTag2 was used. Bound proteins (Pellet) were detected by Western blotting with anti-CF I_m68 antiserum (top panel). To control for equal loading, the membrane was sequentially probed without stripping with anti-Flag and anti-actin antibodies (bottom panel). An asterisk indicates residual signal of the anti-CF I_m68 antibody. Lane 1, total extract from untransfected cells. (C) Recombinant CF I_m68 interacts with NXF1 in vitro. Left, Coomassie-stained SDS-PAGE of the purified recombinant proteins used in the pulldown assay. Right, GST (lane 2), GST-tagged CF I_m25 (lane 3) and NXF1 (lane 4) were tested for interaction with histidine-tagged CF I_m68. Eluted proteins were analyzed by Western blotting with anti-histidine antibody.

CF I_m68 Interacts with the mRNA Export Factor NXF1

The EJC, which is deposited on the mRNA by the splicing reaction, consists of four core components (Y14, Magoh, MLN51, and eIFAIII) and several more peripherally associated proteins (for review see Tange *et al.*, 2004). Complexed proteins include the splicing factors SRm160, RNPS1, Acinus, SAP18 and Pinin, the mRNA export factors UAP56, Aly/REF, and NXF1, and the NMD factor Upf3b. To determine if CF I_m68 may be present in postsplicing complexes, Upf3b-containing mRNPs were immunopurified from extracts of HEK293 cells transiently transfected with Flag–tagged hUpf3b and analyzed by Western blotting with antibodies against EJC proteins, hnRNP proteins, and with anti-CF I_m68 antiserum (Figure 3A). Because several of these factors, including CF I_m, can bind RNA, extracts were treated with RNase A before immunoprecipitation to test for true protein–protein interactions. RNase treatment was shown to be effective by the disappearance of ß-actin mRNA as revealed by RT-PCR (Figure 3A, left). Consistent with previous reports, CBP80, NXF1, REF, and Magoh coimmunopurified with Flag-hUpf3b (Figure 3A, right; Kim *et al.*, 2001; Lejeune *et al.*, 2002; Singh *et al.*, 2007). In addition to these proteins, CF I_m68 can also be detected in Upf3b-containing mRNPs even in the presence of RNaseA, suggesting that it must interact with at least one of the non-RNA components of the complex. As expected, hnRNPA1, which is an abundant component of heterogeneous nuclear RNP (hnRNP) complexes but not of postsplicing complexes (Kim *et al.*, 2001), is not present in the pellet of the coimmunoprecipitation.

Because export of CF I_m68 is not mediated by CRM1 but instead requires mRNA transcription, we asked whether NXF1 might be the export receptor for CF I_m68. Control Flag-tagged CF I_m25, -NXF1, -Upf1, -Upf3b, or the empty Flag vector alone were transiently coexpressed with HA-tagged CF I_m68 in HEK293 cells. Interaction with CF I_m68 was analyzed by immunoprecipitation of the cell lysates with anti-Flag antibodies in the presence of RNase A followed by Western blotting with anti-CF I_m68 antiserum. The expression level of Flag-NXF1 was significantly lower than that of the other Flag-tagged proteins, and especially of Flag-Upf1 (Figure 3B, input, cf. lanes 4 and 5). Nevertheless, the amount of CF I_m68 associated with Flag-NXF1 was far greater than that obtained with either Flag-Upf3b or Flag alone, and equivalent to the amount coprecipitated with Flag-Upf1 (pellet, cf. lanes 8–11). These results suggest that CF I_m68 interacts specifically with NXF1 in vivo, and possibly also with Upf1. Thus, to determine if the interaction between CF I_m68 and NXF1 is a direct one, we performed GST pulldown experiments with purified recombinant proteins. *E. coli*–expressed GST, control GST-CF I_m25, or GST-NXF1 were incubated with purified baculovirus-expressed, histidine-tagged CF I_m68. Bound proteins were analyzed by Western blotting using anti-histidine (Figure 3C). Because GST–NXF1 coprecipitated equivalent amount of CF I_m68 to that coprecipitated by CF I_m25 (cf. lanes 2 and 3), we conclude that CF I_m68 interacts specifically with the mRNA export factor NXF1.

We next aimed to identify the region in CF I_m68 responsible for the interaction with NXF1. We tested the interaction

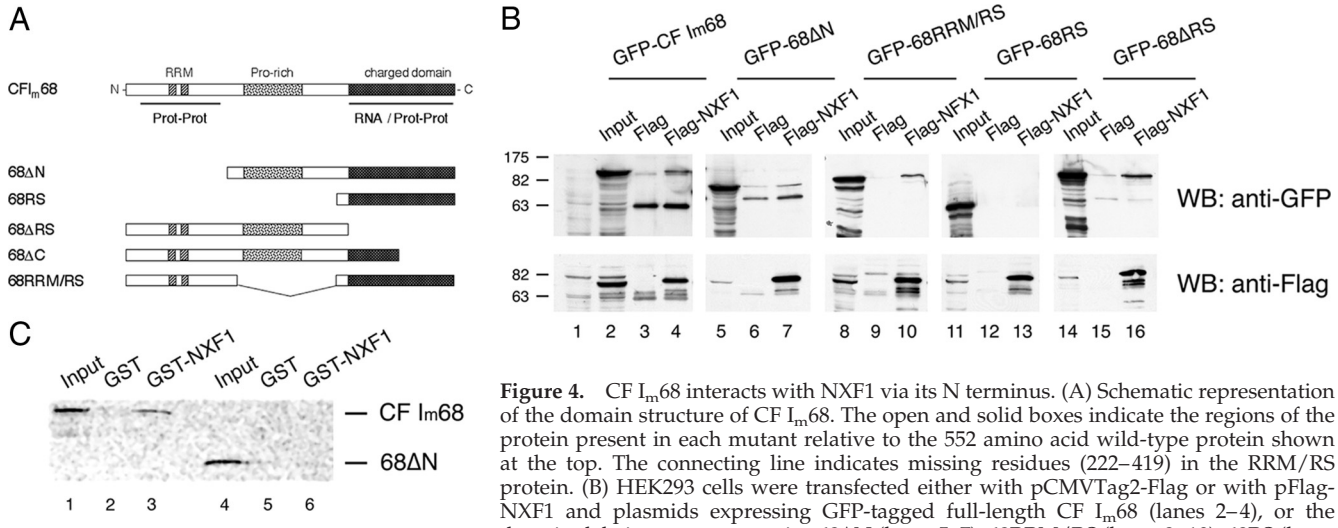

Figure 4. CF I_m68 interacts with NXF1 via its N terminus. (A) Schematic representation of the domain structure of CF I_m68. The open and solid boxes indicate the regions of the protein present in each mutant relative to the 552 amino acid wild-type protein shown at the top. The connecting line indicates missing residues (222–419) in the RRM/RS protein. (B) HEK293 cells were transfected either with pCMVTag2-Flag or with pFlag-NXF1 and plasmids expressing GFP-tagged full-length CF I_m68 (lanes 2–4), or the domain deletion mutant proteins 68ΔN (lanes 5–7), 68RRM/RS (lanes 8–10), 68RS (lanes 11–13), and 68ΔRS (lanes 14–16). Total extracts were immunoprecipitated with anti-Flag antibody and analyzed by Western blotting. Lane 1, extract from untransfected cells. (C) *E. coli*–expressed GST or GST-NXF1 prebound to beads were individually incubated with in vitro–translated, ^{35}S-labeled HA-tagged CF I_m68 or 68ΔN. Bound fractions (lanes 2, 3, 5, and 6) were subjected to SDS-PAGE and analyzed by autoradiography.

by coimmunoprecipitation of Flag-tagged NXF1 with GFP fusions of wild-type and mutant CF I_m68 that were coexpressed in HEK293 cells (Figure 4B). Tested mutants included a deletion of the N-terminal portion of CFI_m68, a region that contains the RRM and was shown to bind the 25-kDa subunit (68ΔN), a deletion of the entire RS-like region at the C-terminus (68ΔRS), the RS-like domain alone (68RS), and a fusion of the RRM and the RS domains (68RRM/RS). As shown in Figure 4B Flag-NXF1 precipitated the fragments 68RRM/RS and 68ΔRS. Deletion of the N-terminal portion impaired the interaction with NXF1, whereas the RS-like domain appeared to interact with NXF1 albeit very inefficiently. Similar results were obtained in GST pulldown experiments in which recombinant GST-CF I_m68 truncations were incubated with Flag-NXF1 expressed in HEK293 cells (data not shown).

To confirm the requirement of the N-terminal region of CF I_m68 for the direct interaction with NXF1, in vitro binding assays were performed. Glutathione beads prebound with GST or GST-NXF1 were incubated with ^{35}S-labeled CF I_m68 or 68ΔN. Bound fractions were resolved and visualized by autoradiography. As shown in Figure 4C, only the full-length protein, but not the N-terminally deleted one, bound NXF1, indicating that the NXF1 interaction region lies within the first 213 amino acids of CF I_m68.

NXF1 can be functionally divided into three domains (Figure 5A; Izaurralde, 2002): the N-terminal half (aa 1-372) interacts with REF (Stutz *et al.*, 2000); the region between aa 371 and 551 binds the essential export cofactor p15 (Katahira *et al.*, 1999; Guzik *et al.*, 2001; Izaurralde, 2002); and the C-terminal domain interacts with components of the NPC (Bachi *et al.*, 2000). Shuttling SR proteins interact with the

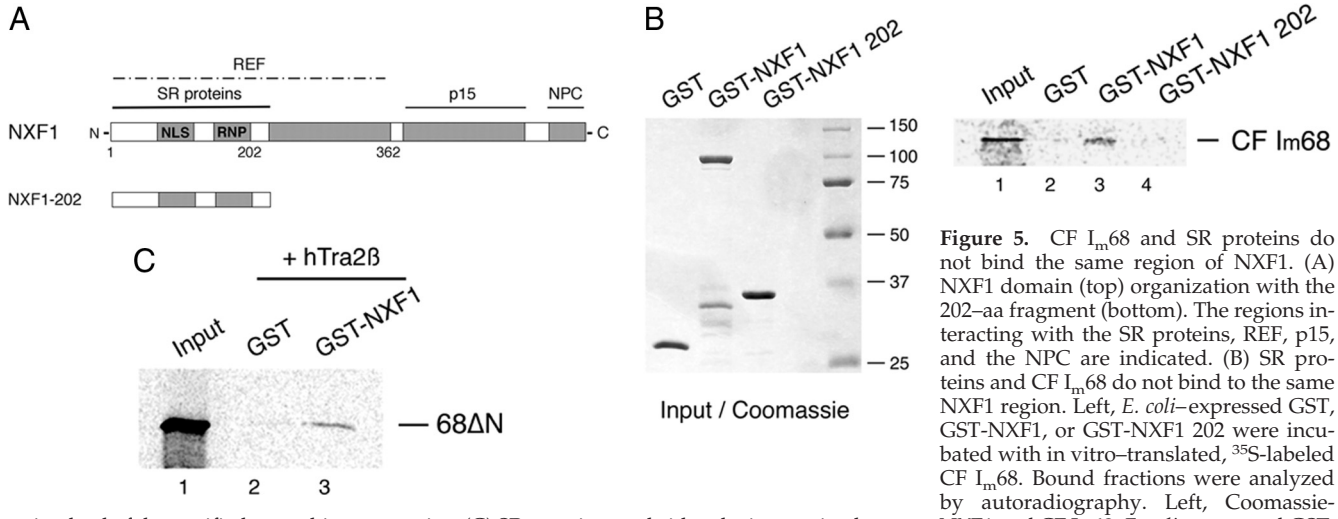

Figure 5. CF I_m68 and SR proteins do not bind the same region of NXF1. (A) NXF1 domain (top) organization with the 202–aa fragment (bottom). The regions interacting with the SR proteins, REF, p15, and the NPC are indicated. (B) SR proteins and CF I_m68 do not bind to the same NXF1 region. Left, *E. coli*–expressed GST, GST-NXF1, or GST-NXF1 202 were incubated with in vitro–translated, ^{35}S-labeled CF I_m68. Bound fractions were analyzed by autoradiography. Left, Coomassie-stained gel of the purified recombinant proteins. (C) SR proteins can bridge the interaction between NXF1 and CF I_m68. *E. coli*–expressed GST, or GST-NXF1 were incubated with in vitro–translated, ^{35}S-labeled CF I_m68ΔN in the presence of recombinant, histidine-tagged hTra2ß as described in *Materials and Methods.* Bound fractions were analyzed by autoradiography.

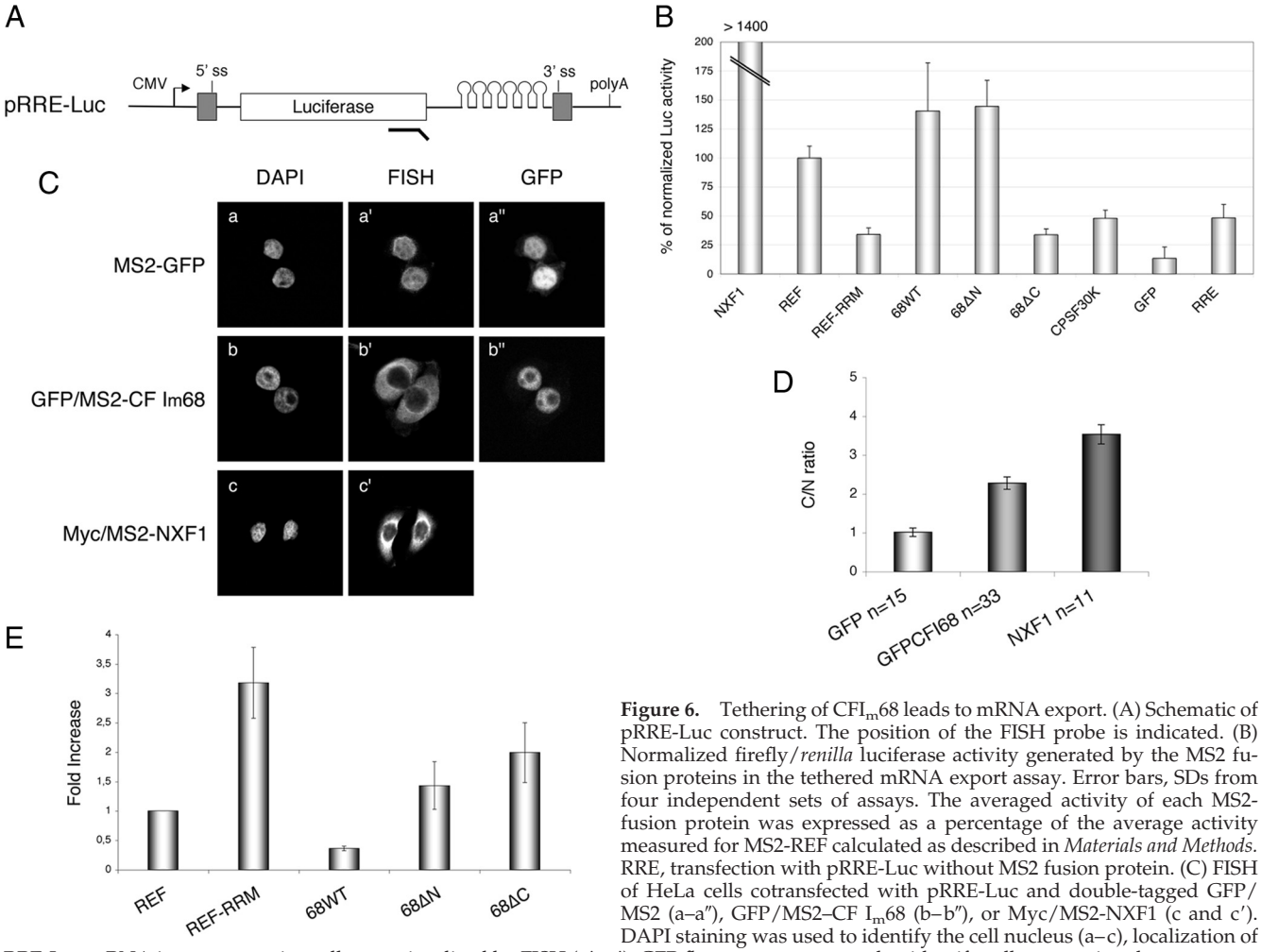

Figure 6. Tethering of CFI_m68 leads to mRNA export. (A) Schematic of pRRE-Luc construct. The position of the FISH probe is indicated. (B) Normalized firefly/*renilla* luciferase activity generated by the MS2 fusion proteins in the tethered mRNA export assay. Error bars, SDs from four independent sets of assays. The averaged activity of each MS2-fusion protein was expressed as a percentage of the average activity measured for MS2-REF calculated as described in *Materials and Methods*. RRE, transfection with pRRE-Luc without MS2 fusion protein. (C) FISH of HeLa cells cotransfected with pRRE-Luc and double-tagged GFP/MS2 (a–a″), GFP/MS2–CF I_m68 (b–b″), or Myc/MS2-NXF1 (c and c′). DAPI staining was used to identify the cell nucleus (a–c), localization of RRE-Luc mRNA in representative cells was visualized by FISH (a′–c′); GFP fluorescence was used to identify cells expressing the exogenous protein (a″ and b″). (D) The average cytoplasmic to nuclear (C/N) ratio for the RRE-Luc mRNA was determined by quantitative analysis of FISH experiments as shown in C for the indicated number of cells per construct. Error bars, SDs. (E) Real-time PCR analysis of the relative amount of RRE-Luc mRNA in the nuclear fractions of cells transfected with the indicated MS2-fusions constructs as determined by real-time RT-PCR. Three experiments were performed and average values are shown.

same domain of NXF1 that binds REF (Huang *et al.*, 2003). To determine if CF I_m68 and SR proteins bind the same domain of NXF1, glutathione beads loaded with GST or GST-NXF1 were individually incubated with in vitro–translated, ^{35}S-labeled, HA-tagged CF I_m68 or 68ΔN. As shown in Figure 5B only full-length NXF1, but not the first 202 aa, which bind shuttling SR proteins (Huang *et al.*, 2003 and data not shown), interacts with CF I_m68.

We previously demonstrated that the C-terminal charged domain of CF I_m68 interacts with shuttling SR proteins (Dettwiler *et al.*, 2004). Thus, we tested whether the C-terminal region of CF I_m68 may mediate the formation of a ternary complex between SR proteins and NXF1. In the presence of the SR protein hTra2ß, GST-NXF1 can indeed efficiently select ^{35}S-labeled 68ΔN, which on its own did not bind NXF1 (cf. Figures 4C and 5C).

CF I_m68 Stimulates mRNA Export

To test if CF I_m68 may contribute to general mRNA export through the interaction with the receptor protein NXF1 (as is the case for REF and some SR proteins), we used a recently described, tethered mRNA export assay (Hargous *et al.*, 2006). In this assay, an mRNA export factor is expressed as a fusion protein with a bacteriophage MS2 coat protein tag and then artificially tethered to a reporter RNA containing the luciferase ORF and six MS2 coat protein–binding sites within an inefficiently spliced intron (Figure 6A). The binding of the export factor leads to the nuclear export of the unspliced RNA that would otherwise be retained in the nucleus, resulting in the expression of luciferase activity. We therefore checked whether tethering CF I_m68 protein in this way would promote export of the reporter RNA. Vectors expressing the different MS2 fusion proteins were transfected into HEK293 cells together with the luciferase reporter construct. Cotransfection of a third vector encoding *renilla* luciferase was used to control the transfection efficiency. As controls, MS2 fusions of GFP, NXF1, REF, and REF-RRM (aa 71–155; Hargous *et al.*, 2006) were tested in the same experiments. As seen in Figure 6B, direct tethering of NXF1 resulted in a very strong luciferase activity. Importantly, tethering of CF I_m68 led to a luciferase expression that was ~10 times lower than that elicited by NXF1, but still higher than that of REF. As expected, REF-RRM did not stimulate export of the RNA Control experiments revealed

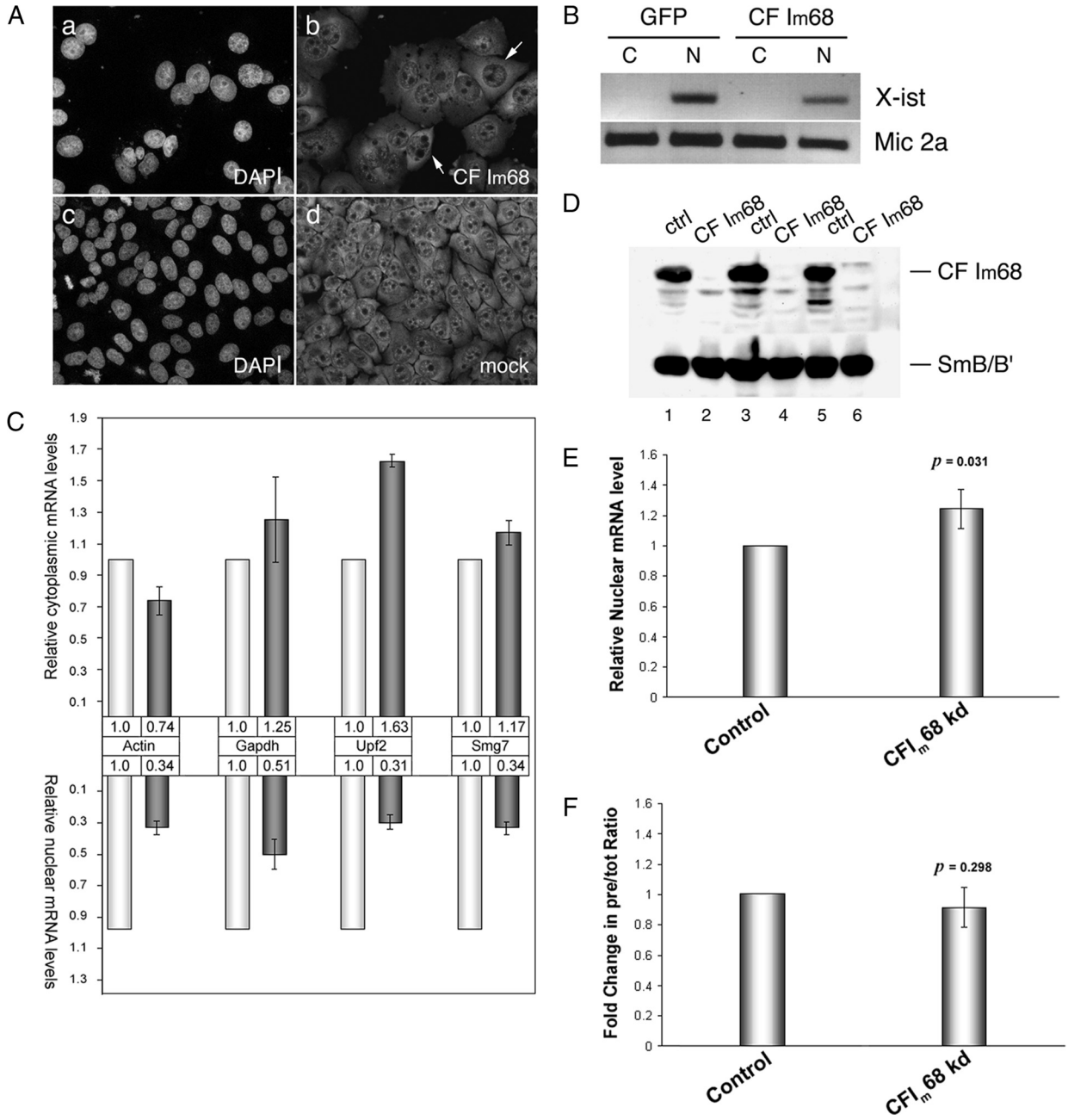

Figure 7. CF I_m68 promotes export of mRNAs. (A) Hela cells growing on coverslips, overexpressing HA-tagged CFI_m68 and a puromycin resistance as well as control cells expressing only the puromycin resistance were fixed with formaldehyde and permeabilized with methanol. Polyadenylated mRNA was visualized by hybridization of a Cy3-labeled oligo(dT)50 probe, whereas the nuclei were stained with DAPI. The panels are projections of Z-stacks from top to bottom of the nuclei. Images were collected on a Leica TCS SP2 AOBS laser scanning confocal microscope equipped with a HCX PL APOlbd.BL 63.0× 1.2W objective (Leica Microsystems). (B) PCR analysis of nuclear and cytoplasmic RNAs of the nuclear X-ist mRNA (top) and the X-encoded Mic 2A transcript (bottom). (C) Overxpression of CF I_m68 stimulates mRNA export. Real-time PCR analysis of the relative amount of ß-actin, Gapdh, Upf2, and Smg7 mRNA levels in the cytoplasmic (top histogram) and nuclear (bottom histogram) fraction of cells expressing HA-tagged EGFP (□) or HA-tagged CFI_m68 (▨). The values were normalized to 18S rRNA levels and then divided by the values obtained in cells expressing the EGFP control. Three independent experiments were performed and average values of three real-time PCR runs with cDNAs from one representative experiment are shown. (D) HeLa cells stably expressing ß-globin mRNA were transfected with a control siRNA or with siRNAs against CFI_m68. Total extract was prepared and analyzed by Western blotting with anti-CFI_m68 and with anti-SmB/B′ antibodies. Three different experiments are shown. (E) CFI_m68 depletion increases nuclear levels of ß-globin mRNA. Real-time RT-PCR analysis of the relative amount of ß-globin mRNA in the nuclear fraction of HeLa cells transfected with a control siRNA or cells depleted of CFI_m68. The indicated values are normalized to 18S rRNA levels. Three experiments were performed and average values are shown. (F) CFI_m68 depletion does not affect pre-mRNA 3′ end processing. Real-time RT-PCR of ß-actin mRNA was performed with probes spanning the 5′UTR or the cleavage site to measure total and unprocessed mRNA, respectively. The ratio of precursor to total mRNA (pre/tot) was calculated to assess the efficiency of 3′ end processing. The processing activity of cells transfected with the control siRNA was set to 1.

that all the MS2 fusion proteins were expressed (Western blot; Supplemental Figure S3) and correctly localized to the cell nucleus (immunofluorescence microscopy, data not shown).

Mutant CF I_m68 proteins were also tested by this assay. Deletion of the N-terminal domain (68ΔN) that is necessary for the direct interaction with NXF1 in vitro did not significantly affect mRNA export, most likely because the protein can still interact with NXF1 via SR protein(s) bound to the C-terminus (see above). In contrast, the deletion of 2/3 of the C-terminal RS motif (68ΔC) strongly reduced luciferase expression. However, because this fragment is known to be imported into the nucleus inefficiently (Dettwiler *et al.*, 2004), this result must be interpreted with caution. In further control experiments, the 30- and the 73-kDa subunits of the cleavage and polyadenylation specificity factor CPSF had no effect when tethered to the reporter RNA (Figure 6B and data not shown, respectively).

The assay described above relies on the measurement of luciferase enzymatic activity and thus requires mRNA translation. To rule out that CF I_m68 might affect the translation efficiency rather than RNA export, we sought to measure directly nuclear and cytoplasmic mRNA levels in the presence of varying amounts of CF I_m68. First, steady-state pRRE-Luc mRNA levels were visualized by FISH; Figure 6C). Quantitation of FISH images for individual cells revealed that the cytoplasmic to nuclear (C/N) ratio of the FISH signal was ~1 for the GFP control, 2.28 for GFP-CF I_m68, and 3.54 for NXF1 (Figure 6D). Additionally, we carried out quantitative reverse-transcription PCR (qPCR) analysis of the relative amount of luciferase mRNA in the nuclear fractions of cells transfected with different MS2-fusion constructs (Figure 6E). Quantitation of the relative nuclear luciferase RNA levels showed that tethering of MS2-CF I_m68 decreased the amount of transcript in the nucleus by ~2.5-fold compared with MS2-REF, whereas the two CF I_m68 deletion mutants (68ΔN and 68ΔC) were less effective in promoting mRNA export. The nuclear fractions were devoid of cytoplasmic contaminations as shown by Western blot analyses for lamin A/C (nuclear) and tyrosine tubulin (cytoplasmic; Supplemental Figure S4). Taken together, these experiments indicate that tethering of CF I_m68 can promote mRNA export.

To further characterize this new function of CF I_m68, we investigated the effect of its overexpression on the export of endogenous mRNAs. HeLa cells were transfected with a selectable plasmid driving CF I_m68 expression under the CMV promoter, and the nucleo-cytoplasmic distribution of polyadenylated RNAs was examined by FISH with a Cy3-labeled oligo(dT) probe (Figure 7A). In cells overexpressing CF I_m68, a slight but appreciable reduction of the amount of nuclear polyadenylated RNA could be observed. To analyze the effect of CF I_m68 overexpression on the nucleocytoplasmic transport of specific mRNAs, nuclear and cytoplasmic fractions were prepared, and RNA was isolated from these extracts. RT-PCR for the nuclear X-inactivation specific (X-ist) mRNA revealed that no nuclear RNA had leaked into the cytoplasmic fractions (Figure 7B). The amounts of four different mRNAs were then measured by qPCR. They are shown in Figure 7C as relative values normalized to the amounts of the same transcripts measured in cells overexpressing HA-EGFP. Importantly, the nuclear concentration of each of these transcripts was reduced two- to threefold, whereas cytoplasmic levels were increased for three of them, with the exception of ß-actin. Nevertheless, even for ß-actin mRNA, the cytoplasmic to nuclear ratio increased ~2.2-fold compared with the control, indicating that CF I_m68 overexpression stimulates mRNA export also in this case.

We also determined the effect of CF I_m68 depletion on mRNA export. Silencing experiments were performed in HeLa cells stably transfected with the ß-globin gene lacking natural introns and therefore expressing cDNA transcripts that do not undergo splicing (Valencia *et al.*, 2008). The assay was validated by depleting NXF1. Two days after transfection of specific siRNAs or of a control siRNA, mRNA levels were analyzed by real-time RT-PCR. NXF1 mRNA was reduced by ≈92% compared with control siRNA-treated cells, and, as a consequence, the level of the nuclear ß-globin mRNA was increased ~2.5 times (Supplemental Figure S5). In the case of CF I_m68, 4 d after transfection of two specific siRNAs, CF I_m68 mRNA was reduced by ≈93% compared with control siRNA-treated cells, whereas the level of the mRNA of the closely related 59-kDa subunit remained unchanged (data not shown). Western blot analysis (Figure 7D) confirmed that the level of the remaining CF I_m68 protein was below detection. Importantly, this silencing of CF I_m68 resulted in a small but statistically significant increase in the nuclear level of ß-globin mRNA (Figure 7E). As above, the purity of these nuclear fractions was assessed by Western blot analyses for lamin A/C and tyrosine tubulin (Supplemental Figure S4). This result supports the idea that CF I_m68 may act in mRNA export, similarly to SR proteins and/or REF, by contributing to the recruitment of NXF1 to the mRNA.

Another variable that could potentially affect the nucleocytoplasmic distribution of mRNAs is inefficient 3′ end processing that would lead to nuclear retention of the unprocessed transcript. However, CF I_m68 depletion did not significantly affect the 3′ end processing efficiency of neither endogenous ß-actin pre-mRNA (Figure 7F) nor of the ß-globin cDNA transcript, which has the strong polyadenylation signal of bovine growth hormone (BGH, data not shown).

In summary, the above experiments indicate a relevant biological role for CF I_m68 in the export of mRNAs.

CF I_m68 Cosediments with Ribosomal Particles

Some of the components of the EJC, including REF and NXF1, dissociate from the mRNA upon entry into the cytoplasm (Le Hir *et al.*, 2001). Therefore, it is possible that CF I_m68 is present in the mRNPs in the nucleus but may then dissociate during or shortly after mRNA export. To gain insight into the remodeling of CF I_m68-containing mRNPs after export, cytoplasmic fractions of HEK293 cells were fractionated across 15–50% sucrose gradients, and the distribution of CF I_m68 was determined by Western blotting. Figure 8A shows that most of the cytoplasmic CF I_m68 was found in lighter complexes at the top of the gradient. However, CF I_m68 could also be detected in the 80S (monosome) region, although to a lesser extent than ASF/SF2. CF I_m68 distribution correlated with that of the nuclear cap-binding complex component CBP80 (Figure 8B). Treatment of cytoplasmic extracts with RNaseA, which induces dissociation of mono- and polyribosomes into ribosomal subunits, led to a redistribution of CF I_m68, SF2/ASF, and rpS6 to the top of the gradient (Figure 8C). Likewise, we found that cosedimentation of CF I_m68 with 80S particles was sensitive to EDTA (data not shown). In contrast, treatment with puromycin, which causes premature termination and thus the disassembly of translating heavy polysomes and an increase of the 80S peak (Sabatini *et al.*, 1971) did not affect CF I_m68 distribution (Supplemental Figure S6). We conclude that CF I_m68 is not associated with translating ribosomes but, like

CBP80 and PABP2, is part of the mRNP particle that is remodeled in the cytoplasm during the initial stages of translation when nuclear proteins are replaced by their cytoplasmic counterparts (Ishigaki *et al.,* 2001; Dostie and Dreyfuss, 2002).

DISCUSSION

Although stimulation of mRNA export by polyadenylation has long been observed, so far little is known about the possible mechanism. In this report we provide first evidence that the 68-kDa subunit of CF I_m, a pre-mRNA 3′ end processing factor, interacts with the mRNA export receptor NXF1 and stimulates mRNA export. On the basis of our observations and on previous data showing that CF I_m68 is associated with BrdU-labeled nascent transcripts (Cardinale *et al.,* 2007), we propose that CF I_m68 is loaded onto the pre-mRNA during cleavage and polyadenylation of the 3′ end of the transcript and remains bound to the mRNA all the way to the cytoplasm where it is removed by the translation machinery. CF I_m68 may thus act as a mark of correct 3′ end maturation and contribute to efficient mRNA export via NXF1 recruitment.

Role of RNA-binding Shuttling Proteins in mRNA Export

Several RNA binding proteins exhibit shuttling activity including the splicing factor U2AF, the polyadenylation factor PABPN1, and members of the hnRNP and SR protein families (for review see Gama-Carvalho and Carmo-Fonseca, 2001). Although U2AF, PABPN1, and the hnRNP proteins

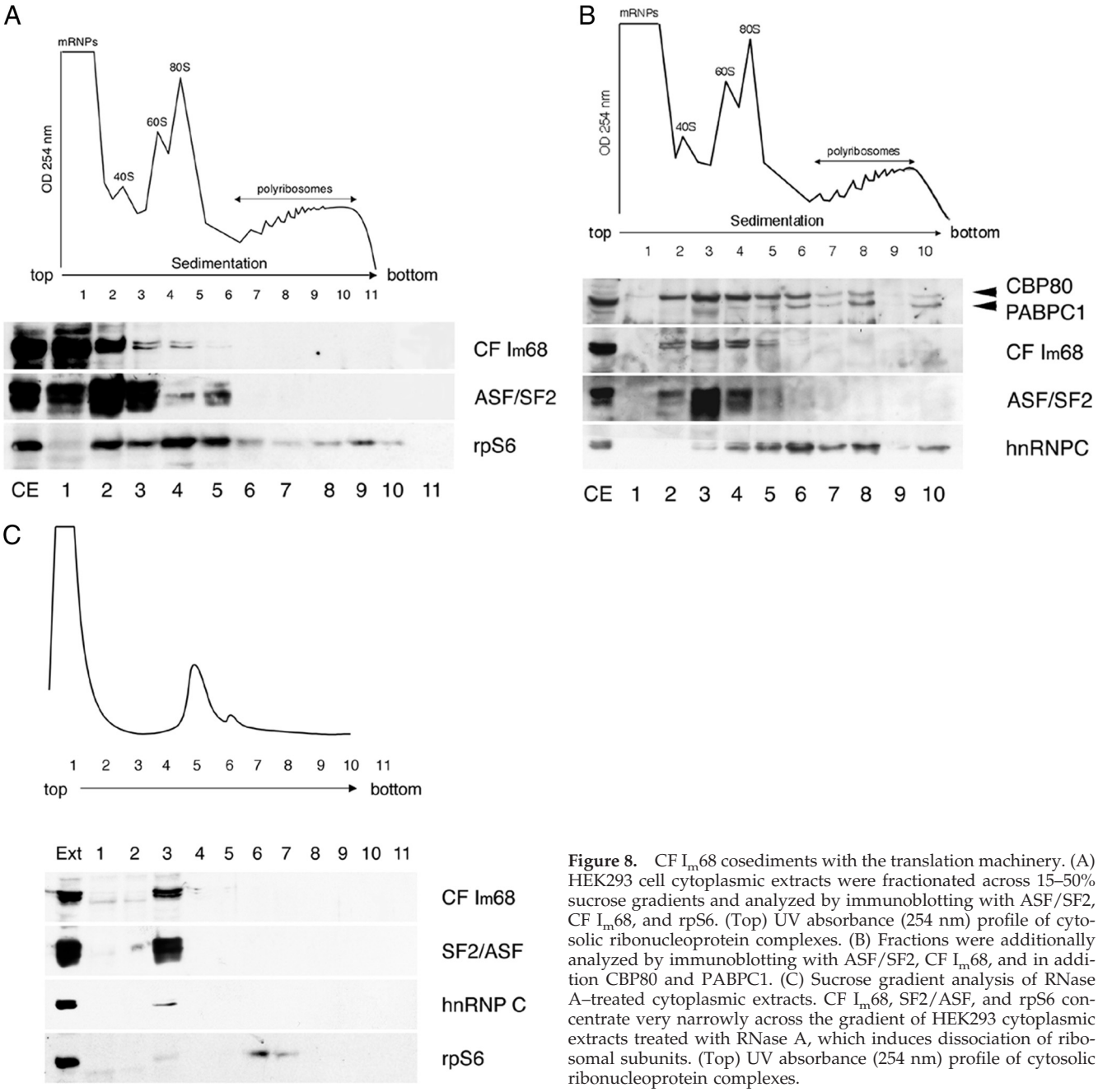

Figure 8. CF I_m68 cosediments with the translation machinery. (A) HEK293 cell cytoplasmic extracts were fractionated across 15–50% sucrose gradients and analyzed by immunoblotting with ASF/SF2, CF I_m68, and rpS6. (Top) UV absorbance (254 nm) profile of cytosolic ribonucleoprotein complexes. (B) Fractions were additionally analyzed by immunoblotting with ASF/SF2, CF I_m68, and in addition CBP80 and PABPC1. (C) Sucrose gradient analysis of RNase A–treated cytoplasmic extracts. CF I_m68, SF2/ASF, and rpS6 concentrate very narrowly across the gradient of HEK293 cytoplasmic extracts treated with RNase A, which induces dissociation of ribosomal subunits. (Top) UV absorbance (254 nm) profile of cytosolic ribonucleoprotein complexes.

were shown to shuttle actively and independently of mRNA traffic, shuttling SR proteins are exported bound to the mRNA. Similar to SR proteins, CF I_m shuttling activity depends on active transcription, suggesting that it leaves the nucleus in association with the mRNA. Consistent with this observation both CF I_m subunits contact the RNA substrate, as demonstrated by UV cross-linking studies (Rüegsegger *et al.*, 1996).

What is the role of shuttling proteins in mRNA export? Shuttling RNA-binding proteins may contribute to the assembly of an export competent mRNP by recruiting mRNA export factors. Consistent with this view U2AF 35-kDa subunit (Zolotukhin *et al.*, 2002), several shuttling SR proteins (Huang *et al.*, 2003; Lai and Tarn, 2004), and now CF I_m have been shown to interact with the mRNA export receptor NXF1. In addition, shuttling RNA-binding proteins may fulfill different roles in the nucleus and in the cytoplasm. This is the case for instance for the shuttling SR protein ASF/SF2 that, besides its well-characterized role in basal and regulated splicing, is involved in mRNA surveillance and in translation (Sanford *et al.*, 2004; Zhang and Krainer, 2004).

Mechanisms for the Recruitment of the mRNA Export Receptor NXF1

In *S. cerevisiae* where only 5% of the genes contain introns, export factor recruitment is coupled to transcription and 3′ end formation rather than to intron splicing. The interaction between the mRNA and the heterodimeric export receptor Mex67/Mtr2 (NXF1/p15 in metazoa) is provided by the RNA-binding protein Yra1 (REF). Yra1 interacts with the transcription elongation complex (THO complex, Strasser *et al.*, 2002; Abruzzi *et al.*, 2004) and requires 3′ end formation to be recruited cotranscriptionally to mRNAs (Lei and Silver, 2002). How the yeast pre-mRNA 3′ end processing complex participates in the recruitment of mRNA export factors is, however, still unknown.

In metazoans, the current model for the recruitment of export factors on the mRNA proposes that adaptor proteins such as REF and the export receptor NXF1 are "deposited" after splicing as components of the EJC (for review see Stutz and Izaurralde, 2003). REF is recruited by UAP56 that binds cotranscriptionally. Before export, NXF1/p15 would bind to REF and thereby release UAP56. However, only NXF1, p15, and UAP56 have been shown so far to be essential for this process (Tan *et al.*, 2000; Herold *et al.*, 2001; Wilkie *et al.*, 2001; Gatfield and Izaurralde, 2002; Wiegand *et al.*, 2002). Recently, an EJC-independent mechanism was proposed for the recruitment of REF based on the observation that UAP56 and REF bind at the 5′ end of the mRNA and interact with the cap-binding complex. In this new model recruitment occurs via an interaction between REF and the cap-binding protein CBP80 (Cheng *et al.*, 2006). The observation that depletion of REF in *Drosophila* cells does not lead to accumulation of poly(A)RNA in the nucleus (Gatfield and Izaurralde, 2002) suggests that alternative mechanisms must exist for the recruitment of NXF1 on mature mRNAs. Consistent with this idea, the shuttling SR proteins SRp20,

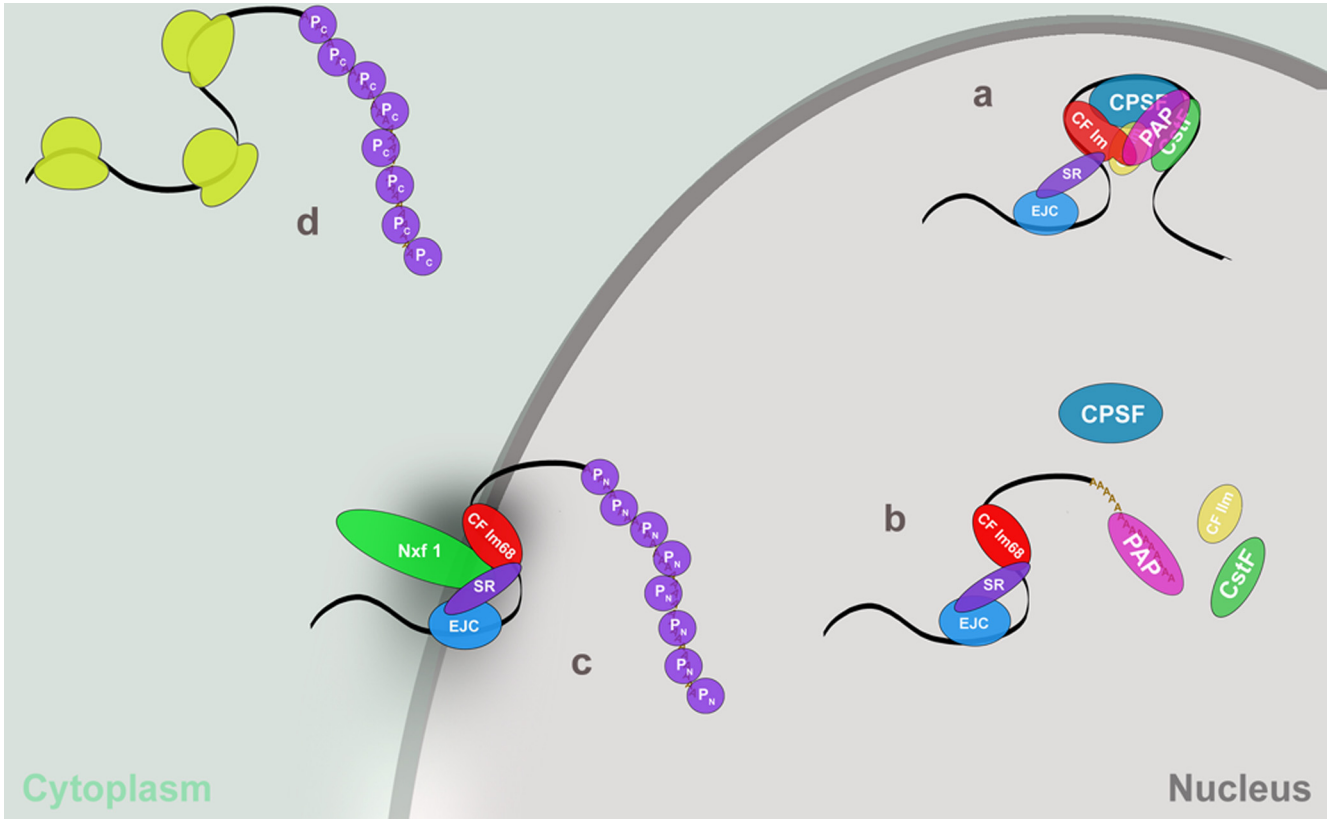

Figure 9. Model of potential role of CF I_m68 in assembly and export of mRNPs. (a) CF I_m is recruited to the pre-mRNA as part of the cleavage and polyadenylation complex. (b) Once correct 3′ end processing has occurred, CF I_m68 remains associated with the mRNA and (c) in conjunction with SR proteins contributes to the recruitment of the export receptor NXF1. (d) In the cytoplasm, the translating ribosomes remove the nuclear proteins that are reimported into the nucleus.

9G8, and ASF/SF2 that can bind to the pre-mRNA independently of the EJC, were shown to act as NXF1-adaptor proteins. Here, we have demonstrated that CF I_m68 interacts with NXF1 and contributes to mRNA export most likely in conjunction with SR proteins. The presence of CF I_m68 may signal that the mRNA has been properly cleaved and polyadenylated (Figure 9) and may be packaged into an export-competent mRNA ribonucleoprotein particle (mRNP) that can recruit the export receptor. This novel observation provides for the first time a molecular connection between 3′ end formation and mRNA export.

In contrast to NXF1, CF I_m68 does not appear to dissociate from the mRNA upon entry into the cytoplasm. Instead, sucrose gradient centrifugation shows that CF I_m68 cosediments with the 80S ribosome. According to current models, the first passage of the 80S ribosome on an mRNA (called "pioneer round" of translation) leads to a major rearrangement of the mRNP composition whereby the remaining nuclear mRNP components are removed and replaced by cytoplasmic factors (for review see Maquat, 2004; Moore, 2005). Thus, our findings that CFI_m68 is released from the mRNP at this stage may have interesting implications not only for mRNA export and translation initiation but also for mRNA quality control mechanisms. For example, the presence of CF I_m bound downstream of a stop codon may contribute to the definition of the proper termination codon in metazoa. Further studies on the role of the factors that bind the 3′ end of the mRNA should provide new important insight into the mechanism that defines premature stop codons and leads to NMD.

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