

# Nup214-Nup88 Nucleoporin Subcomplex Is Required for CRM1-mediated 60 S Preribosomal Nuclear Export\*

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The nuclear pore complex (NPC) conducts macromolecular transport to and from the nucleus and provides a kinetic/hydrophobic barrier composed of phenylalanine-glycine (FG) repeats. Nuclear transport is achieved through permeation of this barrier by transport receptors. The transport receptor CRM1 facilitates export of a large variety of cargoes. Export of the preribosomal 60 S subunit follows this pathway through the adaptor protein NMD3. Using RNA interference, we depleted two FG-containing cytoplasmically oriented NPC complexes, Nup214-Nup88 and Nup358, and investigated CRM1-mediated export. A dramatic defect in NMD3-mediated export of preribosomes was found in Nup214-Nup88-depleted cells, whereas only minor export defects were evident in other CRM1 cargoes or upon depletion of Nup358. We show that the large C-terminal FG domain of Nup214 is not accessible to freely diffusing molecules from the nucleus, indicating that it does not conduct 60 S preribosomes through the NPC. Consistently, derivatives of Nup214 lacking the FG-repeat domain rescued the 60 S export defect. We show that the coiled-coil region of Nup214 is sufficient for 60 S nuclear export, coinciding with recruitment of Nup88 to the NPC. Our data indicate that Nup214 plays independent roles in NPC function by participating in the kinetic/hydrophobic barrier through its FG-rich domain and by enabling NPC gating through association with Nup88.

Selective communication between the nucleus and the cytoplasm in eukaryotes occurs through nuclear pore complexes (NPC),<sup>5</sup> multiprotein assemblies that transverse the nuclear envelope (NE) (1–3). Each NPC is composed of ~30 proteins, collectively termed nucleoporins (4), and displays an 8-fold horizontal rotational symmetry in relation to the NE (5). The

general shape of the NPC is conserved from yeast to humans (4, 6, 7), but individual nucleoporins differ widely in sequence. A large subset of nucleoporins contains long phenylalanine-glycine dipeptide-containing domains (FG repeats), which are thought to form a hydrophobic/kinetic meshwork creating a barrier to most macromolecules while allowing passage of transport receptor complexes (2, 8). These complexes are thought to pass the NPC by interacting with FG repeats, thus permeating the NPC core (8–11). To achieve nuclear transport, proteins and RNAs bind transport-competent receptors either directly or indirectly via adaptor proteins (12).

Directionality of transport through the NPC is determined by the Ran GTP/GDP gradient, which exists between the nucleus and the cytoplasm (13–15), and possibly by the presence of specific high affinity binding sites for transport receptors located at either the nuclear or cytoplasmic faces of the NPC (16). Although the general NPC architecture is symmetric, the localization of several nucleoporins is restricted to either the nuclear or the cytoplasmic face of the NPC (3), supporting the idea that nucleocytoplasmic asymmetry might be established by asymmetric distribution of specific binding sites at the NPC. Indeed, preferential interactions between several transport receptors and specific FG-containing nucleoporins have been described *in vitro* (3). In contrast, recent studies in yeast show that receptor-mediated nuclear transport is not affected when FG repeats of asymmetric nucleoporins are absent (17, 18), indicating that they are not essential for directional transport and that the FG-domains of different nucleoporins may be functionally redundant. Furthermore, imaging of single molecule translocation through the NPC shows that the most kinetically important interactions during nuclear translocation take place in the central pore and that these interactions exhibit the characteristics of unbiased diffusion, indicating that there is no directionality within the NPC itself (19).

Next to permeation, gating is a second proposed mode of energy-dependent NPC translocation. Gating involves conformational changes of the NPC to achieve transport (20) and is supported by conformational states that have been detected under various conditions (21–24). The role of individual nucleoporins in this process is unknown.

The Nup214-Nup88 subcomplex is localized to the cytoplasmic face of the NPC (25, 26). Nup214 is dispensable for *in vitro* NPC assembly and protein import (27), but it is essential in vertebrate cells, and its depletion causes a strong mRNA export defect (28). Nup214 contains two central coiled coils known to interact with Nup88 (25, 29) and a long C-terminal FG repeat that interacts strongly with the transport factor CRM1 *in vitro*

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<sup>5</sup> The abbreviations used are: NPC, nuclear pore complex; NE, nuclear envelope; NES, nuclear export signal; NLS, nuclear localization signal; mAb, monoclonal antibody(s); hNup, human nucleoporin; GFP, green fluorescent protein; eGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; RNAi, RNA interference; shRNA, short hairpin RNA; FKBP, FK506-binding protein; FRB, FKB-rapamycin-binding domain; HA, hemagglutinin.

in a RanGTP- and cargo-stimulated fashion (29–31). These data suggest that Nup214 plays an essential role in CRM1-mediated export. However, it remains to be elucidated as to whether and how NPC asymmetry influences transport processes that are initiated on the opposite side of the NPC. Recent studies proposed that the FG-rich domain of the asymmetric nuclear Nup153 and cytoplasmic Nup214 can cross the NPC providing a binding site to transport receptors and escorting transport complexes through the NPC (32–34).

CRM1 mediates the nuclear export of proteins bearing a nuclear export signal (NES) by binding cooperatively with RanGTP (35–38). The nature of NES-containing cargoes differs widely. The large subunit of the ribosome is exported to the cytoplasm via the CRM1 pathway, assisted by the transport adaptor NMD3 (39–42).

We have compared the roles of the Nup214-Nup88 and Nup358 complexes in different CRM1 export pathways. We show that the CRM1-mediated export of the 60 S ribosomal subunit is dependent on the Nup214-Nup88 subcomplex, whereas that of small NES cargoes remains relatively unaffected. We show that the central domain of Nup214 is required for 60 S export and Nup88 targeting to the NPC, whereas the FG repeats are dispensable.

## EXPERIMENTAL PROCEDURES

**Antibodies**—Anti-hNup358/RanBP2 antiserum, anti-hNup358V, and anti-hNup358F were generously provided by V. Cordes (Karolinska Institute, Stockholm, Sweden) and A. Gast and F. Melchior (Max Planck Institute for Biochemistry, Munich, Germany), respectively. Antibodies to Nup214 (43) anti-hNup88 (BD Transduction Laboratories), monoclonal antibody (mAb) 414 (Eurogentec/Babco), and anti-HA (12CA5) were described previously.

**Plasmid Construction**—pSUPER-358 (43), pSUPER-214, Rev-S1-GFP (44), and HA-Nup153 (45) were described previously. The NLS-eGFP-NES insert containing the SV40 nuclear localization signal (NLS) and protein kinase A inhibitor NES was subcloned from pBSSK (46) into the pcDNA3 vector (Stratagene) using the HindIII-NotI restriction sites. The NLS-eGFP insert was amplified by PCR from pBSSK using the forward primer 5'-CCCCTCGAGGTCGACGGTATC-3' and a reverse primer containing a NotI site, 5'-ATATATATAGCGGCCGCTTAGTTTCTAGACTTGTACAGCTC-3', and subcloned into pcDNA3 by digestion with HindIII and NotI. GFP-NMD3 and rpL29-GFP were a kind gift from U. Kutay (40). To create a RNAi-insensitive Nup214 expressing plasmid, DpnI-mediated site-directed mutagenesis was performed on pBluescriptKS(-)CAN (47) creating four silent mutations in the target sequence TCACATCCGCTAGCAACAC. Wild type and mutated Nup214 coding sequences were subcloned into the EcoRI sites of pcDNA3 (Stratagene). A DNA oligo, which contained AgeI, SacII, and FseI sites, was cloned into the RNAi-insensitive Nup214 XcmI sites, located at positions 6157 and 6253 of the open reading frame, leading to the parental construct. The AgeI and FseI sites were used to perform unidirectional deletions using the ExoIII/S1 deletion kit (Fermentas). To create Nup214-FRB, the FRB domain lacking the HA1 tag

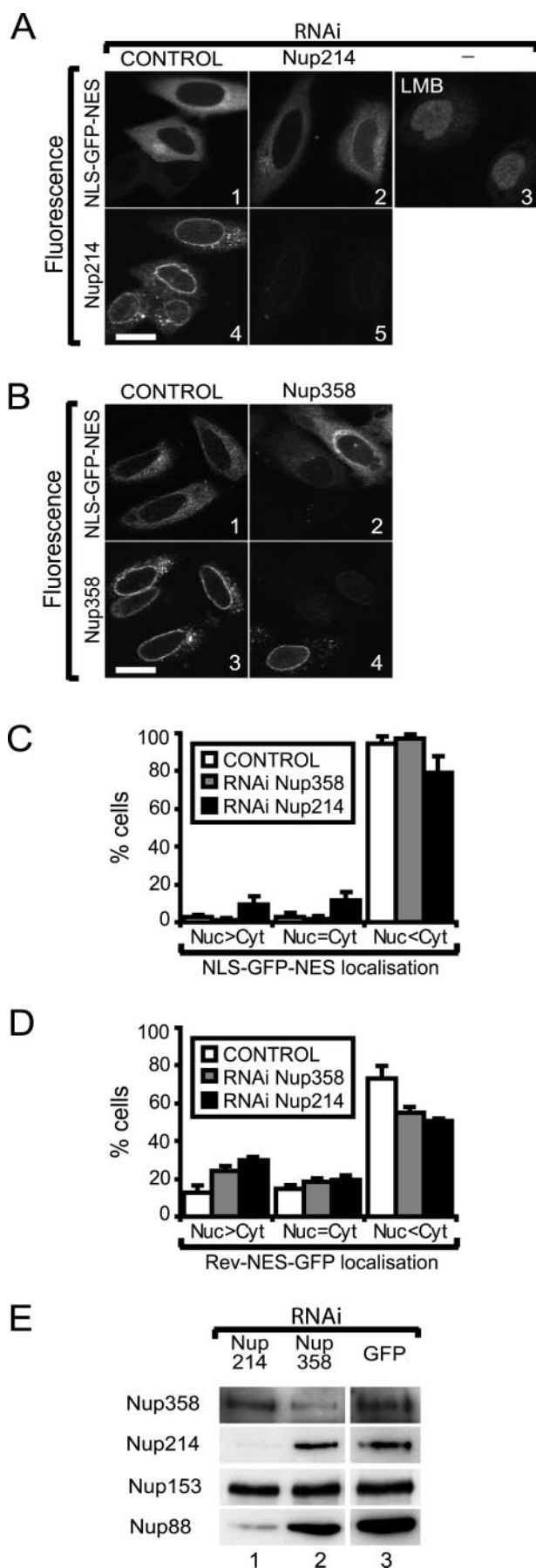
from the plasmid pC<sub>4</sub>-R<sub>H</sub>E (regulated heterodimerization kit, Argent) was PCR-amplified and cloned in-frame into the parental construct using AgeI and SacII sites. To create pcDNA3 HA-Nup214-(585–832), HA-Nup214-(804–1058), and HA-Nup214-(585–1058), Nup214 regions were PCR-amplified on pBluescriptKS(-)CAN (47) and cloned into pcDNA3-HA (48). FKBP lacking the HA1 tag and SV40 NLS was PCR-amplified from the plasmid pC<sub>4</sub>EN-F1E (regulated heterodimerization kit, Argent) to clone into pRev(1.4)-GFP (49) using BamHI and AgeI sites and into GST-NLS-GFP from plasmid pEW103 (kind gift of Erik Wiemer, Erasmus University, Rotterdam, The Netherlands) using BsrGI and SacII sites. All constructs were sequenced for confirmation.

**Cell Culture and Transfections**—Low passage HeLa and MCF-7 cells (ATCC CCL-2) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics at 37 °C and 5% CO<sub>2</sub> in a humidified incubator. Transfections were performed using electroporation on MCF-7 cells as described previously (50) or FuGENE 6 (Roche Applied Science) on HeLa cells according to the manufacturer's instructions. For all RNAi assays in HeLa cells, experiments proceeded for 72 h; the amount of pSUPER plasmids transfected was 106 ng/cm<sup>2</sup> on 70–80% confluent cells. pSUPER-GFP was a generous gift from Rene Bernards, The Netherlands Cancer Institute, Amsterdam. For immunofluorescence experiments, NLS-GFP, NLS-GFP-NES, and GFP-NMD3 were co-transfected at a maximum of 47 ng/cm<sup>2</sup>, pRev-NES-GFP as described (43), pRev-S1-GFP at 21.7 ng/cm<sup>2</sup>, rpL29-GFP at 1.2 ng/cm<sup>2</sup>, pRev-FKBP-GFP at 1 ng/cm<sup>2</sup>, and GST-NLS-GFP-FKBP at 2.3 ng/cm<sup>2</sup>. For all rescue experiments pcDNA3-derived plasmids were co-transfected at 5.3 ng/cm<sup>2</sup> except for the heterodimerization assays, where Nup214-FRB was co-transfected at 7.3 ng/cm<sup>2</sup> maximum. For Western blot analysis, pcDNA3 and pcDNA3-HA-derived plasmids were co-transfected at 26.5 ng/cm<sup>2</sup>. The non-immunosuppressive rapalog AP21967 (regulated heterodimerization kit, Argent) was used at 500 nM for 3 h prior to fixation except for Nup214 overexpression, which was at 250 nM. Leptomycin B was used for 2 h at a concentration of 100 nM.

**Immunofluorescence Staining and Image Analysis**—Indirect immunofluorescence was performed as described previously (43). Images were recorded with Leica TCS NT2 and SP2 confocal microscopes and analyzed using ImageJ software. For subcellular distribution studies, a minimum of 100 cells/condition were scored. The presence of Nup214 at the NE (Fig. 3A, Nup214 expression) was defined as positive when rim intensities were 3-fold higher than average cytoplasmic intensities as determined using Image J software. Nup88 NE staining analysis was performed as described previously (43) on 25 cells/sample.

## RESULTS

**The Nup214-Nup88 Subcomplex Is Dispensable for Basic NES-mediated Nuclear export**—The strong *in vitro* interaction between Nup214 and CRM1 (29–31), suggests that this nucleoporin has an important stimulatory role in NES-mediated nuclear export (16, 30, 31). To test for this possibility, we depleted Nup214 by expression of Nup214-specific shRNAs in human cells and recorded the nucleocytoplasmic localization

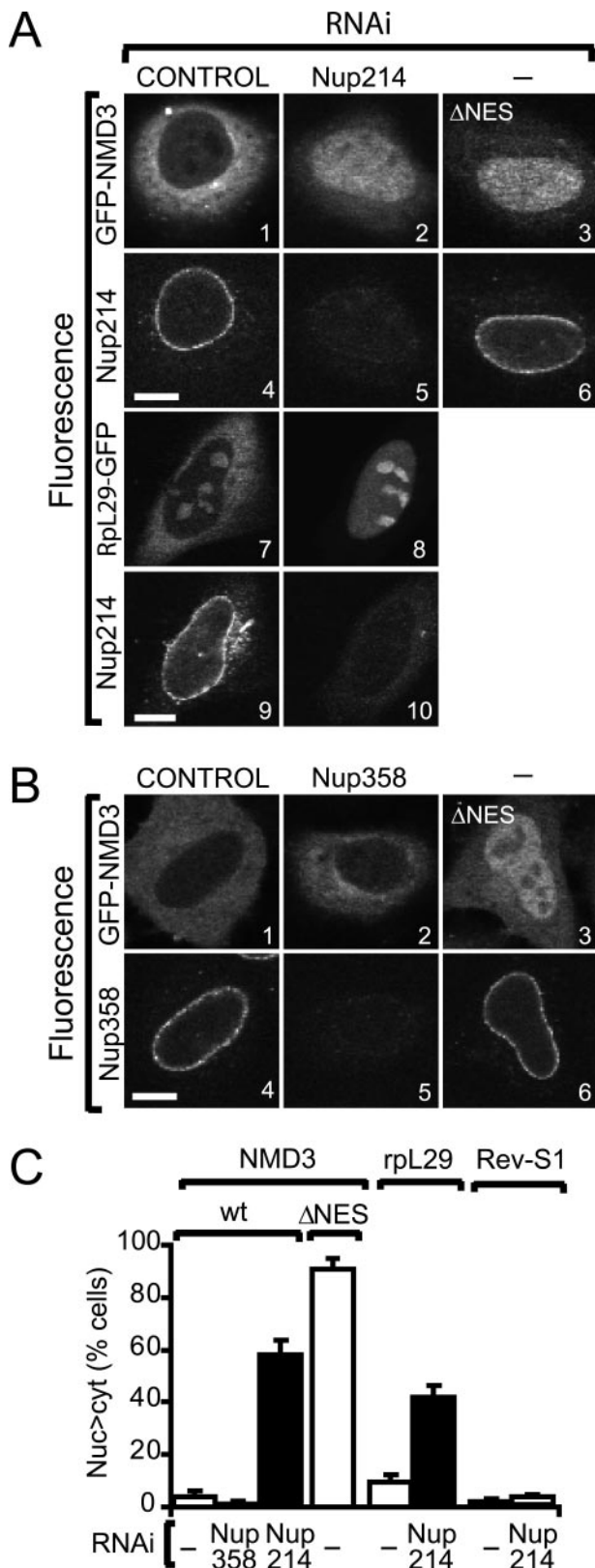


of NES-reporter proteins. To confirm efficient depletion, Western blot analyses were performed on knocked down cells lysates. As shown in Fig. 1E, Nup214-shRNA resulted in strong depletion of Nup214 (lane 1), whereas shRNA directed to Nup358 (lane 2) or GFP (lane 3) had no effect. As expected from previous studies, knockdown of Nup214 caused a strong depletion of Nup88, indicating that the stability of these two nucleoporins is co-dependent (43). We first tested a NES-reporter protein consisting of the NES derived from protein kinase A inhibitor (51) and fused to GFP. The import activity of this protein is provided by an SV40 NLS. In control cells, this reporter protein is excluded from the nuclei, indicating that the NES is active and prevails over the NLS activity. Nuclear accumulation of this protein was detectable when cells were treated with the CRM1 inhibitor leptomycin B, indicating that NLS-GFP-NES is exported via CRM1 pathway (Fig. 1, A3). Energy depletion of transfected cells by chilling on ice resulted in an even distribution of the protein between the nucleus and the cytoplasm, indicating that the reporter protein is small enough to slowly diffuse through the NPC (data not shown). Surprisingly, depletion of the Nup214-Nup88 subcomplex caused only a small export defect (Fig. 1, A and C). Nuclear export of the NES-GFP-NLS reporter protein in cells depleted of Nup358 was unaffected (Fig. 1, B and C). We have previously shown that depletion of Nup358 causes a small reduction in export of a Rev(1.4)-GFP-NES reporter protein (43), which is targeted to the cytoplasm and sensitive to leptomycin B (44, 49). In addition to an NLS, the Rev(1.4) protein also provides nuclear retention activity which permits a more stringent assessment of nuclear export. We repeated the above experiments using this reporter protein. In this case, depletion of either Nup358 or the Nup214-Nup88 subcomplex resulted in a moderate reduction of export efficiency (Fig. 1D). As the Nup214-Nup88 subcomplex is required for anchoring of Nup358 to the NPC (43), the reduction of nuclear export by depletion of Nup214-Nup88 may be largely attributable to co-depletion of Nup358 from the NPC. We concluded that the Nup214-Nup88 subcomplex does not have a major role in NES-dependent nuclear export of these cargoes.

*The Nup214-Nup88 Subcomplex Is Required for CRM1-mediated 60 S Preribosome Export*—Considering the discrepancy between the strong binding *in vitro* between CRM1 and Nup214 and the weak effects of Nup214 depletion on NES-mediated

**FIGURE 1. Wild type levels of the Nup214-Nup88 subcomplex are not required for basic NES-mediated nuclear export.** Shown is the subcellular distribution of the NES reporter proteins NLS-GFP-NES (A–C) and Rev(1.4)-NES-GFP (D) depleted for Nup214-Nup88 (A, C, and D) or Nup358 (B, C, and D) in HeLa and MCF-7 cells. Cells were immunolabeled with anti-hNup214 (A2 and A4) and anti-hNup358F (B2 and B4) primary antibodies and Texas Red-labeled secondary antibodies. Scale bars represent 20  $\mu$ m. C and D, quantification of the subcellular distribution of NLS-GFP-NES and Rev(1.4)-NES-GFP on knocked down cells. 100 fluorescent cells/condition were scored for predominantly nuclear (Nuc>Cyt), equal (Nuc=Cyt), or predominantly cytoplasmic (Nuc<Cyt) GFP fluorescence intensity. The mean distribution is shown, and error bars represent standard errors. E, Western blot of HeLa cells transfected with pS-Nup214 (lane 1), pS-Nup358 (lane 2), and pS-GFP (lane 3). Blots were probed for Nup358, Nup214, Nup153, and Nup88 using anti-Nup358V, anti-hNup214, mAb 414, and anti-hNup88, respectively. Note that knock-down protein levels are an underestimate of true knock-down efficiency because of incomplete targeting of the cell population by transient transfection, especially noticeable for Nup358.





**FIGURE 2. The Nup214-Nup88 subcomplex is required for CRM1-mediated 60 S preribosome export.** A and B, subcellular distribution of GFP-NMD3 and Rpl29-GFP expressing HeLa cells 72 h after co-transfection with pSUPER control (A, panels 1 and 4, 7 and 9, and B, panels 1 and 2) or shRNA expression plasmids targeting Nup214 (A, panels 2 and 5, 8 and 10) or Nup358 (B, B4, B5, and B6). Cells were fluorescently labeled with anti-hNup214 (A4, A5, A6, A9, A10) and anti-hNup358 (B4, B5, and B6) primary antibodies and Texas Red-labeled secondary antibodies. HeLa cells expressing GFP-NMD3<sup>ΔNES</sup> represent maximum nuclear accumulation (A3 and B3). Scale bars, 10  $\mu$ m. C, quantification of results illustrated in A and B showing percentages of cells presenting nuclear accumulation (Nuc>cyt) of GFP-NMD3, GFP-NMD3<sup>ΔNES</sup>, and Rpl29-GFP as well as those for the supraphysiological NES reporter, Rev(1.4)-GFP-S1. Error bars represent standard errors. wt, wild type.

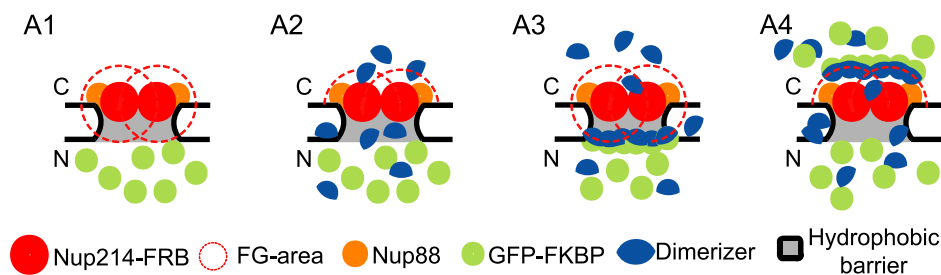
export *in vivo*, we hypothesized that Nup214 might be required for certain classes of CRM1-dependent nuclear export substrates. It has been shown previously that in vertebrates, the large 60 S preribosome subunit is exported via CRM1 and the transport adaptor NMD3 (40, 42). We therefore investigated the role of the Nup214-Nup88 subcomplex in 60 S preribosomal nuclear export by studying the localization of GFP-tagged NMD3 (40).

In control cells, GFP-NMD3 was largely excluded from the nucleus (Fig. 2, A and C). This cytoplasmic localization of NMD3 was strictly dependent on its NES (Fig. 2, A and C). Depletion of the Nup214-Nup88 subcomplex resulted in a striking nuclear redistribution of GFP-NMD3 (Fig. 2, A and C). In contrast, Nup358-deficient cells showed no difference from the control (Fig. 2, B and C). The localization of the NMD3<sup>ΔNES</sup> remained unchanged in Nup214-shRNA- or Nup358-shRNA-expressing cells, indicating that NMD3 nuclear import was not impaired by depletion of the Nup214-Nup88 subcomplex or Nup358 (data not shown). To confirm that nuclear accumulation of GFP-NMD3 reflected a 60 S preribosome export defect, the localization of the preribosomal component rpl29 was assessed in wild type or Nup214-Nup88-depleted cells. Apart from the accumulation at the nucleoli, which is also observed in control cells, depletion of Nup214-Nup88 resulted in a strong nuclear accumulation of GFP-tagged rpl29 (Fig. 2, A, panels 8 and 10, and C). *In vitro*, certain recombinant versions of NMD3 possess a very high affinity for CRM1 (40), ~100-fold higher than regular NESs. To test whether the export defect due to depletion of Nup214-Nup88 was specific for high affinity NESs, we determined the nuclear export driven by an NES of a similar affinity, the supraphysiological S1 NES (44). Depletion of Nup214-Nup88 did not induce nuclear accumulation of this reporter protein, indicating that the effects were not related to high affinity CRM1 binding of NMD3 (Fig. 2C).

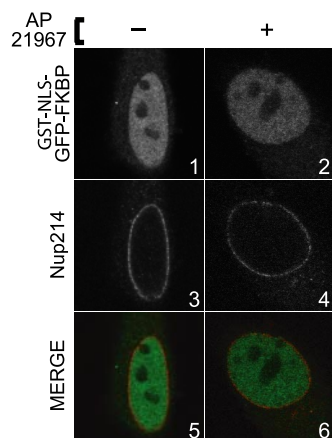
**The FG-repeat Domain of Nup214 Cannot Access the Nucleus**—It has been proposed recently that the FG-domain of Nup214 could access the nuclear compartment providing a binding site for export complexes. This mechanism would explain how a cytoplasmic localized nucleoporin can mediate export (33, 34). To examine this possibility (Fig. 3, A1), we have tested accessibility of the C-terminal domain of Nup214 to the nuclear compartment *in vivo*. For this, we used a rapamycin-dependent heterodimerizer system (52). The small (95 amino acids) FRB protein, one of the two heterodimerizing components, was fused to the C terminus of the RNAi-insensitive Nup214. The other heterodimerizing component, FKBP, was fused to either GST-NLS-GFP or the NES-deficient Rev(1.4)-GFP, two constitutively nuclear proteins that cannot freely diffuse across the NPC. As depicted in Fig. 3A, rapamycin-dependent dimerization would occur only if the C-terminal FRB-containing domain of Nup214 could reach the nuclear compartment.

Expression of Nup214-FRB rescued the effects of shRNA-induced Nup214-Nup88 depletion in Nup214 expression

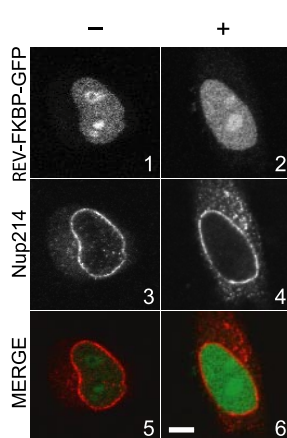
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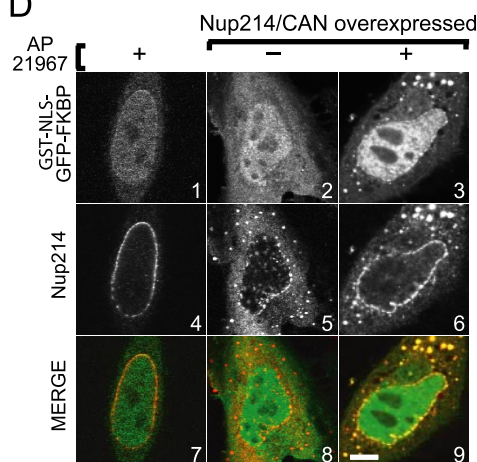
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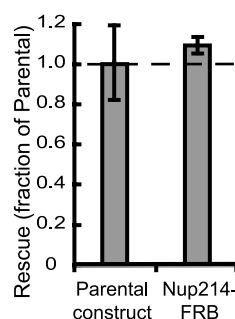
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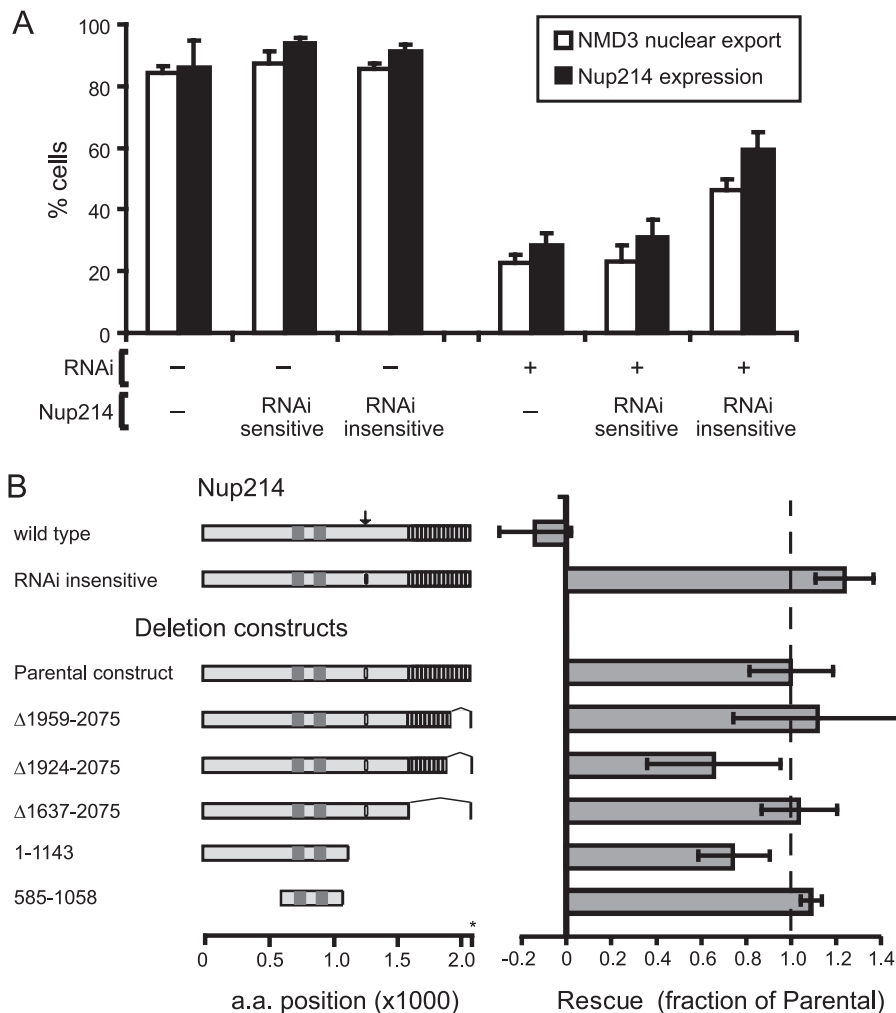
**FIGURE 3. The FG-repeat domain of Nup214 does not access the nucleus.** A, schematic representation of a system to assess the nuclear presence of the FG-repeat domain of Nup214. Nucleoporins Nup214-FRB and Nup88 are represented as red and orange circles, respectively. The dashed red circle around Nup214 represents the approximate radius of an unstructured Nup214 FG-repeat domain to access the nucleus. After the addition of the rapamycin dimerizer (blue ovals in A2), nuclear GFP-FKBP (green circles) would accumulate at the NE only if the Nup214 C terminus has access to the nucleus (A3). Cytoplasmic GFP-FKBP would accumulate at the NE if the FG-repeat domain of Nup214 has access to the cytoplasm (A4). N, nucleus; C, cytoplasm. B–D, subcellular localization of GST-NLS-GFP-FKBP (B and D) or Rev(1.4)-FKBP-GFP (C) after knockdown of endogenous Nup214 and exogenous expression of Nup214-FRB. Note that in D, panels 1–3, GST-NLS-GFP-FKBP is present in the nucleus and cytoplasm. Nup214 is detected by anti-hNup214 and Texas Red-labeled secondary antibody. Rapamycin-derived heterodimerizer (AP21967) was added as indicated. Scale bars, 10  $\mu$ m. E, FRB-tagged Nup214 is active in promoting 60S preribosomal export. Rescue of GFP-NMD3 nuclear export in Nup214-depleted cells by parental (left) or FRB-fused Nup214 proteins. Methods used are as described in the legend for Fig. 4.

(Fig. 3, B, panels 3 and 4, and C, panels 3 and 4) and NMD3 nuclear export (Fig. 3E). This indicates that the Nup214-FRB RNAi-insensitive derivative of Nup214 is correctly expressed, targeted to the NE, and functional. Previous stud-

ies have shown that a fusion protein consisting of a FRB and Rev NES is small enough ( $\sim 11.5$  kDa) and capable of diffusing freely through the NPC, indicating that the FRB component would not prevent the capacity of the Nup214 C terminus to cross the NPC (53). When Nup214-FRB and GST-NLS-GFP-FKBP were highly overexpressed (Fig. 3D, panels 1–6), the two proteins colocalized in cytoplasmic dots in a rapamycin-dependent manner (Fig. 3D, panels 2 and 3, 5 and 6, and 8 and 9), providing a control for rapamycin-induced heterodimerization. Furthermore, small amounts of cytoplasmic GST-NLS-GFP-FKBP were sufficient to induce a visible colocalization with Nup214 (Fig. 3D, panels 1, 4, and 7). However, no rapamycin-induced heterodimerization was detected when the nuclear reporter proteins were confined to the nucleus, using either GST-NLS-GFP-FKBP or Rev(1.4)-GFP-FKBP protein (Fig. 3, B, panels 2, 4, and 6, and C, 2, 4, and 6). These results indicate that the FG-repeat domain of Nup214 is not able to access the nuclear compartment from its cytoplasmic site.

**Nup214 FG Repeats Are Dispensable for 60S Preribosomal Nuclear Export**—To determine which region of Nup214 was required for preribosomal nuclear export, we designed several deletion constructs of Nup214 and expressed them in Nup214-Nup88-depleted cells. To ensure expression of the reintroduced Nup214 proteins, we designed four silent point mutations in the nucleotide sequence that is targeted by the Nup214 shRNA. To determine the extent of rescue that can be obtained in this setup, we transfected HeLa cells expressing GFP-NMD3 under normal or Nup214-Nup88-depleted conditions with plasmids expressing either RNAi-sensitive

or -insensitive Nup214 (Fig. 4A). In control cells, efficient nuclear export of NMD3 was found in 84% of the cells, and 86% of the cells showed a clear NE staining of Nup214. These scores were not significantly altered when wild type or



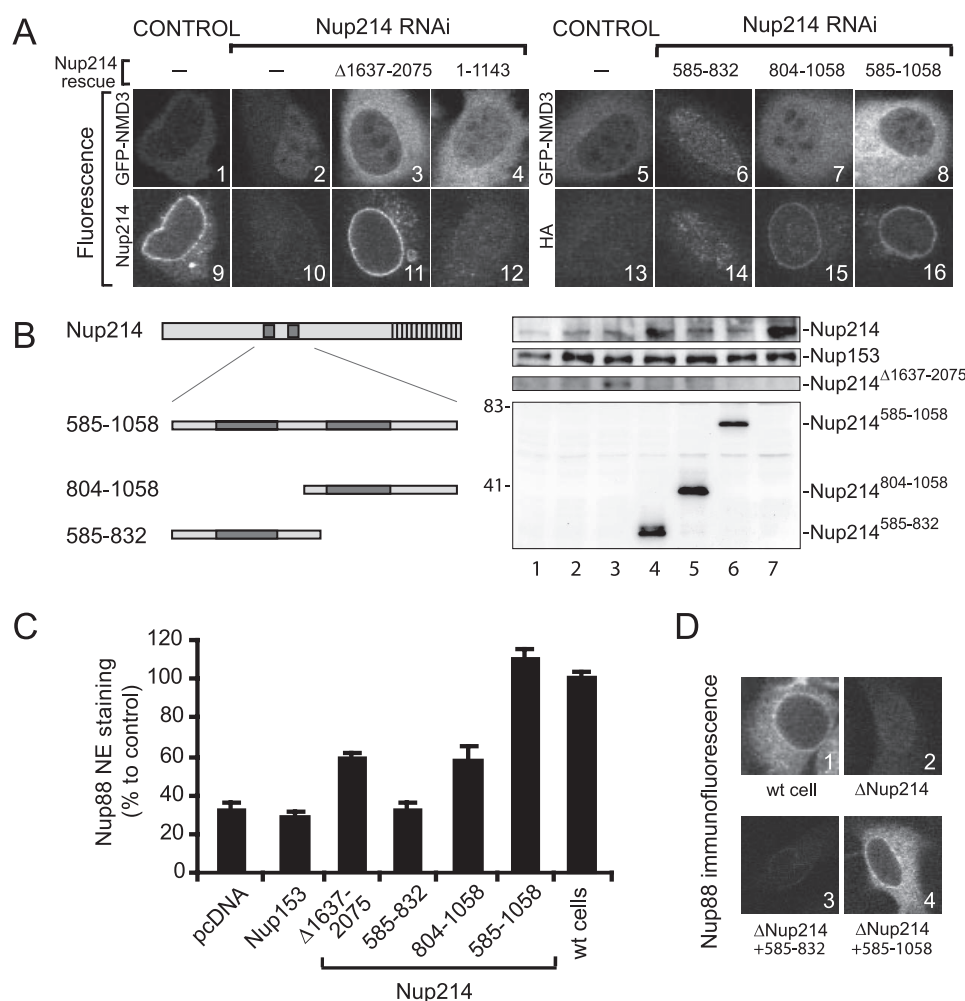
**FIGURE 4. Nup214 FG repeats are dispensable for 60S preribosomal nuclear export.** *A*, rescue of GFP-NMD3 nuclear export and Nup214 expression in Nup214-Nup88-depleted HeLa cells by exogenous Nup214. HeLa cells were transfected with Nup214-shRNA expression plasmids (*right*) or control plasmids (*left*) and co-transfected with RNAi-sensitive or -insensitive versions of a Nup214 expression plasmid as indicated below the graph. White bars represent the percentage of cells showing cytoplasmic GFP-NMD3 staining greater or equal to nuclear staining. Black bars represent the percentages of cells showing Nup214 staining at the nuclear envelope. Error bars represent standard errors. *B*, rescue of GFP-NMD3 nuclear export in Nup214-Nup88-depleted HeLa cells by Nup214 deletion mutants. Nup214 deletion constructs are represented as horizontal bars. Dark boxes, indicate central coiled-coil domains; vertical bars, FG repeats; arrow, RNAi target. A black oval denotes a mutated RNAi target. Amino acid (a.a.) positions of Nup214 are shown at the bottom (asterisk). Bars graph at the right show the percentage of rescue obtained for each construct relative to the parental deletion construct (dashed line). Error bars represent standard error.

RNAi-insensitive Nup214 was exogenously expressed. Upon depletion of Nup214-Nup88, only 23% of the cells showed efficient nuclear export of GFP-NMD3. Concomitantly, the presence of Nup214 at the NE was reduced to 28% of cells. Neither GFP-NMD3 export nor the expression of Nup214 was significantly enhanced when an RNAi-sensitive Nup214 mRNA was overexpressed, indicating that the exogenous Nup214 transcript was recognized and degraded by the RNAi machinery. In contrast, when the RNAi-insensitive version was reintroduced, 46% of the cells were able to export GFP-NMD3. This coincided with a significant increase of Nup214 expression (Fig. 4A). These data indicated specific rescue of shRNA-mediated depletion of Nup214-Nup88 by exogenous Nup214 DNA constructs and defined the dynamic range of the assay to be roughly from 25 to 50% of wild type.

We next tested GFP-NMD3 nuclear export of Nup214-Nup88-depleted cells that lacked FG repeats to a varying extent (Fig. 4B). These derivatives were created by ExoIII deletion from a parental construct that had a small deletion from amino acid position 2055–2076 of the Nup214 sequence. This parental construct was able to rescue NMD3 export to levels comparable with the full-length rescue plasmid. Interestingly, most FG-repeat deletions rescued to similar levels as the parental construct. Two constructs encoding Nup214 versions lacking the entire FG-repeat domain (Nup214(Δ1637–2075) and Nup214(1–1143)), which contains the CRM1 binding site, were not significantly perturbed in their capacity to rescue NMD3 export. We conclude that Nup214, but not its FG-repeat region, is essential for 60S preribosomal export.

**Nup214 Central Coiled-coil Domains Are Sufficient for 60S Preribosomal Nuclear Export**—Thus far, our data have indicated that the Nup214 FG domain cannot access the nuclear compartment. In addition, this region seems dispensable for 60S preribosomal export. These facts suggest that Nup214 does not interact directly with the 60S export complex. To further test this possibility, we expressed three versions of the central Nup214 coiled-coil domains. These domains are required to mediate interaction with Nup88 and with the NPC (54, 55). Incorporation of the HA1-tagged coiled-coil domains into the NPC was analyzed by immunofluorescence and confocal microscopy imaging in Nup214-depleted HeLa cells. As shown previously (54), although the first and second Nup214 coiled coils showed no or low NE staining (Fig. 5A, panels 14 and 15, respectively), the protein containing both domains was targeted to the NE very efficiently (Fig. 5A, panel 16). Analogously, Nup88 NE staining was found as high as wild type levels only when the construct containing both coiled coils of Nup214 was expressed (Fig. 5, C and D). Next, we analyzed NMD3 export by confocal microscopy imaging. Although cells expressing the first or second coiled-coil domains (Fig. 5B) of Nup214 elicited no or little rescue on NMD3 export assays (Fig. 5A, panels 6 and 7), cells expressing the complete central domain (residues 585–1058) rescued NMD3 export capacity to the same extent as the Nup214 RNAi-insensitive construct (Figs. 4B and 5A, panel 8). Western blot analysis of HeLa cell extracts expressing shRNAi targeting Nup214 and co-trans-





**FIGURE 5. Nup214 central coiled-coil domains are sufficient for 60 S preribosomal nuclear export.** *A*, subcellular distribution of GFP-NMD3 expressing HeLa cells 72 h after co-transfection with pSUPER control (panels 1 and 9, 5 and 13) or pS-Nup214; and with empty pcDNA (panels 2 and 10) or Nup214 rescue plasmids expressing Nup214-Δ1637-2075 (panels 3 and 11), Nup214-1-1143 (panels 4 and 12), Nup214-585-832 (panels 6 and 14), Nup214-804-1058 (panels 7 and 15), and Nup214-585-1058 (panels 8 and 16). Cells were fluorescently labeled with anti-hNup214 (panels 9 and 12) and anti-HA 12CA5 (panels 13 and 16) primary antibodies and Texas Red-labeled secondary antibodies. *B*, Western blot of HeLa cells transfected with pSUPER empty (lane 7) or pS-Nup214 (lanes 1–6). HA-Nup153 (lane 2), Nup214-Δ1637-2075 (lane 3), Nup214-585-832 (lane 4), Nup214-804-1058 (lane 5) and Nup214-585-1058 (lane 6) were co-transfected. Blots were probed for Nup214 and Nup153 using mAb 414, for Nup214-Δ1637-2075 using anti-hNup214, and for HA1 using 12CA5. *C*, Nup214 coiled-coil region is sufficient to target Nup88 to the NPC. This is a graphic representation of fluorescence levels of endogenous Nup88 as a percentage of the empty pSUPER negative control after knockdown of Nup214 and co-expression of the indicated plasmids. Representative images are shown in *D*. Note that the absence of Nup214 reduces endogenous levels of Nup88 (see also Fig. 1E).

fectured with HA-Nup153, Nup214-Δ1637-2075, Nup214-585-832, Nup214-804-1058, or Nup214-585-1058 (Fig. 5B, lanes 2–6, respectively) showed that endogenous Nup214 levels continued to be significantly reduced, indicating that their expression did not interfere with Nup214 RNAi. HA-Nup153 expression also did not rescue NMD3 export (data not shown). These results indicate that Nup214 function in 60 S export is mediated by the central domain of Nup214, which interacts with Nup88.

## DISCUSSION

In this study we have assessed the role of the Nup214-Nup88 complex in CRM1-mediated nuclear export. Human CRM1 was

first identified as a Nup214 co-precipitating protein that interacted specifically with the C-terminal FG repeat of this nucleoporin (29). *In vitro*, this interaction is enhanced by RanGTP and cargo, suggesting a role in translocation through the NPC or disassembly of export complexes (30, 31). It was therefore surprising that depletion of the Nup214-Nup88 subcomplex had little or no effect on CRM1-dependent nuclear export of simple export cargoes. In yeast, a relatively strong *in vitro* interaction between Nup159 and Xpo1 exists as well (18), suggesting that this interaction has an important evolutionary conserved function. However, removal of the high affinity domain in Nup159 does not significantly affect nuclear export of an NES-GFP-NLS reporter protein (18) or cell viability. Furthermore, studies in yeast indicate that a significant fraction of FG repeats can be removed from the NPC before cell viability is compromised (17). Therefore, the *in vivo* significance of the high affinity CRM1/Nup214 interaction remains unknown, but it may function to attenuate rather than stimulate CRM1-mediated nuclear export (46).

In addition to contributing to the hydrophobic inner core of the NPC (8), the FG-repeat region of Nup214 has recently been proposed to move cargo through the NPC from the nuclear to the cytoplasmic face of the NE (33, 34). In fact, the FG-repeat region of Nup214 is able to cross the NPC by itself (56) and is predicted to be unstructured (57) and long enough to cross the NPC from a cytoplasmic anchoring point. In

addition, overexpression of Nup214 results in a presence of this nucleoporin at both sides of the NPC (58). But, irrespective of such a system operating, our Nup214 depletion data indicate that it is not essential for CRM1-mediated nuclear export in cultured cells. To examine whether the C-terminal tail of Nup214 reaches the nuclear face of the NPC *in vivo*, we provided GFP reporter proteins and the C-terminal tail of Nup214 with rapamycin-dependent heterodimerizing tags. Using this system, we only detected NPC localization of the reporter proteins when they were in the cytoplasm, indicating that the FG-repeat region of Nup214 accesses the cytoplasm but not the nucleoplasm under our *in vivo* conditions.

We found that the presence of the Nup214-Nup88 subcomplex was required for CRM1-mediated nuclear export of 60 S pre-ribo-

somal subunits. This indicates that different cargoes served by the same transport receptor have different nucleoporin requirements, which complements earlier observations that different nucleoporins serve distinct nucleocytoplasmic transport pathways (59–62). Because depletion of Nup214 has no general effect on nuclear protein import (27) or export (this study), the observed preribosomal nuclear export defect is likely not a consequence of pleiotropic effects on general protein transport pathways. Also, 60 S export was rescued by Nup214 versions that cannot bind Dbp5. Dbp5 interaction with Nup214–Nup159 is required for mRNA export (63, 64), suggesting that the observed 60 S export defects are not primarily caused by general inhibition of mRNA export.

The mechanism by which large ribonuclear protein complexes translocate through the NPC remains largely unknown. The size of a 60 S preribosomal particle (25 nm) is ~100-fold that of a GFP molecule and close to the upper NPC size limit for a nondeformable cargo (65), suggesting that a significant conformational change of the NPC should occur during its translocation. Recent analysis using cryoelectron tomography of functional *Dyctiostelium* NPCs has revealed distinct structural states correlating with a variable central volume that likely represent large cargoes in transit (24).

In yeast, nuclear export of 40 S and 60 S preribosomes was reported to require the Nup159p–Nup82p–Nsp1p subcomplex (63), which is the proposed yeast homologue of the vertebrate Nup214–Nup88–Nup62 subcomplex (55). Both Nup214–Nup88–Nup62 and Nup159–Nup82–Nsp1 subcomplexes are associated through interactions of coiled-coil domains (54, 55), and these domains in Nup159p and Nsp1p are necessary and sufficient for cell viability (66, 67). We find that in vertebrate cells, the requirement of the Nup214–Nup88 subcomplex for 60 S preribosomal export is dependent on the central coiled-coil domain that contains the Nup88 and possibly Nup62 interaction domains but is not dependent on its N terminus nor its large FG-repeat region. Because of the structural characteristics of the Nup214 central domain and its role in correctly positioning the cytoplasmic Nup214–Nup88 subcomplex, it is likely part of the cytoplasmic structures of the NPC. Furthermore, this region lacks FG repeats, thought to be essential for CRM1 interaction. Therefore, we consider it unlikely that a direct interaction between Nup214 and the 60 S preribosomal export complex is required for 60 S export. We propose, rather, that the Nup214–Nup88 core domain plays a structural role in large scale conformational changes required for 60 S preribosome export, perhaps functioning in a hinge-like manner. This is consistent with deletion analysis in yeast, which indicates that only the coiled-coil domain of Nup159, the closest yeast homologue of Nup214, is required for 40 S preribosomal nuclear export (63). Further evidence that the Nup214–Nup88 subcomplex may be required for nuclear export of large cargoes is the strong mRNA export defect of depletion of Nup214 and Nup88 (28) or their yeast equivalents, Nup159 and Nup82 (59, 60, 68, 69).

In conclusion, we have shown that the Nup214–Nup88 subcomplex is required for CRM1-mediated export of a specific cargo, the 60 S preribosome, in a process independent of strong CRM1–FG interactions.

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