Nuclear export of the large (60S) ribosomal subunit depends on the adapter protein Nmd3 to provide a nuclear export signal (NES). The leucine-rich NES is recognized by the export receptor Crm1 to mediate export via interaction with the nuclear pore complex (NPC). Here, we show that certain mutant Nmd3 proteins that are impaired for binding to the 60S subunit accumulate at the nuclear envelope. These mutant proteins also show enhanced binding to Crm1, both in vivo and in vitro. Although their interaction with the NPC is dependent on recognition of the NES by Crm1, their interaction with Crm1 is not strictly dependent on RanGTP. Using a collection of GFP-tagged nucleoporin mutants, we identified several nucleoporins, including components of the Nup82 complex that copurified with the mutant Nmd3. The Nup82 complex is on the cytoplasmic face of the NPC and has previously been shown to be important as a terminal binding site for Crm1-mediated export. Mutations in the Nup82 complex led to accumulation of wild-type Nmd3 in the nucleoplasm, suggesting that the interaction of mutant Nmd3 with the Nup82 complex reflects a defect in the bona fide export pathway for the 60S subunit. These results suggest that in the absence of the ribosome, Nmd3 is not efficiently released from Crm1 at the NPC.

The nucleocytoplasmic transport of most proteins and RNAs is mediated by karyopherins (1–3), a conserved group of soluble factors dedicated to the unidirectional movement of cargo through the nuclear pore complex (NPC) in the nuclear envelope (4, 5). 14 karyopherins have been identified thus far in yeast (6), and they all share a common structural organization, including an N-terminal Ran-binding loop, a central nucleoporin-binding domain, and a C-terminal cargo-binding motif (2). Karyopherins can be subcategorized as either importins or exportins (3, 7) that carry cargo into or out of the nucleus, respectively. The binding of exportins to their substrates in the nucleus depends on the formation of a ternary complex with RanGTP that enhances the exportin-cargo interaction (8). Upon translocation to the cytoplasm, hydrolysis of GTP on Ran, stimulated by a cytoplasmic GTPase-activating protein (GAP), results in a conformational change that disassembles the export complex (9). The directionality of export is controlled by the high concentration of RanGTP in the nucleus versus cytoplasm and the requirement for RanGTP to stabilize the interaction of exportins with their cargo in the nucleus.

Among exportins, Crm1 recognizes the greatest diversity of substrates, ranging from ribosomes to cell cycle regulators (10, 11). Crm1 binds cargo molecules that possess leucine-rich nuclear export sequences (NESs) (12–14). Upon formation of an export complex with RanGTP, Crm1 transports its cargo through the NPC. In yeast, NPCs are composed of ~30 proteins that are arranged in hierarchical subcomplexes (15, 16). One such subcomplex composed of Nup82, Nup159, and Nsp1 (the “Nup82 complex”) is located on the cytoplasmic side of the NPC. Conditional mutations in this complex cause accumulation of ribosomal subunits in the nucleoplasm (17). In mammalian cells Crm1 shows particularly strong binding to Nup214, the homologue of yeast Nup159 (14, 18). Moreover, Nup214 acts as a terminal-binding site for Crm1 prior to export complex disassembly at the NPC (19). While the Nup82 complex has also been implicated in the export of mRNPs, this function is physically separable from that of ribosome export (17).

Nmd3 is the export adapter for the 60S ribosomal subunit, bridging the interaction between the subunit and Crm1 (20–23). The nucleocytoplasmic shuttling of Nmd3 is driven by a nuclear localization sequence (NLS) and a NES, both of which are located in the C terminus of the protein. The leucine-rich NES (amino acids 496–505) is predicted to form an amphipathic helix with the hydrophobic residues critical for Crm1 interaction aligned on one surface (reviewed in Refs. 11 and 24). Deletion or mutation of this NES results in the nuclear accumulation of both Nmd3 and nascent 60S ribosomal subunits (20, 21, 25).

Although the role of Nmd3 in 60S export is well documented (26), the molecular details of how the subunit is recruited to the NPC are lacking. Here, we identify a class of Nmd3 mutants that fail to bind to 60S subunits and accumulate at the NPC in a Crm1-dependent fashion. The localization of these mutant Nmd3 proteins at the NPC implies that the ribosome contributes to disassembly of Crm1 from the pre-60S subunit at the NPC.
TABLE 1
Strains used in this study

| Strain     | Genotype and notes                  | Source     |
|------------|-------------------------------------|------------|
| AJY1539    | MATa leu2 ura3 his3Δ1 met15Δ0 cmr1(TS39C)-HA | This study |
| AJY1848    | MATa leu2Δ0 ura3Δ0 his3Δ1 lys2Δ0 NIC96-mRFP-KanMX6 | (32)       |
| AJY1849    | MATa leu2Δ0 ura3Δ0 his3Δ1 NIC96-mRFP-KanMX6 cmr1(TS39C)-HA | This study |
| AJY2155    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NIC96-GFP-HIS3 | (32)       |
| AJY2154    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP159-GFP-HIS3 | (32)       |
| AJY2156    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP1100-GFP-HIS3 | (32)       |
| AJY2157    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP116-GFP-HIS3 | (32)       |
| AJY2158    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP49-GFP-HIS3 | (32)       |
| AJY2161    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP82-GFP-HIS3 | (32)       |
| AJY2162    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP85-GFP-HIS3 | (32)       |
| AJY2163    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP53-GFP-HIS3 | (32)       |
| AJY2164    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP84-GFP-HIS3 | (32)       |
| AJY2165    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP57-GFP-HIS3 | (32)       |
| AJY2166    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP60-GFP-HIS3 | (32)       |
| AJY2167    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP60-GFP-HIS3 | (32)       |
| AJY2168    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP133-GFP-HIS3 | (32)       |
| AJY2169    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP145-GFP-HIS3 | (32)       |
| AJY2170    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP157-GFP-HIS3 | (32)       |
| AJY2171    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP70-GFP-HIS3 | (32)       |
| AJY2172    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP125-GFP-HIS3 | (32)       |
| AJY2173    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP125-GFP-HIS3 | (32)       |
| AJY2174    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP125-GFP-HIS3 | (32)       |
| AJY2175    | MATa leu2Δ0 ura3Δ0 his3Δ1 met15Δ0 NUP125-GFP-HIS3 | (32)       |
| AJY2176    | MATa leu2Δ0 ura3Δ0 his3Δ1 nup120-Clonat’ (pA1608) | This study |
| BY4741     | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Research Genetics |
| BJ564      | MATa ura3-52 leu2Δ1 trpl his3Δ200 pep4Δ:His3 prb1Δ1.6R can1 | E. Jones |
| LG220      | MATa trp1-1 his3Δ200 leu2Δ1 | (43)       |
| Nup82-2MA8 | MATa his3Δ3-200 leu2Δ-3,112 lys2-801 trp1-1 ura3-52 nup82-HIS3/pNUP82-108(LEU) | (45)       |
| LGY109     | MATa RAT7::HIS3 his3Δ200 ura3-52 leu2Δ1/plasmid pL4G (RAT7) | (37)       |
| VDPY122    | MATa RAT7::HIS3 his3Δ200 ura3-52 leu2Δ1/pplasmid pVDP17 (RAT7ΔΔΔR) | (37)       |

TABLE 2
Plasmids used in this study

| Plasmid   | Relevant markers and notes | Source or reference |
|-----------|---------------------------|---------------------|
| pAJ414    | URA3 2p. NMD3-13xcmyc | This study         |
| pAJ516    | URA3 2p. nmd3ΔN167-13xcmyc | This study         |
| pAJ538    | LEU2 CEN NMD3-14xcmyc | (21)               |
| pA135     | LEU2 CEN NMD3-GFP | (32)               |
| pA690     | URA3 2p. GAL10-GST-NMD3 | This study         |
| pA693     | URA3 2p. GAL10:GST-nmd3ΔN167 | This study         |
| pA752     | LEU2 CEN nmd3[AA]-13xcmyc | This study         |
| pA754     | LEU2 CEN nmd3[AA]-GFP | (32)               |
| pA1298    | LEU2 CEN nmd3[1397TL259S,1296P]-13xcmyc | This study |
| pA1403    | LEU2 CEN nmd3[1397TL259S,1296P]-3xcmyc | This study |
| pA1404    | LEU2 CEN nmd3[1263P,318I]-13xcmyc | This study         |
| pA1405    | LEU2 CEN nmd3[1263P,318I] -13xcmyc | This study         |
| pA1406    | LEU2 CEN nmd3[1263P,318I] -GFP | This study         |
| pA1407    | LEU2 CEN nmd3[1263P,318I] -GFP | This study         |
| pA1408    | LEU2 CEN nmd3[1263P,318I] -GFP | This study         |
| pA1412    | LEU2 CEN nmd3[1263P,318I] -AAA-GFP | This study         |
| pA1413    | LEU2 CEN nmd3[1263P,318I] -AAA-GFP | This study         |
| pA1414    | LEU2 CEN nmd3[1263P,318I] -AAA-GFP | This study         |
| pA1515    | LEU2 CEN nmd3[1263P,318I] -AAA-GFP | This study         |
| pA1516    | LEU2 CEN nmd3[1263P,318I] -AAA-GFP | This study         |
| pA1536    | LEU2 CEN nmd3[1263P,318I] -GFP | This study         |
| pA1538    | GST-NSe -GFP | This study         |
| pA1541    | CRM1-His | This study         |
| pKW581    | Hist-RAN(GSP1) | (43)               |
| pL1599    | LEU2 CEN NMD3-supra NES-GFP | This study         |
| pL1600    | LEU2 CEN nmd3[1397TL259S,1296P -supra NES]-GFP | This study |
| pL1608    | URA3 CEN NUP120-GFP | This study         |

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Media—The strains used are listed in Table 1. AJY1849 was made by crossing AJY1848 with AJY1539. Nup120Δ::KanMX was switched to Nat' as described (41).

Plasmids are listed in Table 2. pAJ414 was made by moving the Ehel-HindIII fragment from pAJ408 (21) into SmaI-HindIII-cut pRS416. pAJ516 (nmd3ΔN167-myc) was made by PCR amplification of NMD3 with the oligo 5'- GAATTCAAGAG-AACATTTTTGTGTGTGG and ligating to the EcoRI site at nt 4 in NMD3 using pAJ538. pAJ690 (GST-NMD3) was made by inserting NMD3 into pEG(KG) (42). pAJ693 was constructed by moving the deletion from pAJ516 to pAJ690. pAJ1298, pAJ1403 and pAJ1404 were isolated as described (25). To create plasmids pAJ1406, pAJ1407, and pAJ1408, pAJ1298, pAJ1403, and pAJ1404, respectively, were cut with PciI and SpeI and co-transformed into wild-type strain W303 with BsgI-BspHI-digested pAJ582. pAJ1412, pAJ1413, and pAJ1414 were made by swapping the BglII-HindIII fragment from pAJ754 into pAJ1406, pAJ1407, and pAJ1408 (respectively). To construct pAJ1515 and pAJ1516, the oligomeric c-Myc tag from pAJ538, as a PciI-HindIII fragment, was ligated into

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the same sites of pAJ1412 and pAJ1413. pAJ1536 was generated by ligating the Pacl-EagI fragment from pAJ582 into the same sites of pAJ516. pAJ1538 was made by ligating the complementary oligonucleotides 5’-AGCTTGGCTGTTGATATCAAGCCTGAAATTTCAAGGCTAATTCATG) and 3’-GATCCAAGTGAATTAGCCCTTGAAATAGCAGTGTTGATGATATCAACAACAGCATAAA, into BamHI-cut pGEX4T-3. To create pAJ1541, CRM1 was PCR amplified from its genomic locus using 5’-GCCGGGATCCATGAAGGAAATTGG and 3’-GGCCGGGATCCATCAAGTGGTGAAGG, digested with BamHI and NotI, and ligated into the same sites of pET-Dan196–223 and 5’-CATAAAAGGTGGAAGAAACACTGTCCAGTTTATG and 5’-CATAAAACGTCAGTTTCTCAGCTTTATG for nmd3196–223 and cloned into the appropriate vectors.

The supraphysiological NES mutations were made by PCR using the sense oligo 5’-CGAATTCTGAGCTAGAAGGATCTGGAAACATCTTTATG and the antisense oligo 5’-GACTCTGAAGAAAACACTGTCCAGTTTATG into column, washed with lysis buffer and eluted with 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10% glycerol, 1 mM dithiothreitol, and protease inhibitors), resuspended in ten volumes of lysis buffer and sonicated. Following clarification, Ni-NTA resin was added, and the suspension was rocked for 1 h. The suspension was loaded into a column, washed with lysis buffer, and bound proteins were eluted by heating in Laemmli buffer. One-fourth of the eluate from each reaction was used for SDS-PAGE.

**Purification of Recombinant Fusion Proteins**—The GST-Nmd3 fusion proteins were expressed and purified from yeast strain BJ5464 containing pAJ690 or pAJ693. Briefly, cultures were diluted to A600 ~ 0.15 in SC-ura (raffinose) media, incubated for 6 h at 30 °C, and induced for 6 h with 1% galactose. Cells were washed once in lysis buffer (20 mM Tris, pH 7.5, 500 mM KCl, 10% glycerol, 1 mM dithiothreitol, and protease inhibitors), resuspended in 5 volumes of lysis buffer and lysed as described above. Following clarification, 1/10 bed volume of glutathione-Sepharose 4B was added to the extract, and the suspension was rocked for 1 h. The suspension was then loaded into column, washed with lysis buffer and eluted with 50 mM glutathione in 50 mM Tris-HCl, pH 8.0. Fractions containing GST-Nmd3 were pooled and dialyzed into storage buffer (50 mM Tris-HCl, pH 7.5, 200 mM KCl, 10% glycerol, 10 mM MgCl2, and 1 mM β-mercaptoethanol).

GST-(PKI)NES was purified in the following manner. A culture of Stratagene codon + (RLI) containing pAJ1538 was diluted to an A600 ~ 0.1 in Luria broth containing 75 μg/ml ampicillin and 30 μg/ml chloramphenicol and cultured at 37 °C to an A600 ~ 0.4. Expression was induced with 125 pmol isopropyl-1-thio-β-D-galactopyranoside for 5 h at 30 °C. Cells were washed once in lysis buffer (20 mM Tris, pH 7.5, 300 mM KCl, 10% glycerol, 1 mM dithiothreitol, and protease inhibitors), resuspended in ten volumes of lysis buffer and sonicated. Following purification steps were as described for the GST-Nmd3.

Purification of Ccm1-His6 was as follows. A culture of Stratagene codon + (RLI) containing pAJ1541 (CRM1-His6) was diluted to an A600 ~ 0.1 in Luria broth containing 75 μg/ml ampicillin and 30 μg/ml chloramphenicol and cultured at 37 °C to an A600 ~ 0.4. Expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 5 h at 25 °C. Cells were washed once in lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 10% glycerol, 15 mM imidazole, and protease inhibitors), resuspended in ten volumes of lysis buffer and sonicated. Following purification steps were as described for the GST-Nmd3.

**Immunoprecipitations**—Overnight cultures were diluted to A600 ~ 0.15 in fresh glucose-containing dropout media, cultured at 30 °C and visualized as described (28).

**Fluorescence Microscopy**—Overnight cultures of AJY1539, AJY1848, or AJY1849 expressing GFP-tagged Nmd3 alleles were diluted to 0.15 in fresh glucose-containing dropout media, cultured at 30 °C and visualized as described (28).

**Identification of Mutant Nmd3 Proteins That Accumulate at the Nuclear Pore Complex**—In a screen for nmd3 loss-of-function mutants (25), we identified three mutants that exhibited unusual localization (Fig. 1A). In contrast to wild-type Nmd3-GFP, which was distributed throughout the nucleus and cytoplasm, the Nmd3(V340D)-GFP, Nmd3(L263P,F318I)-GFP, and Nmd3(V340D)-GFP mutant proteins accumulated in a striking ring-like pattern around the nucleus (Fig. 1A). Mutant c-Myc-tagged Nmd3 proteins behaved similarly (data not shown). To
determine whether these nmd3 mutants were accumulating at the nuclear envelope, we co-expressed the GFP-tagged mutant proteins with Nic96-mRFP, a marker for nuclear pore complexes. Indeed, the mutant Nmd3-GFP proteins co-localized with Nic96-mRFP, with one mutant, Nmd3(I139T, L259S, L296P)-GFP, showing particularly strong envelope enrichment (Fig. 1B). To facilitate detection of Crm1 in subsequent experiments described below we used a strain expressing the crm1T539C allele, which was also epitope-tagged with HA (27, 28). This allele of CRM1 renders yeast sensitive to leptomycin B but is not reported to alter CRM1 function otherwise. However, we found that the nuclear envelope accumulation of the mutant Nmd3 proteins described here was enhanced in this allele of CRM1, possibly because of the HA tag rather than the T539C mutation. Nevertheless, the NPC localization of Nmd3(I139T, L259S, L296P)-GFP could be observed in wild-type cells, where the fluorescence around the nuclear rim typically formed only a partial ring (Fig. 1C). Enhancing the affinity of the NES of mutant Nmd3 for CRM1 (Nmd3(I139T, L259S, L296P-supraNES, see below), led to a significant increase in the NPC localization of mutant Nmd3 in CRM1 wild-type cells (Fig. 1C).

These three NPC-localizing nmd3 mutants contained point mutations within two regions of Nmd3 implicated in 60S binding (25) (Fig. 2A). nmd3(V340D) was initially characterized as a strong loss of function mutant that was significantly impaired for binding to 60S subunits and could not support growth when expressed as the only copy of Nmd3 (25, 28). To examine whether the other NPC localized mutants were also defective for 60S binding, we performed immunoprecipitations (IPs) from cells expressing c-Myc-tagged versions of the mutants, monitoring Rpl8 as a reporter for 60S subunits. The NPC-localized mutants exhibited a dramatic deficit in 60S binding relative to wild-type Nmd3 (Fig. 2B). These results suggest that mutant Nmd3 proteins that cannot bind to 60S subunits accumulate at NPCs.

Accumulation of Mutant Nmd3 Proteins at the NPC Is Crm1-dependent—As the dissociation of export cargo from Crm1 and RanGTP at the nuclear envelope is typically very rapid, cargo is generally not observed at the NPC using standard microscopic techniques. However, Engelsma et al. (29) identified NES-like peptides from a pool of randomly generated synthetic peptides, that exhibited a marked nuclear envelope accumulation in permeabilized HeLa cells. These synthetic NESs showed unusually strong interaction with Crm1, leading to their description as “supraphysiological” (29). These results led us to consider that the NPC localization of the Nmd3 mutant proteins may similarly depend on increased binding to Crm1. As an initial test of this idea, we performed co-immunoprecipitation experiments using c-myc-tagged Nmd3. Western blotting against HA revealed a significant and specific increase (~8-fold) in Crm1 with the mutant proteins relative to wild-type Nmd3 (Fig. 2C).
The enhanced interaction with Crm1 and the nuclear envelope enrichment of the mutant Nmd3 proteins suggest that they are recruited to the nuclear pore complex in a Crm1-dependent manner.

To test this more directly, we mutated the NES of the mutant Nmd3 proteins to disrupt Crm1 binding. We have previously shown that an Nmd3 mutant containing three point mutations within its NES (I193T, L259S, L296P) (Fig. 3A) exhibits a strong nucleoplasmic accumulation (28), mimicking the effects seen for wild-type Nmd3 in the presence of the Crm1-specific inhibitor leptomycin B (LMB) (21, 30, 31). Using this NES mutation, we asked whether disrupting Crm1 interaction also led to loss of NPC localization. As seen in Fig. 3B, this was indeed the case; introduction of NES mutations resulted in nucleoplasmic localization of the mutant Nmd3 proteins, with no detectable nuclear envelope enrichment. Consistent with this result, the mutant proteins possessing wild-type NESs also relocated to the nucleoplasm upon treatment with LMB (data not shown). These results demonstrate that the NPC localization of the mutant Nmd3 proteins depends on Crm1 and suggest that it reflects an arrest in the export pathway.

To demonstrate that the physical interaction between the NPC-localized mutant proteins and Crm1 was indeed disrupted by the introduction of mutations in the NES, we tested the ability of these proteins to co-immunoprecipitate Crm1. As predicted, the introduction of mutations in the NES of the mutant proteins significantly reduced their ability to co-immunoprecipitate Crm1 (Fig. 3C, compare lanes 4 and 6 with lanes 5 and 7), whereas the NES mutation by itself had no apparent effect on 60S binding (Fig. 3C, lane 3). This finding further supports the idea that NPC enrichment of the mutant Nmd3 proteins is dependent upon a classical Crm1-NES interaction.

Mutant Nmd3 That Accumulates at the Nuclear Envelope Interacts Directly and Stably with Crm1 in Vitro—In vivo, Crm1 binds to NESs cooperatively with RanGTP and the formation of stable Crm1-NES interactions in vitro also typically requires RanGTP. The co-immunoprecipitation of Crm1 with cargo from extracts is not typically observed, because the rapid dissociation of the ternary complex upon RanGTP hydrolysis. Thus, the stable interaction of mutant Nmd3 with Crm1 in extracts suggests that Crm1 can bind to mutant Nmd3 even after dissociation of Ran. To test if Crm1 can bind Nmd3 independently of RanGTP, we carried out in vitro binding assays using recombinant Crm1, RanGTP, and GST fusion proteins of wild-type or mutant Nmd3. Because we were not able to purify sufficient quantities of the GST-tagged Nmd3 (I139T, L259S, L296P), we used an N-terminal truncation mutant (Nmd3A167) that also showed loss of 60S binding and NPC accumulation (Fig. 4, A and B). Full-length wild-type Nmd3 and Nmd3A167 were produced as GST fusion proteins in yeast, and the well characterized NES from protein kinase A inhibitor (PKI), fused to GST was purified from E. coli.

GST-(PKI)NES, GST-Nmd3, or GST-Nmd3A167 were incubated with Crm1 in the presence or absence of RanGTP. The GST fusion proteins were recovered from the reactions on glutathione-Sepharose beads. Bound proteins were separated by SDS-PAGE followed by immunoblotting for Crm1. As shown in Fig. 4C, GST-(PKI)NES exhibited weak interaction with Crm1 in the absence of RanGTP (lane 1), but showed greatly enhanced interaction (~20-fold) in the presence of RanGTP (lane 2), consistent with the formation of a stable ternary complex. Similarly, wild-type GST-Nmd3 bound Crm1 in

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**FIGURE 2. Nmd3 NE mutants are deficient for 60S binding and show increased binding to Crm1.**

A, cartoon showing point mutations within Nmd3 (I139T, L259S, L296P, V340D). Dark gray shading indicates regions implicated in 60S subunit binding (25). B, α-cmyc immunoprecipitations were conducted in extracts prepared from strain AJY1539 (crm1[T539C]) harboring either empty vector (pRS415), pAJ538 (NMD3-cmyc), or pAJ1404 (nmd3[L263P,F318I]-cmyc). Immunoprecipitated proteins were resolved on an 8% SDS-PAGE gel followed by Western blotting for the various Nmd3 alleles using α-cmyc or for the 60S protein Rpl8 using anti-Rpl8. C, α-cmyc immunoprecipitations were from extracts of strain AJY1539 (crm1[T539C]-HA) harboring either empty vector (pRS415), pAJ538 (NMD3-cmyc), pAJ1298 (nmd3[I139T,L259S,L296P]-cmyc), pAJ1404 (nmd3[L263P,F318I]-cmyc), or pAJ1404 (nmd3[L263P,F318I]-cmyc). Immunoprecipitated proteins were resolved on a 10% SDS-PAGE gel followed by Western blotting for the mutant Nmd3 proteins using α-cmyc and for Crm1 using anti-HA.
a RanGTP-dependent manner (compare lanes 3 and 4). In contrast GST-Nmd3ΔN167 was capable of stably binding to Crm1 in the absence of RanGTP, with no significant increase in Crm1 binding in the presence of RanGTP (lanes 5 and 6). This finding supports the characterization of a supraphysiological interaction between Crm1 and Nmd3ΔN167 in vivo. The stability and RanGTP independence of this interaction likely leads to the observed enrichment of the mutant Nmd3 protein at the NPC, mimicking a disassembly intermediate of the export complex.

Converting the NES of Nmd3 to a Supraphysiological NES—The accumulation of NMD3(I139T,L259S,L296P) was reminiscent of the behavior of a supraphysiological NES previously described (29). However, unlike the supraphysiological NES, the mutations in Nmd3 that increased binding to Crm1 were not within the NES. To test if the NPC localization could be enhanced by mutations in the NES as well, we mutated the NES of yeast Nmd3 to mimic the supraphysiological NES identified previously (496LLDELDEMTL505 → 496LLDLFDELSV505) (Fig. 5A and Ref. 29). Although the mutations introduced into the NES of yeast Nmd3 were based on a modified NES from human cells, these mutations led to increased NPC accumulation of Nmd3(I139T,L259S,L296P) in yeast, and correspondingly reduced levels of the protein in the cytoplasm and nucleoplasm. Additionally, this mutant Nmd3 was mildly dominant negative pertaining to the export of proteins from the nucleus. The stability and RanGTP independence of the interaction between Crm1 and Nmd3ΔN167 support the characterization of a supraphysiological interaction in vivo, which likely leads to enrichment of the mutant protein at the NPC, mimicking a disassembly intermediate of the export complex.

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are mechanisms to regulate its functional interaction with the Nmd3 in the context of the nascent 60S subunit in which there

Therefore, we argue that a very strong NES can be tolerated when expressed as the sole copy of NMD3 (data not shown).

NES supports growth at a rate comparable to wild-type cells and is dependent on specific interactions with nucleoporins. The correlation between nucleoporins known to be important for 60S subunit export with those co-purified with mutant Nmd3, suggests that the interaction of mutant Nmd3 with specific Nups represent interactions that are physiologically relevant for 60S export.

Within the NPC, distinct subcomplexes of nucleoporins can be identified, (36) and references therein. Among these is the Nup82 complex, composed of Nup82, Nup159, Nic96, and Nup85) had previously been implicated in the nuclear export of preribosomal particles (17, 33–35). The correlation between nucleoporins known to be important for 60S subunit export with those that co-purified with mutant Nmd3, suggests that the interactions of mutant Nmd3 with specific Nups represent interactions that are physiologically relevant for 60S export.

When expressed in the crm1(T539C) strain (Fig. 5C), suggesting that either Crm1 was being titrated out by binding to mutant Nmd3 or that critical binding sites on the NPC were blocked by accumulation of mutant Nmd3 with Crm1. The introduction of the supraphysiological NES into wild-type Nmd3 did not result in an obvious accumulation at the NPC (Fig. 5B), nor was it dominant negative for growth (data not shown). However, the signal for this Nmd3 protein was somewhat reduced in the nucleoplasm (Fig. 5B), suggesting an increased rate of export from the nucleus. These results are consistent with our observation that wild-type Nmd3 harboring a supraphysiological NES supports growth at a rate comparable to wild-type cells when expressed as the sole copy of NMD3 (data not shown). Therefore, we argue that a very strong NES can be tolerated on Nmd3 in the context of the nascent 60S subunit in which there are mechanisms to regulate its functional interaction with the export machinery (see “Discussion”). In contrast, the introduction of a supraphysiological NES into Nmd3(I139T,L259S,L296P), which is impaired for 60S binding, further enhanced the stability of Nmd3-Crm1 complexes arrested at the NPC, likely titrating available Crm1 from the cellular pool.

**Mutant Nmd3 Associates with Specific Nucleoporins**—We anticipated that the NPC-localized mutant Nmd3 proteins might co-purify with a distinct subset of nucleoporins (Nups), providing insight into the manner in which the wild-type Nmd3 interacts with NPC during 60S export. To this end, we expressed mutant or wild-type Nmd3 proteins in cells containing GFP fusions to various nucleoporins obtained from the GFP-tagged strain collection (32). Particular emphasis was given to those nucleoporins that had previously been implicated in 60S export (17, 33, 34). For analysis of Nup120, which was absent from the GFP library, we expressed NUP120-GFP from a low copy vector in a nup120 deletion strain. C-Myc-tagged versions of either wild-type Nmd3 or Nmd3(I139T,L259S,L296P) were expressed in each of the GFP-tagged strains. Nmd3-bound complexes were immunoprecipitated, and the presence of GFP-tagged nucleoporins was monitored by Western blotting.

Among the 21 NPC-associated proteins examined, only four were enriched in the immunoprecipitations with Nmd3 (I139T,L259S,L296P) (Table 3, Fig. 6A and data not shown). These four nucleoporins (Nup82, Nup159, Nic96, and Nup85) had previously been implicated in the nuclear export of preribosomal particles (17, 33–35). The correlation between nucleoporins known to be important for 60S subunit export with those that co-purified with mutant Nmd3, suggests that the interaction of mutant Nmd3 with specific Nups represent interactions that are physiologically relevant for 60S export.

**Mutations in the NUP82 Complex Affect Nmd3 Export and Binding to the NPC**—If the binding of mutant Nmd3 to the Nup82 complex reflects a physiologically relevant interaction of Nmd3 with the NPC then mutations in this complex would be expected to affect the localization of wild-type Nmd3. To examine this, we monitored Nmd3-GFP in conditional nup82Δ108 and nup159/ram7-1 mutants. Because these mutants cause significant changes in nuclear morphology at elevated temperature (17), cells were cultured at the semipermissive temperature of 26 °C. Although Nmd3-GFP showed a slight nuclear bias in wild-type cells (Fig. 7A), it accumulated strongly in the nucleoplasm in both nup82Δ108 and nup159/
rat7-1 mutants, even at permissive temperature (Fig. 7A), indicating that the Nup82 complex is important for Nmd3 export. It has been shown previously that Crm1 interacts strongly with the FG repeats of NUP214 (Nup159 in yeast) (14, 19), however, only the conserved coiled-coil C terminus of Nup159 is required for viability (37). In addition, only the carboxyl terminus of Nup159 is required for 40S subunit export (17). We examined the localization of wild-type Nmd3p as well as mutant Nmd3 with an enhanced NES in cells expressing only the coiled-coil domain of Nup159 (rat7/H9004N/H9004R). In contrast to the strong nuclear accumulation of Nmd3 in rat7-1 cells, Nmd3 did not accumulate in the nuclei of rat7/H9004N/H9004R cells (Fig. 7B). These results show that the C terminus of Nup159 is sufficient for Nmd3 export. In contrast, the FG repeats, while not essential for export, are required for the nuclear envelope accumulation of mutant Nmd3. Transient docking of the export complex on the FG repeats of Nup159, while not essential, may provide an appropriate environment.

**TABLE 3**

NPC-related proteins that are enriched in Nmd3(I139T,L259S,L296P)-bound complexes

| NPC-associated factor | Localization | NPC subcomplex | Enrichment in Nmd3(T/S/P) IP | Role in ribosome export |
|-----------------------|--------------|----------------|-----------------------------|-------------------------|
| Nup49                 | Symmetric    | Nic96          | No                          | Yes*                    |
| Nup53                 | Symmetric    | Nic96          | No                          | No                      |
| Nup57                 | Symmetric    | Nup53          | No                          | No                      |
| Nup59                 | Symmetric    | Nup53          | No                          | No                      |
| Nup60                 | Nuclear      |                | No                          | No                      |
| Nup82                 | Coiled-coil  | Nup82          | Yes                         | Yes                     |
| Nup84                 | Symmetric    | Nup84          | No                          | No                      |
| Nup85                 | Symmetric    | Nup84          | Yes                         | Yes                     |
| Nup100                | Cytoplasmic-biased | Nup84   | No                          | No                      |
| Nup116                | Cytoplasmic-biased | –         | No                          | Yes                     |
| Nup120                | Symmetric    | Nup84          | No                          | No                      |
| Nup133                | Symmetric    | –              | No                          | Yes                     |
| Nup145                | Symmetric    | Nup84          | No                          | No                      |
| Nup157                | Symmetric    | Nup84          | No                          | No                      |
| Nup159                | Cytoplasmic  | Nup82          | Yes                         | Yes                     |
| Nup170                | Symmetric    | Nup53          | No                          | No                      |
| Nic96                 | Symmetric    | Nic96          | Yes                         | Yes*                    |
| Nsp1                  | Symmetric    | Nup82,Nic96    | No                          | Yes                     |
| Gle1                  | Cytoplasmic-biased | –         | No                          | No                      |
| Seh1                  | Symmetric    | Nup84          | No                          | No                      |
| Sec13                 | Symmetric    | Nup84          | No                          | No                      |

* Ref. 33.
* Ref. 34.

**FIGURE 7.** Mutations in the Nup82 complex block export of Nmd3. A, Nmd3-GFP (pAJ582) was expressed in wild-type (AJY1539), nup82Δ108, or nup159 (rat7-1ts, LG230) cells cultured at 26 °C. GFP fluorescence and DIC images are shown. B, coiled-coil domain of Nup159 is sufficient for proper localization of Nmd3. GFP-tagged Nmd3 (pAJ582) or Nmd3(I139T,L259S,L296P)-supraNES was expressed in WT (RAT7) cells or cells expressing only the coiled-coil domain of Rat7/Nup159 (rat7ΔNΔR). GFP and Hoechst fluorescence of live cells are shown.
to enhance the disassembly of the export complex (see “Discussion”).

DISCUSSION

In a screen for NMD3 loss-of-function mutants, we unexpectedly identified a class of mutant Nmd3 proteins that accumulate at the nuclear envelope (NE) and are deficient for 60S binding. Surprisingly, the mutations did not map to the conserved nucleocytoplasmic shuffling sequences. The NE localization was, however, Crm1-dependent, as the aberrant localization could be disrupted by point mutations in the NES of Nmd3 or by the Crm1 inhibitor, leptomycin B (LMB) (data not shown). Crm1 was also enriched in immunoprecipitations with the NE mutants, indicating a stable interaction between these two factors in vivo. Co-immunoprecipitation assays with mutant Nmd3 identified several nucleoporins in three of the five major NPC subcomplexes. Among these were components of the Nup82-Nup159-Nsp1 complex, present on the cytoplasmic face of the NPC. The loss of accumulation of mutant Nmd3 at the nuclear envelope in certain nup159 mutants suggests that the Nup82 complex is a binding site for the Nmd3-Crm1 complex. The interaction of mutant Nmd3 with Nic96 and Nup85, nucleoporins in two other subcomplexes of the NPC may reflect additional binding sites of the 60S export complex at the NPC. Interestingly, an interaction between Crm1 and Nup85 or Nic96 has not been reported previously, suggesting that these interactions are specific to the mutant Nmd3, possibly by modifying the affinity of Crm1 for these nucleoporins.

Mutant Nmd3 Arrestrs on the Cytoplasmic Face of the NPC and Resembles an Export Complex Disassembly Intermediate—The stable association of Nmd3(I139T,L259S,L296P) with Crm1 and the Nup82 subcomplex of the NPC is reminiscent of the behavior of a supraphysiological NES peptide that shows high affinity for Crm1 independent of RanGTP (29). Englesma et al. (2004) demonstrated that synthetic peptides that bind Crm1 independently of RanGTP arrest at the NPC, dependent on Nup358-RanBP2, a RanBP1 homologue that is bound to the cytoplasmic face of the NPC by Nup88 (Nup82 in yeast) and Nup214 (Nup159 in yeast) (38). As yeast does not have a Nup358 homolog, the interaction between Nmd3(I139T,L259S,L296P) and Nup82 and Nup159 is likely to be analogous to the behavior reported for the synthetic NESs in the vertebrate system. Intriguingly, Crm1 has also been shown to interact strongly with Nup214 (Nup159) when bound to both cargo and RanGTP, leading to the idea that Nup214 may serve as a terminal binding site for Crm1-bound complexes following export (14, 18, 19). As RanGTP is rapidly converted to RanGDP and released from export complexes in the cytoplasm by RanGAP and RanBP1, it is also likely that the Crm1/Nmd3(I139T,L259S,L296P) interaction on the cytoplasmic face of the NPC persists in the absence of RanGTP, thus implying a direct and stable interaction between Crm1 and mutant Nmd3. In the absence of the 60S subunit, Nmd3(I139T,L259S,L296P) may keep Crm1 in a conformation that mimics an intermediate of disassembly of the 60S export complex at the NPC.

Although we have shown that mutations in the Nup82-Nup159-Nsp1 complex block Nmd3 export, only the coiled-coil domain of Nup159 (Rat7), and not the FG repeats of Nup159, is essential for viability. Because the coiled-coil domain of Nup159 is thought to be necessary for the structure of the NPC but not for interactions with transport receptors, the different phenotypes of rat7-1 and rat7ΔNAR (expressing only the coiled-coil domain) suggest that the export block in rat7-1 cells results from a loss of structural integrity of the NPC. The coiled-coil domain of Nup159 is also the only region of Nup159 required for 40S export in yeast (17) and for export of human Nmd3 (39). Interestingly, the rat7ΔNAR mutant did not accumulate mutant Nmd3 at the nuclear envelope, suggesting that its accumulation is dependent on domains of Nup159 other than the coiled coil domain. Even though binding of the 60S export complex to Nup159 is clearly not necessary for 60S export, transient tethering to Nup159 may enhance disassembly of the 60S export complex by arresting it in an environment with a local high concentration of RanGAP and RanBP1. A failure to capture the export complex at this step would not necessarily impair 60S export, but could result in its release into the cytoplasm where disassembly by the soluble fraction of RanGAP and RanBP1 would be less efficient (38).

Regulation of Nmd3-Crm1 Interaction—As noted above, efficient Crm1-dependent export depends on an optimum balance between affinity for Crm1 and ability to release Crm1. The high intrinsic affinity of Nmd3 for Crm1, revealed by the nmd3 mutants presented here, and its RanGTP independence, would necessitate an additional mechanism for disrupting Crm1 binding once the subunit has docked at the NPC. Because mutant Nmd3 that does not bind to 60S subunits shows Crm1-dependent enrichment at the NPC, we suggest that the 60S subunit itself contributes to proper disassembly of the complex. This could be through release of a factor from the 60S-Nmd3-Crm1 complex that was needed for presentation of the auxiliary Crm1 binding site while in the nucleus. Alternatively, proper disassembly could be mediated by the recruitment of a factor (or ribosomal protein) to the subunit, or a conformational change in the subunit at the cytoplasmic face of the NPC, thus altering the nature of the Nmd3-60S interaction and allowing folding of the protein to block strong Crm1 binding. We have suggested previously that, in vivo, Nmd3 binds recycling 60S subunits in the cytoplasm, as well as nascent subunits in the nucleolus (40). Because the unusually strong interaction with Crm1 appears to be independent of RanGTP, it would be necessary to prevent this binding in the cytoplasm, to avoid unproductive interaction with Crm1.

Acknowledgments—We thank P. Silver, C. Cole, and E. Marcotte for providing strains and N.-J. Hung for assistance with microscopy.

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