Mapping the Functional Domains of Yeast NMD3, the Nuclear Export Adapter for the 60 S Ribosomal Subunit*

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Nuclear export of the large ribosomal subunit requires the adapter protein Nmd3p to provide a leucine-rich nuclear export signal that is recognized by the export receptor Crm1. Nmd3p binds to the pre-60 S subunit in the nucleus. After export to the cytoplasm, the release of Nmd3p depends on the ribosomal protein Rpl10p and the GTPase Lsg1p. Here, we have carried out a mutational analysis of Nmd3 to better define the domains responsible for nucleocytoplasmic shuttling and ribosome binding. We show that mutations in two regions of Nmd3p affect 60 S binding, suggesting that its binding to the subunit is multivalent.

Yeast Nmd3p is a 59-kDa shuttling protein that is required for export of 60 S ribosomal subunits out of the nucleus (1, 2). Nmd3p binds to nascent 60 S subunits in the nucleus and provides a leucine-rich nuclear export signal (NES)4 that is recognized by the export receptor Crm1. This export function of Nmd3p is conserved in metazoans (3, 4). However, Nmd3p homologs are also present in archaea, suggesting a more ancient function of this protein that predates the development of the nuclear envelope. We have recently shown that release of Nmd3p depends on the loading of the essential large subunit Rpl10p (5, 6). Thus, Nmd3p may stabilize an intermediate of ribosome assembly prior to Rpl10p loading. Interestingly, in the euryarchaea, Nmd3p is expressed as a fusion to an eIF5A-like domain (7). Although the function of eIF5A in translation is not well established, the association of Nmd3 and eIF5A in archaea suggests that they have related functions.

Yeast Nmd3p is 518 amino acids in length. The leucine-rich NES is within its C-terminal 50 amino acids (amino acids 470 – 518) (1, 2) and is predicted to form an amphipathic α-helix. Upstream of the NES is a putative coiled-coil region (aa 426 – 465) that could support intra- or intermolecular protein interactions, possibly to facilitate interaction of the NES or NLS with transport receptors. It has been suggested that this domain contains a secondary NES (NES2) (2) that can functionally substitute for the primary NES. Additionally, Nmd3p contains a highly basic region (aa 399 – 419) that is necessary for efficient import of Nmd3p into the nucleus (1) in a Kap123p-dependent manner (8). These C-terminal shuttling motifs are found in the eukaryotic Nmd3 proteins but not in their archaean counterparts. The N-terminal 27 kDa of Nmd3p comprises the domain that is conserved in all archaean Nmd3 proteins. An additional 9 kDa is conserved among the euryarchaeotic proteins that also contain a C-terminal eIF5A-like domain (7). The highly conserved N-terminal domain contains four Cys-α-Cys repeats that are probably zinc binding motifs reminiscent of Type IV zinc fingers (9) and treble clef motifs (10). This potential zinc-coordinating domain in Nmd3p is probably required for protein-RNA and/or protein-protein interaction with the 60 S subunit; however, this has not been established experimentally. In lieu of three-dimensional structural information for Nmd3p, we present a mutational analysis of the functional organization of Nmd3p, utilizing point mutations as well as deletion mutations.

EXPERIMENTAL PROCEDURES

Strains—Strains used in this work were AJY2110 (MATa ura3-50 his3Δ1 leu2Δ0 lys2Δ0 nmd3Δ:kanMX/pAJ112), AJY1539 (MATa leu2-3 ura3 his3Δ1 met15Δ0 CRM1(T539C)-HA) (5), and W303 (W303 MATa leu2-3,112 his3Δ1 ura3-1 trp1-1 ade2-1 can1-100 SSD1-d). Unless otherwise noted, standard yeast genetics methods and media were used as described in Ref. 11. All strains were grown at 30 °C unless otherwise indicated in rich medium (yeast extract-peptone) or dropout medium (synthetic complete) containing either 2% glucose or 1% galactose.

Plasmids—Plasmids used in this work are listed in Table 1. pAJ582 (NMD3-GFP) was made by amplifying GFP with 5′ oligonucleotide AJO230 (AGAGAATGGAGTCAAGAACACAC-ACCCGTTGAACTCAGCGAGCGATCCGGGTATAATTAA) and 3′ oligonucleotide AJO307 (GCCGAAGCTTGGCCGCGGCGTCGACATGTCCAAGAAGTCGTA) using pFA6a-GFP(S65T)-KanMX6 (12) as template, digesting with Pael and HindIII, and ligating it into the same sites of pAJ538. pAJ583 and pAJ584 were made similarly, using pAJ534 (nmd3ΔA50) and pAJ535 (nmd3Δ100) (1), respectively. Unless otherwise noted, Myc denotes 13 tandem copies of the c-Myc epitope. pAJ367 was made similarly to pAJ536 (NMD3Δ120) (1). To make pAJ752 (NMD3AAA-myc), 5′ oligonucleotide AJO247 (CGGAATTCACGAGCGGGTTAACGACCGTTGAACTCAGCGAGCGATCCGGGTATAATTAA) was used to amplify NMD3AAA from the genomic DNA of W303 using the Pael and HindIII restriction sites.

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4 The abbreviations used are: NES, nuclear export signal; NLS, nuclear localization signal; aa, amino acids; DAPI, 4’,6-diamidino-2-phenylindole; GFP, green fluorescent protein.
Mutational Analysis of NMD3

~CGAGCTCTGCTGCTGGGTCGGCTGCTGAGATTCAACGGGTGTGTTCTCGACTCCAT-
TTT~) into BamHI-HindIII-cut pAJ667. Moving oligonucleotides AJO364 (GATCCCCACAAATCAACATGATTAATGTCATTTCATCTGCCTCGTCGGCTAATTCTACAGCGTTGATTT) and AJO307 (GCGAGATCCGGGCTGCAGCTCGTCTTCATCAATGTTT), respectively, incorporating the underlined mutations. This product was amplified with AJO247 (CGGAATTCCTGTCCAGTTTATGGATC) and AJO885 (GTTCTCGACTTCTTCTGCTGCTTATCATC) was reamplified with AJO247 and AJO887 (GACTTAATTAACCCGGGCTGCCTGAGATCCGGGTGTGTTCTCGACTCCATC). The resulting product was cut with BglII and PacI and cloned into the same sites of pAJ534.

Cysteine Mutants—Each of the eight conserved cysteines was individually replaced with serine using fusion PCR and replacing the wild-type NMD3 open reading frame in pAJ538. All mutations were confirmed by sequencing.

Loss of Function Mutants—The NMD3 open reading frame was mutagenized by PCR using TaqDNA polymerase and primers AJO106 (GCCGCTCGAGACACCATGGAATCCGCTGCTGAGATTCAACGGGTGTGTTCTCGACTCCATTT) and AJO305 (TCCCCGGGCTGCTGAGATCCGGGCTGCAGCTCGTCTTCATCAATGTTT), respectively, incorporating the underlined mutations. To make pAJ571 (NMD3Δ123-myc), 5’ oligonucleotide AJO247 (CGGAATTCCTGTCCAGTTTATGGATC) and 3’ oligonucleotide AJO388 (GCAAGCTTTAGATTAGATTAATGCATTTCGCGCTGGCCTCGAGCATCTCATC) were used to amplify nucleotides 843–1516 of NMD3. This product was then amplified with AJO247 (CGGAATTCCTGTCCAGTTTATGGATC) and AJO388 (GCAAGCTTTAGATTAGATTAATGCATTTCGCGCTGGCCTCGAGCATCTCATC) respectively, incorporating the underlined mutations. To make pAJ571 (NMD3Δ123-myc), 5’ oligonucleotide AJO247 (CGGAATTCCTGTCCAGTTTATGGATC) and 3’ oligonucleotide AJO388 (GCAAGCTTTAGATTAGATTAATGCATTTCGCGCTGGCCTCGAGCATCTCATC) were used to amplify nucleotides 843–1516 of NMD3. This product was then amplified with AJO247 (CGGAATTCCTGTCCAGTTTATGGATC) and AJO388 (GCAAGCTTTAGATTAGATTAATGCATTTCGCGCTGGCCTCGAGCATCTCATC) respectively, incorporating the underlined mutations. To make pAJ571 (NMD3Δ123-myc), 5’ oligonucleotide AJO247 (CGGAATTCCTGTCCAGTTTATGGATC) and 3’ oligonucleotide AJO388 (GCAAGCTTTAGATTAGATTAATGCATTTCGCGCTGGCCTCGAGCATCTCATC) were used to amplify nucleotides 843–1516 of NMD3. This product was then amplified with AJO247 (CGGAATTCCTGTCCAGTTTATGGATC) and AJO388 (GCAAGCTTTAGATTAGATTAATGCATTTCGCGCTGGCCTCGAGCATCTCATC) respectively, incorporating the underlined mutations.

Indirect Immunofluorescence—Indirect immunofluorescence was performed as described previously (1). Antibodies used were monoclonal α-c-Myc (9E10) for the primary antibody (1:1000 dilution; Covance), and the secondary antibody was Cy2-conjugated α-mouse antibody (1:300 dilution; Jackson IRL). DNA was stained with DAPI. Fluorescence was visualized on a Nikon E800 microscope fitted with an x 100 objective and a
Diagnostic Instruments SPOT II camera controlled with SPOT software. Images were prepared using Adobe Photoshop 5.0.

**GFP Localization**—Culture conditions are given in the corresponding figure legends. Cultured cells were fixed with a 1:9 volume of 37% formaldehyde for 40 min. The cells were washed twice with 0.1 M potassium phosphate, pH 6.6, and resuspended in 0.1 M potassium phosphate, pH 6.6, 1.2 M sorbitol. 0.05% Triton X-100 was added to permeabilize cells. After 4 min, 1 μg/ml DAPI was added to stain nuclei. After an additional 2 min, cells were washed twice with PBS and visualized as described for indirect immunofluorescence.

**Immunoprecipitations**—For immunoprecipitations of Myc-tagged Nmd3 proteins, cultures were grown as indicated in the respective figure legends. All subsequent steps were carried out at 0–4 °C. Cells were thawed and washed in immunoprecipitation buffer (10 mM Tris, pH 7.6, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin and pepstatin A). The cells were resuspended in one volume of immunoprecipitation buffer, and extracts were made by glass bead lysis (5 × 50 s with 1-min intervals on ice). Insoluble material was pelleted by centrifugation at 15,000 × g for 10 min at 4 °C. 1.5 μl of α-c-Myc (9E10 monoclonal; Covance) antibody was added to equal A260 units of sample supernatants and rocked for 1 h at 4 °C. 30 μl of bovine serum albumin-blocked protein A-agarose beads (Invitrogen) were then added, and rocking was continued for an additional 1 h. Beads were washed three times with immunoprecipitation buffer and eluted in 50 μl of 1× Laemmli sample buffer without β-mercaptoethanol. Proteins were run on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose for Western blotting using α-c-Myc or α-Rpl8p antibodies.

**Leptomycin B (LMB) Treatment**—For LMB treatment, overnight cultures were diluted to an A260 of ~0.1 and incubated for 6 h at 30 °C. They were then concentrated 10-fold in fresh medium. LMB (from M. Yoshida or LC Laboratories) was added at 6 h at 30 °C. The cells were then concentrated 10-fold in fresh medium. LMB (from M. Yoshida or LC Laboratories) was added at 6 h at 30 °C. They were then concentrated 10-fold in fresh medium.

**Results**

**Deletion Analysis**—A series of c-Myc-tagged N-terminal and C-terminal truncations of Nmd3p (Fig. 1A) were expressed in yeast from low copy centromeric (CEN) plasmids under control of the NMD3 promoter. All of the N-terminal deletions tested (Δ123, Δ167, Δ192) were unable to complement an nmd3 deletion (data not shown). The C-terminal truncations (nmd3Δ50, nmd3Δ100, and nmd3Δ120) have been described previously (1). Selected truncated proteins were tested for their ability to bind to 60 S subunits using a co-immunoprecipitation assay. Samples were analyzed by SDS-PAGE, followed by Western blotting for Nmd3p and for Rpl8p as a reporter for 60 S subunits. Removal of up to 120 amino acids from the C terminus of the protein did not affect binding to 60 S subunits (Fig. 1B). However, removal of an additional 74 amino acids (NMD3Δ194) completely disrupted 60 S interaction in this assay. Deletion of 123 amino acids from the N terminus of Nmd3p, including the first three Cys-X-X-Cys motifs, led to reduced binding to 60 S subunits, whereas deletion of 167 or more amino acids, including all four Cys-X-X-Cys motifs, completely abolished 60 S binding as indicated by the lack of co-immunoprecipitation of Rpl8p (Fig. 1B). From these results, we conclude that the N terminus of Nmd3p (aa 1–398), which serves in archaeal Nmd3p orthologs, is required for 60 S binding.

**Nuclear Export**—The C-terminal 33 amino acids of Nmd3 contain a leucine-rich NES (Fig. 2, A and B) (1, 2). Deletion of this region was lethal in some strain backgrounds (1) but supported extremely weak growth in the S288c-related background BY4742 (Fig. 2D and data not shown). A helical projection of this sequence shows its predicted amphipathic structure (Fig. 2C). We introduced point mutations into the NES that were predicted to disrupt the hydrophobic face of the amphipathic helix (Fig. 2B). Changing the hydrophobic residues Leu496 and Leu497 had little effect on cell growth, whereas changing Ile493, Leu497, and Leu500 all to alanine significantly impaired growth. Remarkably, changing the single leucine at position 505 to alanine also had a profound affect on growth (Fig. 2C). To ask what is sufficient for nuclear export, we subcloned amino acids 489–501 (PQINIDELLDEL) and 485–505 (DEDAPQINIDELLDEMTL) separately into a reporter composed of the 175-kDa cytoplasmic protein Xrn1p fused to the well characterized NLS of SV40 large T antigen and GFP. The Xrn1p-NLS-GFP reporter (13) alone was nuclear, and the addition of the shorter peptide (aa 489–501) did not change this distribution. On the other hand, the longer peptide (aa 485–505) was functional as an NES, since it led to cytoplasmic accumulation of the reporter (Fig. 2B). Interestingly, the double point mutation (L496A,L497A) blocked nuclear export of the Xrn1 reporter (Fig. 3), although it did not block export of full-length Nmd3 (Fig. 4). The triple point mutation significantly inhibited export of both the reporter (Fig. 3) and full-length
Nmd3 (Fig. 4). Since the double mutation blocked export of the Xrn1 reporter but not in the context of full-length Nmd3p, we conclude that sequences outside its NES contribute to export activity. Based on sequence alignment and conservation, we previously suggested that Nmd3p contains a noncanonical NES. However, the importance of L505 for nuclear export leads us to propose a slightly different alignment that is a perfect match to canonical leucine-rich NESs (see Fig. 2C).

It has been suggested that Nmd3p contains a second NES (aa 445–456) (2) within a region predicted to form a short coiled-coil (aa 446–468). Deletion of this domain resulted in a reduction in cell growth rate (Fig. 2D) but did not lead to accumulation of the protein within the nucleus (Fig. 4). On the other hand, combining the coiled-coil deletion with the double point mutation in the NES (L496A,L497A) severely impaired growth, although this NES mutant alone grew at nearly wild-type rates (Fig. 2D). In addition, this mutant protein accumulated in the nucleus (Fig. 4). Combining the coil deletion with the triple point mutation was lethal (Fig. 2D).

Although the coiled-coil region does not support efficient export, it does significantly enhance the function of the canonical NES. Because coiled-coils are typically protein-protein interaction motifs, this region of Nmd3p may enhance the function of the canonical NES by recruiting an additional factor that stabilizes the interaction of Nmd3p with Crm1p. Residual interaction with this factor in the absence of the canonical NES may account for the weak export activity of this region.

Nuclear Localization—We previously showed that deletion of the C-terminal 120 amino acids of Nmd3p was lethal and resulted in a cytoplasmic localization of the protein, due to removal of sequences that are necessary for nuclear localization (1). Amino acids 399–418 are highly basic (50% Arg and Lys), typical of an NLS. To test if this region was entirely responsible for NLS activity, an internal deletion encompassing these residues was made. This mutant protein localized to the cytoplasm but accumulated in the nucleus (Fig. 4). LMB blocks Crm1 interaction with its ligands (15, 16), thereby trapping molecules dependent on Crm1 export in the nucleus. Furthermore, this mutant was viable, albeit slowly growing (Fig. 2D). These results indicate that nuclear import was impaired but not abolished in this mutant.

Interestingly, when fused to GFP as a reporter, amino acids 399–418 were not sufficient to localize GFP to the nucleus (data not shown), but a larger region (aa 387–434) was sufficient (1). This larger region included a highly conserved patch of hydrophobic residues immediately upstream of the basic region implicated in NLS activity. Nmd3p deleted of the hydrophobic patch (aa 384–398) or both the hydrophobic patch and the basic region (aa 383–418), was non-complementing (Fig. 2D) but retained 60 S binding (data not shown). However, only removal of the entire region (aa 384–418) resulted in a complete inability of Nmd3p to localize to the nucleus, even in the presence of LMB (Fig. 4). Thus, the hydrophobic patch (aa 384–398) and the basic region (aa 399–418) are necessary and sufficient for nuclear localization activity.
Nmd3 Loss of Function Mutants—Deletion analysis identified the shuttling sequences of Nmd3p and demonstrated that the conserved amino-terminal domain was responsible for 60 S subunit binding. However, it did not provide finer mapping of residues within this domain that are responsible for binding to the 60 S subunit. To address this, we screened for missense mutations that led to loss of function, with the expectation that among these would be mutants with reduced 60 S binding. We used PCR mutagenesis and a colony color-based plasmid shuffle technique to identify mutants (see “Experimental Procedures”). From this screen, we identified 16 independent missense mutants (Table 1). These mutations can be grouped roughly into three classes: mutations of cysteines within the Cys-X_{2}-Cys motifs; mutations clustered in the region of aa 80–115; and mutations of hydrophobic residues in the region of aa 230–340. Several mutants showed incomplete loss of function. In addition, because several mutants appeared to adversely affect growth of wild-type cells, we scored all mutants for dominant negative effects when expressed in wild-type cells (Table 2). Interestingly, both loss of function and dominant gain of function mutants were obtained by different substitutions at His^{108} (Table 2). Although these mutants did not show obvious differences in 60 S binding by an immunoprecipitation assay, H108R suppresses lsg1 and rpl10 mutants (see below). We have shown that one mechanism of suppression of rpl10 and lsg1 mutants is by facilitating release of Nmd3p from 60 S subunits (5). Thus, the dominant H108P mutant may impair release of Nmd3p from 60 S subunits in the cytoplasm.
Mutational Analysis of NMD3

The loss of function mutants were tested for their ability to bind to 60 S subunits by assaying for co-immunopurification of 60 S subunits with c-Myc-tagged mutant Nmd3 proteins. The mutants showed variable levels of 60 S binding with a general correlation between the degree of loss of 60 S binding and the lack of complementation (Fig. 5 and Table 2). All mutants that retained partial in vivo function also retained 60 S binding, whereas mutants that showed a significant loss of 60 S binding were unable to complement an nmd3 deletion mutant.

Cysteine Mutants—The screen for loss of function mutants identified five mutations that affected three of the universally conserved cysteine residues (Cys19, Cys21, and Cys46). Because of the high frequency of cysteine mutants, we decided to mutate each of the eight cysteines in these putative zinc binding motifs to serine, an amino acid of similar size but unable to coordinate Zn$^{2+}$ ion. The viability of the C146S mutant may indicate that this residue is not absolutely essential for zinc binding. Changing Cys146 to arginine, a bulkier residue that could sterically block Zn$^{2+}$ binding or cause charge repulsion, resulted in a more severe growth defect (data not shown). As with the random loss of function mutants, the cysteine mutants showed a close correlation between 60 S binding and ability to complement an nmd3 deletion (Fig. 6).

**Table 1**

Plasmids used in this study

| Plasmid | Description | Reference |
|---------|-------------|-----------|
| pAJ377  | NMD3Δ133-myc LEU2-CEN |          |
| pAJ414  | NMD3-myc URAS3-2a |          |
| pAJ515  | nmd3Δ2-myc URAS3-2a |          |
| pAJ516  | nmd3Δ3-167-myc URAS3-2a |          |
| pAJ517  | nmd3Δ192-myc URAS3-2a |          |
| pAJ534  | NMD3Δ40-myc LEU2-CEN |          |
| pAJ535  | NMD3Δ100-myc LEU2-CEN |          |
| pAJ536  | nmd3Δ120-myc LEU2-CEN |          |
| pAJ537  | nmd3Δ194-myc LEU2-CEN |          |
| pAJ538  | NMD3-myc LEU2-CEN |          |
| pAJ582  | NMD3-GFP LEU2-CEN |          |
| pAJ583  | NMD3Δ50-GFP LEU2-CEN |          |
| pAJ584  | NMD3Δ100-GFP LEU2-CEN |          |
| pAJ670  | XR11-NLS-GFP URAS3-2a |          |
| pAJ671  | XR11-NLS-GFP [N189–201] URAS3-2a |          |
| pAJ673  | XR11-NLS-GFP [N83–95] URAS3-2a |          |
| pAJ676  | XR11-NLS-GFP [N189–201] URAS3-2a |          |
| pAJ677  | XR11-NLS-GFP [N83–95] URAS3-2a |          |
| pAJ678  | XR11-NLS-GFP [N83–95] URAS3-2a |          |
| pAJ751  | NMD3ΔAA-myc LEU2-CEN |          |
| pAJ752  | NMD3AAA-myc LEU2-CEN |          |
| pAJ753  | NMD3AAA-GFP LEU2-CEN |          |
| pAJ754  | NMD3AAA-GFP LEU2-CEN |          |
| pAJ1358 | NMD3ΔNLS [N399–418] myc LEU2-CEN |          |
| pAJ1359 | NMD3ΔNLS [N384–418] myc LEU2-CEN |          |
| pAJ1360 | NMD3ΔNLS [N384–418] myc LEU2-CEN |          |
| pAJ1416 | NMD3ΔC446–468-myc LEU2-CEN |          |
| pAJ1423 | NMD3ΔCC4AA-myc LEU2-CEN |          |
| pAJ1424 | NMD3ΔCC4AA-myc LEU2-CEN |          |
| pAJ1593 | NMD3L505A-myc LEU2-CEN |          |

**Table 2**

NMD3 loss of function alleles

| Loss of function allele | Δnmd3 complementation | Dominant negative | Cellular localization without LMB | With LMB | 60 S binding | rp10[G161D] suppression | LSG1[K349T] suppression |
|------------------------|-----------------------|-------------------|----------------------------------|----------|-------------|------------------------|------------------------|
| Wild type              | ++ +                  |                   | n/c                             | n/c      | n + + +     | –                      | –                      |
| C35R                   |                       |                   | c (foci)                         | n/c      | + + +       | –                      | –                      |
| C56R,Y,S               | –                     |                   | c (foci)                         | n/c      | + + +       | –                      | –                      |
| L82P                   | +                     |                   | n/c                             | n/c      | + + +       | –                      | –                      |
| E106K                  | +                     |                   | n/c                             | n/c      | + + +       | –                      | –                      |
| H108P                  | –                     | + +                 | n/c                             | n/c      | + + +       | –                      | –                      |
| H108R                  | +                     | + +                 | n/c                             | n/c      | + + +       | –                      | –                      |
| S109F                  | –                     | –                   | c (foci)                         | n/c      | – + + +     | –                      | –                      |
| C146R                  | +                     | –                   | c (foci)                         | n/c      | – + + +     | –                      | –                      |
| S230P                  | –                     | + +                 | c (foci)                         | n/c      | – + + +     | –                      | –                      |
| V340D                  | –                     | + +                 | c (foci)                         | n/c      | – + + +     | –                      | –                      |
| I103V, Q142R, Q160H    | +                     | –                   | c (foci)                         | n/c      | + + +       | + + + +                | –                      |
| I139T, L259S, L296P    | –                     | –                   | c (foci)                         | n/c      | + + +       | + + + +                | –                      |
| L263F, F3181           | ND*                   | –                   | c (foci)                         | n/c      | – + + +     | + + + +                | –                      |

* n, nucleoplasmic; c, cytoplasmic.
* ND, not determined.

5 M. West, J. Hedges, K-Y. Lo, and A. W. Johnson, unpublished results.
Plasmids harboring suppressing NMD3 mutants were isolated and sequenced (Table 3). Suppressing mutations mapped predominantly to two regions of Nmd3p (Fig. 7A). The first region (approximately aa 80–115) contains several basic residues predicted by hydrophobic cluster analysis (20) to form the edge of a β-strand and could represent an RNA binding surface. The second region (approximately aa 290–380) spans the region in which suppressor mutations were identified previously (Fig. 7A) (17). The strongest suppressor, I112T,I362T, contained two mutations, one in each domain. Subcloning each mutation revealed that both contribute to suppression of rpl10[G161D] (Fig. 7C).

We have recently shown that the cytoplasmic GTPase Lsg1p as well as Rpl10p are required for release of Nmd3p from 60 S subunits in the cytoplasm (5). Furthermore, we have proposed that Lsg1p facilitates Rpl10p loading into the subunit, thereby releasing Nmd3p. We also showed that nmd3[I112T,I362T] and nmd3[L291F] can suppress mutations in both RPL10 and LSG1. Consequently, we tested our panel of nmd3 mutants isolated from the rpl10 mutant screen for their ability to suppress rpl10[G161D] and the LSG1[K349T] mutant that contains a mutation in the essential lysine of the Walker A motif necessary for coordinating Mg²⁺. In general, we found that mutations that suppressed rpl10[G161D] also suppressed LSG1[K349T] with the strongest suppressor being NMD3[I112T,I362T] (Fig. 7B).

Several of the mutations identified in the screen for loss of function mapped to the same domains as the suppressor mutations. Because several of these showed only partial loss of function, we tested them for their ability to suppress rpl10[G161D] and LSG1[K349T]. Interestingly, two of these mutants (L82P and H108R) suppressed both rpl10 and lsg1 mutants. NMD3[L282P] showed relatively weak suppression of rpl10 and lsg1, whereas H108R suppressed the rpl10 and lsg1 mutants to a greater extent (Fig. 7B). The mapping of suppressor mutations to the same regions in which we found loss of function mutants suggests that suppression is due to a partial loss of function in Nmd3p. Mutations in both suppressor domains were found to affect 60 S binding, and nmd3[I112T,I362T] has a reduced affinity for 60 S subunits in vivo and in vitro (5). We suggest that these two domains represent two 60 S binding domains within Nmd3p (Fig. 8).

### DISCUSSION

A complete understanding of the function of Nmd3p and its interaction with the large ribosomal subunit will require structural information about Nmd3. In lieu of this, we have carried out a comprehensive mutational analysis of Nmd3 to map and more clearly define the functional domains of this protein. We show that the N-terminal portion of Nmd3, conserved in all eukaryotes and archaea, contains two domains that contribute to ribosome binding. This region of the protein also contains four Cys-X₇-Cys repeats, found in all Nmd3 orthologs and predicted to coordinate two Zn²⁺ ions. Analysis of each of these cysteines suggests that the first four act together to coordinate one Zn²⁺, whereas the second four coordinate a second Zn²⁺

| Suppressor allele | Δnmd3 complementation | Rpl10[G161D] suppression | LSG1[K349T] suppression |
|-------------------|-----------------------|--------------------------|-------------------------|
| Empty             | −                     | −                        | −                       |
| WT                | +++                   | +                        | +                       |
| T112T,I362T       | +++                   | ++                       | +++                     |
| I112T             | +++                   | +                        | +                       |
| I362T             | +++                   | ++                       | +                       |
| R113G             | +++                   | +                        | +++                     |
| L291F             | +++                   | +                        | +                       |
| L359P             | +++                   | +                        | +++                     |
| N332D,Y379H       | +++                   | +++                      | +++                     |
| V349H,G360D,E476D | +++                   | +++                      | ND⁴                     |

⁴ ND, not determined.

**TABLE 3**

NMD3 suppressor alleles

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**FIGURE 5.** Coimmunoprecipitation of 60 S subunits with nmd3 loss of function mutants. The indicated mutants were expressed with oligomeric C-terminal tags from centromeric vectors in AJY1539. Untagged wild-type Nmd3p was used as a negative control. Extracts were prepared and immunoprecipitated as described under “Experimental Procedures.” Immunoprecipitated proteins were separated by SDS-PAGE, and the presence of tagged mutant Nmd3p or Rpl8p as a ribosomal marker was assessed by Western blotting using anti-c-Myc or anti-Rpl8p, respectively.

**FIGURE 6.** Analysis of cysteine mutants. A, the indicated cysteine residues of Nmd3p were individually replaced with serine. The mutant proteins were expressed from LEU2 centromeric vectors in AJY2104 (nmd3 expressing NMD3 from pAJ112). The ability of the mutant Nmd3 proteins to support growth was assayed by plating 10-fold serial dilutions on 5-fluoroorotic acid-containing plates. B, the mutant proteins were expressed with C-terminal oligomeric c-Myc tags in the wild-type strain AJY1539. Extracts were prepared, and the mutant Nmd3 proteins were immunoprecipitated. The levels of co-immunoprecipitating 60 S subunits, indicated by Rpl8p, were determined by quantitative Western blotting for Rpl8p and Nmd3-Myc using a Li-Cor Odyssey infrared imaging system. The Rpl8p signals, relative to the corresponding Nmd3 signals, were normalized to that of the wild-type Nmd3 control. WT, wild type; IB, immunoblot; IP, immunoprecipitation.

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The first 60 S binding domain is contained within an insert between the third and fourth Cys-X$_2$-Cys repeats. Consequently, the second zinc may organize this binding domain.

As shown previously, the domains C-terminal to the ribosome-binding domain, regulate protein shuttling and are unique to the eukaryotic Nmd3 proteins. These domains, in order, are an NLS, a coiled-coil, and a canonical leucine-rich NES. The NLS of Nmd3 is composed of a highly basic domain (aa 399–418) as well as a short and highly conserved hydrophobic patch immediately upstream (aa 384–398). Together, these two motifs are sufficient for the nuclear localization of an exogenous reporter. Deletion of the coiled-coil region or point mutations in this region (data not shown) did not affect nuclear import. This is in contrast to the case with human Nmd3, where mutations in a large region, spanning the basic region and the coiled coil region, affected import, leading to the conclusion that human Nmd3 contains a complex NLS (4). In addition, human Nmd3 contains nucleolar localization signals within this region that are required for distinct nuclear and nucleolar accumulation. We did not observe obvious differences in subnuclear localization with mutants of yeast Nmd3, which does not show obvious enrichment within the nucleolus.

It has been reported that Kap123 is the primary importer for Nmd3 (8), since the fusion of this NLS to GFP makes the nuclear accumulation of GFP Kap123-dependent. However, unlike Nmd3, Kap123 is not essential, and in our hands, the nuclear import of full-length Nmd3 is not obviously affected by deletion of KAP123, suggesting that an additional import pathway(s) can efficiently replace Kap123.

Nuclear export of Nmd3 depends on its canonical leucine-rich NES. This NES can confer nuclear export activity on exogenous proteins (1, 2). Here, we show that some mutations are well tolerated in the NES when it is in the context of full-length Nmd3 but not when fused to an exogenous reporter. This suggests that other regions of Nmd3 enhance its NES function. One such region appears to be the coiled-coil, since deletion of this region sensitizes the NES point mutants. Nmd3 export is mediated by Crm1, which recognizes the leucine-rich NES.

From our mutational analysis of Nmd3, we conclude that its interaction with the 60 S subunit is divalent, although additional, weaker contacts may also exist. Such a multivalent interaction could provide a means of ensuring that only properly assembled subunits are exported. In the milieu of ribosome assembly, any single interaction between Nmd3 and constituents of the 60 S subunit may not be strong enough for stable interaction with Nmd3. By requiring the correct spatial organization of more than one binding surface, stable interaction with Nmd3 could then be divalent, ensuring that only properly assembled subunits are exported.

FIGURE 7. Mutations that suppress rpl10 and LSG1 mutants. A, schematic of Nmd3 showing the positions of various mutations. B, separate screens were carried out for nmd3 loss of function mutants as well as for mutants that suppressed rpl10[G161D]. These mutants were also tested for their ability to suppress lsg1 mutants. The growth suppression for several of these mutants is shown. (See Tables 2 and 3 for a complete list of mutants.) The nmd3 mutants were expressed in AJY1657 (rpl10G161D), and serial dilutions were plated and incubated at 35 °C (restrictive temperature) or 25 °C (permissive temperature). The mutants were also co-expressed with the dominant negative LSG1[K349T] mutant and plated on galactose plates to induce mutant LSG1 expression. C, both mutations in NMD3[I112T, I362T] contribute to suppression.

FIGURE 8. Schematic diagram portraying divalent binding of Nmd3p to 60 S subunits. The coordination of two Zn$^{2+}$ ions is based on the phenotypes of individual cysteine mutants within the Cys-X$_2$-Cys motifs. BD1 and BD2, the proposed 60 S binding domains of Nmd3p, based on the observation that suppressor mutations and mutations that weaken Nmd3p-60 S interaction map to these regions. The numbers below Nmd3p are the amino acid positions of the approximate boundaries of the indicated domains. CC, coiled-coil.

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strictly dependent on the completion of multiple assembly events on the ribosome, thereby utilizing structural proofreading of the subunit for quality control of exported subunits (21). Along this line of thought, it is also possible that the final steps of assembling the Nmd3 binding site are facilitated by Nmd3 by induced fit. Although there is presently no evidence for this, nascent pre-60 S subunits are highly unstable in the absence of Nmd3 (22), possibly because of failure of correct assembly.

Last, one can imagine that the release of Nmd3 from the subunit in the cytoplasm may be driven by a conformational change in the subunit that alters the spatial organization of its multivalent binding site. We have shown recently that the release of Nmd3 is dependent on loading the ribosomal protein Rpl10 and the activity of the GTPase Lsg1 (5, 6). We proposed that the proper accommodation of Rpl10 into the subunit in the cytoplasm depends on Lsg1, whose GTPase activity may induce a conformational change in the subunit as Rpl10 loads. Rpl10 binds in a deep cleft between the central protruberance and the GTPase stalk, and its presence in the subunit appears to organize this region. Bacterial ribosomes lacking L16, the bacterial counterpart to Rpl10, are in a conformation distinct from intact ribosomes (23). The change in conformation upon Rpl10 loading or the presence of Rpl10 itself could alter the organization of the Nmd3 binding site, disrupting one of its interaction surfaces to initiate release of Nmd3. The release of Nmd3 then may not be the target of Lsg1 function but rather the consequence of conformational changes that are the consequence of Rpl10 loading.

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