Nmd3p Is a Crm1p-dependent Adapter Protein for Nuclear Export of the Large Ribosomal Subunit

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Abstract. In eukaryotic cells, nuclear export of nascent ribosomal subunits through the nuclear pore complex depends on the small GTPase Ran. However, neither the nuclear export signals (NESs) for the ribosomal subunits nor the receptor proteins, which recognize the NESs and mediate export of the subunits, have been identified. We showed previously that Nmd3p is an essential protein from yeast that is required for a late step in biogenesis of the large (60S) ribosomal subunit. Here, we show that Nmd3p shuttles and that deletion of the NES from Nmd3p leads to nuclear accumulation of the mutant protein, inhibition of the 60S subunit biogenesis, and inhibition of the nuclear export of 60S subunits. Moreover, the 60S subunits that accumulate in the nucleus can be coimmunoprecipitated with the NES-deficient Nmd3p. 60S subunit biogenesis and export of truncated Nmd3p were restored by the addition of an exogenous NES. To identify the export receptor for Nmd3p we show that Nmd3p shuttling and 60S export is blocked by the Crm1p-specific inhibitor leptomycin B. These results identify Crm1p as the receptor for Nmd3p export. Thus, export of the 60S subunit is mediated by the adapter protein Nmd3p in a Crm1p-dependent pathway.

Key words: nuclear export • ribosome • Crm1p • Nmd3p • *Saccharomyces cerevisiae*

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Introduction

Eukaryotic ribosome biogenesis requires the transport of cargo into and out of the nucleus through the nuclear pore complex. Ribosomal proteins, synthesized in the cytoplasm, are imported into the nucleus, where their assembly into ribosomal subunits in the nucleolus is coordinated with extensive processing of the rRNAs (Kressler et al., 1999; Venema and Tollervey, 1999). The assembled subunits then must be exported to their site of function in the cytoplasm. Most nuclear export events depend on the small GTPase Ran and specific transport receptors that recognize nuclear export signals (NESs)[1] on the cargo molecules (for reviews see Rout et al., 1997; Mattaj and Englmeier, 1998; Görlich and Kutay, 1999; Nakielny and Dreyfuss, 1999; Pemberton et al., 1998). NESs can be integral components of the cargo molecules or can be provided in trans by adapter proteins that bridge the interaction between cargo and receptors (Mattaj and Englmeier, 1998). These receptors must also interact with the nuclear pore complex to mediate vectorial transfer across the nuclear envelope.

Ribosomal subunit export is a major cellular activity in rapidly growing cells and nuclear/cytoplasmic transport of macromolecules has been a field of great interest. Nevertheless, little is known about nuclear export of ribosomal subunits. Microinjection experiments in *Xenopus* oocytes showed that subunit export is energy dependent and receptor mediated (Bataille et al., 1990). In the yeast *Saccharomyces cerevisiae*, mutations affecting the function of Gsp1p (Ran) inhibit ribosomal subunit export (Hutchison et al., 1969; Hurt et al., 1999; Moy and Silver, 1999). Mutations in several nucleoporins, the structural proteins of the nuclear pore complex, as well as overexpression of the tRNA export factor Los1p (Sarkar and Hopper, 1998), also affect ribosomal subunit export (Hurt et al., 1999; Moy and Silver, 1999). Other export factors in yeast include Crm1p(Xpo1p), required for the export of leucine-rich NES-containing proteins, and Mex67p, an essential mediator of mRNA export (Stade et al., 1997; Segref et al., 1997; Sarkar and Hopper, 1998). However, previous work has not demonstrated that these transport proteins are required for ribosomal subunit transport (Hurt et al., 1999; Moy and Silver, 1999). In addition to the receptors for ribosomal subunit export, the export signals for the ribosomal subunits have not been identified. Furthermore, it is

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[1] *Abbreviations used in this paper:* aa, amino acid; DAPI, 4′-6′-diaminodino-2-phenylindole; GFP, green fluorescent protein; NES, nuclear export signal; NLS, nuclear localization sequence; oligo; oligonucleotide; PKI, cAMP-dependent protein kinase inhibitor.

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The Journal of Cell Biology, Volume 151, Number 5, November 27, 2000 1057–1066
http://www.jcb.org/cgi/content/full/151/5/1057

not known if the NESs for ribosomal subunit export are intrinsic to the subunits or are provided in trans by adapter proteins.

We have been studying the role of Nmd3p in 60S subunit biogenesis in *S. cerevisiae*. We showed previously that a temperature sensitive *nmd3* mutant failed to accumulate 60S subunits at nonpermissive temperature (Ho and Johnson, 1999). We found that the kinetics of rRNA processing in the mutant were similar to the kinetics observed in wild-type cells, however nascent 25S rRNA was extremely unstable, displaying a half-life of only four minutes. On the other hand, 60S subunits that were made before the shift to nonpermissive temperature were stable at nonpermissive temperature (Ho and Johnson, 1999). We interpreted these results to indicate that Nmd3p was required for a late step in the 60S biogenesis pathway after initial subunit assembly in the nucleolus, but was not required for maintaining the integrity of mature cytoplasmic subunits involved in translation.

Nmd3p cosediments on sucrose gradients in the position of free 60S subunits. More recently, we have shown that Nmd3p binds directly to 60S subunits and that 60S subunits can be coimmunoprecipitated with Nmd3p (Ho et al., 2000). This coimmunoprecipitation is specific for free 60S subunits; 40S subunits are not coimmunoprecipitated with Nmd3p. By pulse-chase–labeling ribosomal proteins, followed by coimmunoprecipitation of 60S subunits with Nmd3p, we have shown that nascent 60S subunits are bound by Nmd3p (Ho et al., 2000), which is consistent with its role in biogenesis of the 60S subunit. We show here that Nmd3p shuttles and that it is an essential adapter protein that provides the NES to direct nuclear export of nascent 60S subunits via the Crm1p pathway.

Materials and Methods

Strains and Media

The following strains were used in this study: CH1305 (*MATa ade2 ade3 leu2 lys2-801 ura3-52*) (Kranz and Holm, 1990), MNY7 (*MATa CRM1::KANr leu2⁻ his3⁻ trp1⁻ ura3⁻*/ pDC-*CRM1*), and MNY8 (*MATa CRM1::KANr leu2⁻ his3⁻ trp1⁻ ura3⁻*/pDC-*CRM1T539C*) (Neville and Rosbash, 1999). Standard yeast genetic methods and selective growth media were as described previously (Kaiser et al., 1994).

Plasmid Constructs

The 5′ oligonucleotide (oligo) CTAGTCTAGACTCGAGAAAATGCATCATCATCATCATCATTCCATGGAATTCACACCTATAGATCC and the 3′-deletion oligos CGCGGATCCGTTTGTATAGGTTAACACT, CGCGGATCCGAGCCATTCTCTTTAACTT, and CGCGGATCCGCACAAGAACTACATCAGG were used in PCR with wild-type *NMD3* as template to amplify *NMD3*Δ50, *NMD3*Δ100, and NMD3Δ120, respectively. The products were digested with BamHI and ligated into pAJ408 (*NMD3::13-myc* in pRS315 [CEN *LEU2*]) (Ho et al., 2000), which replaces the corresponding fragment of wild-type *NMD3*. RPL25 was amplified by PCR from genomic DNA using oligos 5′-CGCGGATCCAAAATGGCTCCATCTGGTAT and 5′-CGCTCTAGAAATGTAACCGATTCTGTTAG. The resulting product was digested with BamHI and XbaI and ligated into the same sites of pTS395 (*GAL10::GFP URA3* CEN). *GAL10::NMD3*Δ100 was made by replacing the HpaI to BglII fragment of *NMD3*Δ100 on a CEN *LEU2* plasmid with a *GAL10::NMD3*-containing SmaI to BglII fragment from pAJ118 (Ho and Johnson, 1999). The 5′-oligo CGCGGATCCTTAAATATCGATTATGTGCC and the 3′-oligos GCGAAGCTTATTGAGCTCTTGAATTGTAATCT and CGCAAGCTTGCGGCCGCTTACTGCTGAGATTCAACGGGTGT were used in PCR reactions to amplify the nuclear localization sequence (NLS) and the NLS plus NES of *NMD3,* respectively. The PCR products were digested with BamHI and HindIII and cloned into the same sites of pTD125 (*GFP* CEN *URA3*) to make green fluorescent protein (GFP)–NLS and GFP–NLS–NES fusions, respectively. Gene fusions were made by PCR to express cAMP-dependent protein kinase inhibitor$(PKI)_{WT}$ NES and PKI_{P12} NES fused to NMD3Δ100. The 5′-oligos CGCGAATTCTCCAATGAATTAGCCTTGAAATTAGCAGGTCTTGATATCAACAAGACAATGGAATTTACACCTATAGATCCGCAC and CGCGAATTCTCCAATGAATTAGCCTTGAAATTAGCAGGTGCTGATATCAACAAGACAATGGAATTTACACCTATAGATCCGCAC and the 3′-oligo CTGCATCCAGTATACACACCCA were used to amplify NMD3Δ100. The resulting PCR products, containing PKI_{WT} NES and PKI_{P12} NES on the 5′-end of *NMD3*, were digested with EcoRI and ligated into c-myc–tagged *NMD3*Δ100. Plasmid pAJ412, expressing c-myc–tagged full-length Nmd3p from a *URA3* centromeric vector, was made by ligating the *NMD3*-containing EheI to HindIII fragment from pAJ408 into the SmaI and HindIII sites of pRS416 (Sikorski and Hieter, 1989). Plasmid pAJ235 expressing GST-Nmd3p, under control of the galactose-inducible *GAL1* promoter, was made by ligating the *NMD3*-containing XhoI to HindIII fragment from pAJ118 into the SalI and HindIII sites of pEG(KT) (Mitchell et al., 1993).

Fluorescence Experiments

Indirect immunofluorescence was carried out as described previously (Heyer et al., 1995), using anti–c-myc mAb (Covance) and Cy3-conjugated goat anti–mouse antibody (Amersham Pharmacia Biotech) as primary and secondary antibodies, respectively. Overnight cell cultures were diluted into fresh medium and grown for 4 h before fixation for indirect immunofluorescence or direct visualization of GFP fluorescence. For experiments requiring galactose induction, cells were grown overnight in medium containing 2% raffinose. Cultures were then diluted into fresh medium containing 2% raffinose and grown for an additional 4 h before the addition of galactose to 2%. Cells stained with 4′-6′-diaminodino-2-phenylindole (DAPI) were grown for 40 min in the presence of DAPI at a concentration of 2.5 μg/ml. Fluorescence was visualized using a ZEISS Axiophot microscope fitted with a 100× objective and a Princeton Electronics MicroMAX CCD camera controlled with the IPLab Spectrum *P* software package from Signal Analytics Corp. Captured images were then prepared using Adobe Photoshop® 5.0.

Immunoprecipitation

50-ml cultures of cells were grown and induced as described in the legend to Fig. 3. Extracts were prepared in 20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 10% glycerol, 0.1% NP-40, and 1 mM PMSF and clarified by centrifugation. Extracts were preincubated with protein A beads for 30 min and the beads were then removed. Anti–c-myc mAb was then added and the samples were mixed at 4°C for 2 h. Protein A beads were added and the samples mixed for an additional 30 min. The beads were pelleted by centrifugation, washed three times in extraction buffer, and bound proteins were eluted by heating in SDS-PAGE loading buffer.

Results

Nmd3p is an essential 59-kD protein (Belk et al., 1999; Ho and Johnson, 1999). The NH_2-terminal 43 kD of Nmd3p is conserved from Archaea to Eukarya, but not Eubacteria, and contains four putative Zn^{2+} binding motifs similar to those found in some ribosomal proteins (Rivlin et al., 1999). The eukaryotic members of this protein family contain COOH-terminal extensions consisting of a highly basic cluster followed by hydrophilic and acidic domains (Fig. 1 a). Based on these motifs, we constructed a series of COOH-terminal deletions of Nmd3p. Nmd3Δ50 lacked the COOH-terminal acidic 50 amino acid (aa). Nmd3Δ100 lacked the COOH-terminal 100 aa, but retained the basic cluster. Nmd3Δ120 lacked the COOH-terminal 120 aa, including the 20 aa basic cluster. All mutants were tagged on the COOH-terminal, with 13 tandem copies of the c-myc epitope (Longtine et al., 1998), and expressed from low-copy centromeric plasmids from the *NMD3* promoter. Full-length Nmd3p containing this epitope tag was functional

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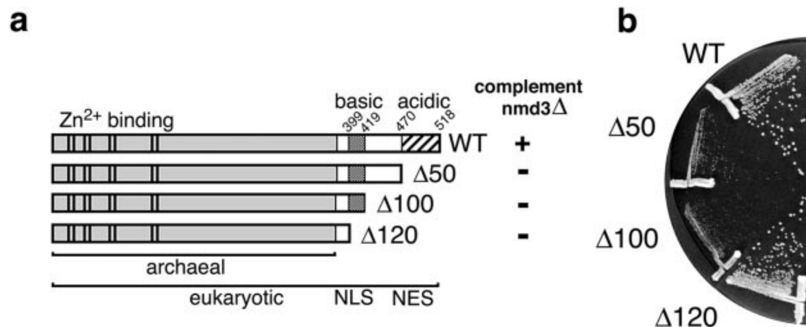

Figure 1. Phenotype of Nmd3p COOH-terminal truncation mutants. (a) A diagram of Nmd3p wild-type and deletion mutants showing the amino-terminal domain conserved in archaebacterial and eukaryotic proteins (gray) containing four Zn^{2+}-binding motifs (cys-x_2-cys), the basic cluster (aa 399–419, stippled), and the acidic COOH terminus (aa 476–518, hatched). NLS and NES indicate approximate positions of NLS and NES, respectively. Numbers indicate aa positions. Complementation was assayed by plasmid shuffle and indicates the ability to replace a plasmid-borne wild-type *NMD3* in an *nmd3::TRP1* disruption strain. (b) The proteins depicted in panel a were tagged with the c-myc epitope and expressed from centromeric vectors in the wild-type yeast strain CH1305 (Kranz and Holm, 1990). Transformants were streaked for single colonies on a selective plate and incubated for 2 d at 30°C.

(Ho et al., 2000) and bound 60S subunits in vivo, as determined by a coimmunoprecipitation assay (Ho et al., 2000). All three truncation mutants were unable to complement an *nmd3* null mutation in a plasmid shuffle assay (Fig. 1 a).

Dominant Mutant Nmd3 Proteins Are Localized to the Nucleus and Inhibit 60S Subunit Biogenesis

Nmd3Δ50 and Nmd3Δ100 inhibited growth when expressed at low levels in *NMD3* wild-type cells (Fig. 1 b). Untagged versions of these truncated proteins inhibited growth only when overexpressed (data not shown), consistent with an earlier observation that overexpression of Nmd3p lacking the COOH-terminal 100 aa inhibited growth (Belk et al., 1999). Although the addition of the multiple c-myc tag to the deletion mutants enhanced their dominant-negative phenotype, this was not specific to the c-myc epitope, as GFP-tagged Nmd3p truncations behaved similarly (data not shown). It is possible that COOH-terminal fusions stabilize the truncated proteins, leading to increased cellular levels of these proteins. Expression of wild-type Nmd3p or Nmd3Δ120 from low-copy plasmids did not adversely affect the growth of wild-type cells. However, Nmd3Δ120 also did not complement the temperature sensitivity of an *nmd3* null mutant, indicating that it was nonfunctional.

Nmd3p is required for a late step in 60S biogenesis (Ho and Johnson, 1999). To determine if the dominant-negative mutants also affected 60S subunit biogenesis, we carried out polysome analysis on sucrose density gradients. Wild-type strains expressing the dominant-negative alleles Nmd3Δ50 and Nmd3Δ100 from low-copy vectors showed reduced 60S subunit levels (Fig. 2 a, Δ50 and Δ100, respectively) and indicate a defect in 60S subunit biogenesis. Similar results had been reported for overexpression of untagged Nmd3Δ100 (Belk et al., 1999). Polysomes from cells bearing Nmd3Δ120 were identical to those from cells containing an empty vector (data not shown), again indicating that Nmd3Δ120 was nonfunctional. Since the production of 60S and 40S subunits is tightly coordinated (Warner, 1989), the specific drop in 60S, but not 40S subunit levels, indicated a defect in the biogenesis of the large subunit, rather than a general downregulation of ribosome biogenesis.

We next examined the intracellular localization of the full-length and truncated proteins. Wild-type Nmd3p was cytoplasmic, as we reported previously (Ho and Johnson, 1999). Surprisingly, Nmd3Δ50 and Nmd3Δ100 were localized to the nucleus (Fig. 2 b, Δ50 and Δ100, respectively), whereas Nmd3Δ120 was cytoplasmic (Fig. 2 b, Nmd3Δ120). These results suggested that the COOH terminus of Nmd3p contains an NES, and the nuclear localization of the mutant proteins lacking the COOH-terminal 50 or 100 aa resulted from a loss of the NES. Furthermore, the nuclear localization of the mutant proteins indicated that Nmd3p also contains a NLS. Since Nmd3Δ120 was cytoplasmic (Fig. 2 b), the 20 aa basic cluster, deleted in Nmd3Δ120 (KKLYQRKSKKSRHWKLKRMA), comprises part of the NLS of Nmd3p (see below). We considered the possibility that Nmd3p does not normally enter the nucleus, and that the Nmd3Δ50 and Nmd3Δ100 were gain of function mutants in which a cryptic NLS was revealed. However, the addition of the strong NLS from SV-40 large T antigen to full-length Nmd3p did not result in nuclear accumulation or a dominant-negative phenotype, whereas a control protein containing this NLS did accumulate in the nucleus (data not shown). The lack of nuclear accumulation of Nmd3p containing the SV-40 NLS probably reflects the rapid export of Nmd3p due to its NES. Consequently, we conclude that the dominant phenotype of Nmd3Δ50 and Nmd3Δ100 was not due to a gain of function from a cryptic NLS. From this analysis of truncation mutants, Nmd3p appears to contain both an NLS and an NES, making it capable of shuttling.

Dominant Mutant Nmd3 Proteins Inhibit 60S Subunit Export

Our results thus far suggested that deletion of an NES from Nmd3p led to retention of the truncated Nmd3p in the nucleus, resulting in inhibition of 60S subunit biogenesis. Since Nmd3p binds directly to 60S subunits (Ho et al., 2000) and is required for a late step in 60S subunit biogenesis, it seemed plausible that the failure to export Nmd3p to the cytoplasm blocked ribosome export from the nucleus. Thus, export itself may be a biogenesis step requiring Nmd3p. An assay for 60S ribosomal subunit export in yeast has been described recently that utilizes a fusion of

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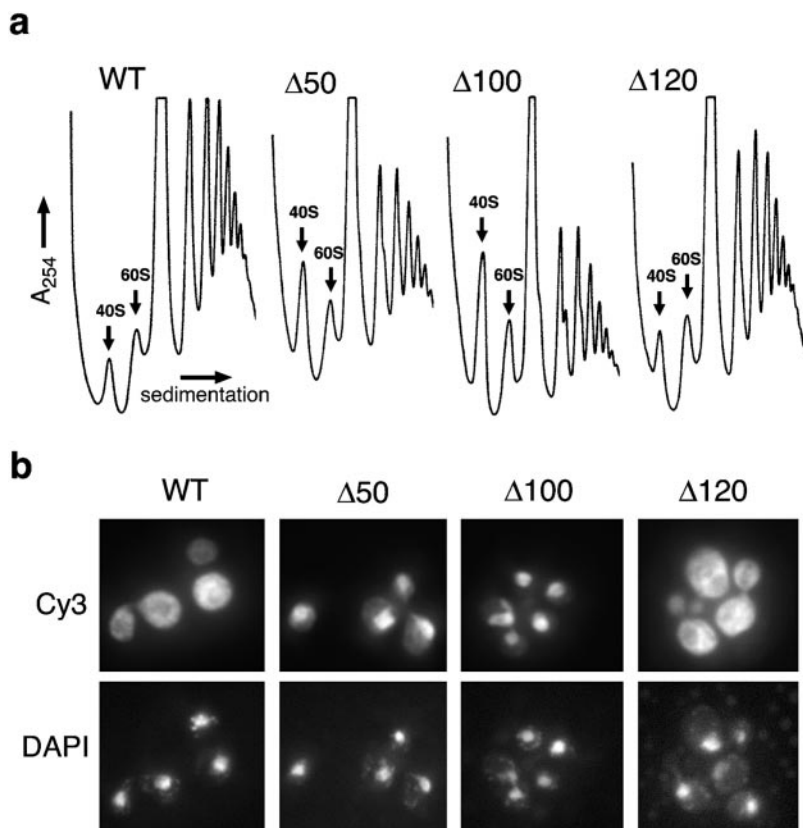

Figure 2. Dominant Nmd3p truncation mutants inhibit 60S biogenesis and are localized to the nucleus. (a) Extracts, prepared from cultures of the transformants shown in Fig. 1 b, were fractionated by ultracentrifugation in 7–47% sucrose gradients, as described previously (Ho et al., 2000). The positions of free 40S and 60S subunits are indicated. The relative peak heights of free subunits are a sensitive indicator of total subunit levels and, hence, biogenesis defects. (b) Indirect immunofluorescence of the c-myc–tagged proteins in yeast. Cy3, rhodamine channel for c-myc–tagged proteins; DAPI, UV channel indicating the position of nuclei.

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GFP to the large ribosomal subunit protein L25 (L25–GFP) (Hurt et al., 1999). This fusion protein is functional and is incorporated into ribosomal subunits, providing a means of monitoring ribosome localization. We reasoned that if Nmd3Δ100 trapped nascent 60S subunits in the nucleus, we should be able to observe nuclear retention of L25–GFP in the presence of Nmd3Δ100. We focused on Nmd3Δ100, since it displayed a stronger dominant phenotype than Nmd3Δ50 (data not shown). Nmd3Δ100 and L25–GFP were put under control of the galactose-inducible *GAL10* promoter and introduced on plasmids into wild-type yeast. Cells coexpressing L25–GFP and wild-type Nmd3p served as a control. Protein expression was induced by the addition of galactose, and the localization of L25–GFP was monitored by fluorescence microscopy. The induction of Nmd3Δ100 led to the accumulation of L25–GFP in the nucleus (Fig. 3 a). In contrast, L25–GFP was not retained in the nucleus and was evident in the cytoplasm in cells expressing wild-type Nmd3p (Fig. 3 c). These results demonstrate that the induction of Nmd3Δ100, which lacks an NES and therefore accumulates in the nucleus, blocked the export of L25–GFP. L25–GFP is a functional protein and can be incorporated into 60S subunits (Hurt et al., 1999). Furthermore, ribosomal proteins that are not incorporated into subunits are often unstable (Woolford and Warner, 1991). Thus, the nuclear retention of L25–GFP likely reflects retention of the entire 60S subunit (see below). In support of this conclusion, we found that when L25–GFP expression was induced in temperature-sensitive *nmd3-4* mutant cells after shift to nonpermissive temperature, no L25–GFP signal was observed, whereas L25–GFP was stably incorporated into cytoplasmic ribosomes if expression was induced before the temperature shift (data not shown). Since *nmd3-4* mutants only affect the stability of nascent 60S subunits, L25–GFP is unstable under conditions that prevent its incorporation into stable subunits.

The binding of Nmd3p to 60S subunits is direct and does not require additional proteins to bridge this interaction (Ho et al., 2000). We have used the 60S-binding activity of Nmd3p to coimmunoprecipitate free 60S subunits bound to c-myc–tagged Nmd3p from cell extracts (Ho et al., 2000). To determine if L25–GFP was incorporated into the ribosomal subunits bound by Nmd3Δ100, we carried out such an immunoprecipitation experiment with Nmd3Δ100. Extracts were prepared from cells coexpressing L25–GFP and Nmd3Δ100, either with or without a c-myc tag. Nmd3Δ100 was immunoprecipitated and the pellet fraction was analyzed by SDS-PAGE and by Western blot for the presence of Nmd3Δ100, L25–GFP, and the wild-type 60S subunit protein L12. We found that both L12 and L25–GFP were coimmunoprecipitated with c-myc–tagged Nmd3Δ100, but not from extracts containing Nmd3Δ100 without the epitope tag (Fig. 4 a). Coomassie blue staining of the gel-separated proteins showed the typical profile of low molecular weight 60S subunit proteins (Fig. 4 b). The identification of these proteins as 60S subunit proteins has been described previously (Ho et al., 2000). These results provide physical evidence that: (a) Nmd3Δ100 binds to 60S subunits, (b) L25–GFP was incorporated into ribosomal subunits, and (c) the L25–GFP–containing subunits trapped in the nucleus were bound by Nmd3Δ100. Since Nmd3Δ100 is blocked for export, but binds to 60S subunits, it is likely that the nuclear retention of the 60S subunits is due to the physical interaction with the truncated Nmd3Δ100 protein trapped in the nucleus.

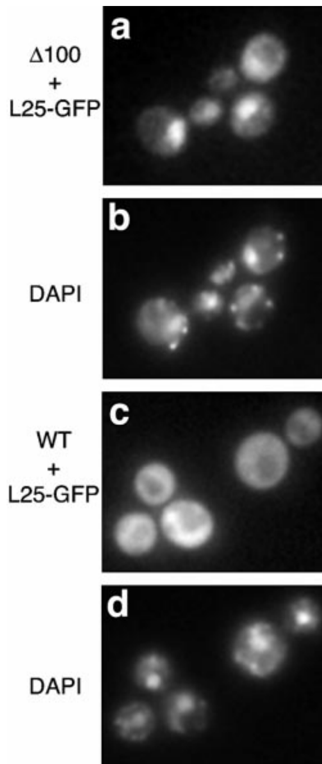

Figure 3. Nmd3Δ100 traps 60S ribosomal subunits in the nucleus. Galactose-inducible L25–GFP and Nmd3Δ100 (13-myc–tagged) or full-length Nmd3p were introduced into wild-type cells (CH1305) on plasmids. Cultures were grown in the presence of raffinose and induced by the addition of galactose. DAPI was added at this time to 2.5 μg/ml. 3 h after induction, the localization of (a and c) L25–GFP or (b and d) DAPI was visualized by fluorescence microscopy. (a and b) Cells coexpressing Nmd3Δ100 and L25–GFP. (c and d) Cells coexpressing full-length Nmd3p and L25–GFP. Similar results were obtained with Nmd3Δ100 without epitope tag (data not shown).

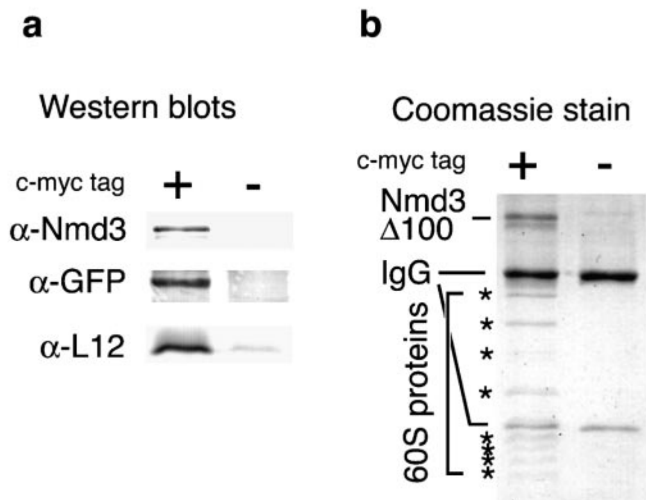

Figure 4. Nmd3Δ100 coimmunoprecipitates 60S subunits containing L25–GFP. (a) Extracts were prepared from cells coexpressing L25–GFP and Nmd3Δ100 with 13-myc (+) or Nmd3Δ100 without 13-myc tag (−). The same amount of total protein from each extract was used. Immunoprecipitation was carried out by the addition of anti–c-myc antibody and protein A beads, and the immunoprecipitated samples were analyzed by SDS-PAGE and Western blot analyisis. Nmd3, Nmd3Δ100; GFP, L25–GFP; L12, wild-type 60S subunit protein L12. (b) Gel-separated proteins from panel a were stained with Coomassie blue. Nmd3Δ100, immunoglobulin heavy and light chains (IgG), and ribosomal proteins (asterisks) are indicated. The identity of the low molecular weight proteins as 60S subunit proteins was confirmed by direct comparison with purified 60S subunits by SDS-PAGE and by Western blot analysis with multiple antibodies specific for 60S proteins (data not shown).

Nmd3p Shuttles

To test if the putative transport signals of Nmd3p could direct the cellular localization of other proteins, we fused the putative NLS (aa 387–435) and the putative NLS plus NES (aa 387–518) to GFP. GFP containing the putative NLS alone (GFP-NLS) was predominantly nuclear (Fig. 5), whereas free GFP was present throughout cells (data not shown). Thus, aa 387–435 are sufficient for directing the nuclear localization of proteins and comprise an NLS. The addition of the putative NES-containing COOH terminus of Nmd3p to GFP-NLS (GFP+NLS+NES) relocalized the protein to the cytoplasm (Fig. 5), suggesting that the COOH terminus of Nmd3p can act as an NES. It could be argued from these experiments alone that the putative NES of Nmd3p simply masked the function of the NLS and, therefore, did not act as an NES. However, subsequent experiments showed that both full-length Nmd3p and GFP+NLS+NES could be trapped in the nucleus in a Crm1p-dependent fashion (see below), demonstrating that these proteins shuttle and contain a functional NES.

Rescue of 60S Biogenesis by the Addition of a Heterologous NES on Nmd3Δ100

The dominant inhibition of 60S subunit biogenesis by Nmd3Δ100, without overexpressing the mutant protein, was likely due to competition between the truncated mutant protein and wild-type Nmd3p for binding to nascent 60S subunits in the nucleus. Because Nmd3Δ100 accumulated in the nucleus, its localized concentration was greater than that of wild-type Nmd3p, which is in the nucleus only transiently, allowing for efficient competition even without overexpression. (Nmd3Δ120 was not dominant negative, presumably because it lacked both the NLS and NES and could not enter the nucleus.) We surmised that nascent 60S subunits bound by mutant Nmd3Δ100 become trapped in the nucleus due to the lack of an export signal. If Nmd3Δ100 sequesters nascent 60S subunits in the nucleus, we should be able to simultaneously restore export of Nmd3Δ100 and 60S subunit production by appending a heterologous NES to Nmd3Δ100. We fused the leucine-rich NES of cAMP-dependent protein kinase inhibitor (PKI_{WT}) (Wen et al., 1995; Stade et al., 1997) to the NH_2 terminus of Nmd3Δ100 (Fig. 6 a). We used a mutant PKI NES (PKI_{P12}), which contained a single aa change that inactivates its NES function (Wen et al., 1995), as a negative control. Nmd3Δ100 containing the PKI_{WT} NES was localized to the cytoplasm (Fig. 6 b), whereas Nmd3Δ100, which contained the PKI_{P12} NES remained in the nucleus (Fig. 6 b). More importantly, Nmd3Δ100, which contained a functional NES, complemented an *nmd3-4* temperature-sensitive mutant (Fig. 7 a) and was able to replace *NMD3* in an *nmd3::TRP1* disruption mutant (data not shown).

Since an *nmd3-4* mutant is unable to produce 60S subunits at nonpermissive temperature (Ho and Johnson, 1999), complementation of this mutant suggested that Nmd3Δ100, containing a functional NES from PKI, supported 60S biogenesis. To determine if this was true, we examined 60S subunit levels on sucrose gradients. After incubation at nonpermissive temperature for 3 h, *nmd3-4* cells containing an empty vector displayed a significant decrease of 60S subunit levels compared with cells bearing a wild-type copy of *NMD3* (Fig. 7 b, vector compared with *NMD3*). The 60S subunit defect was enhanced with longer incubation times and resulted in the arrest of cell growth

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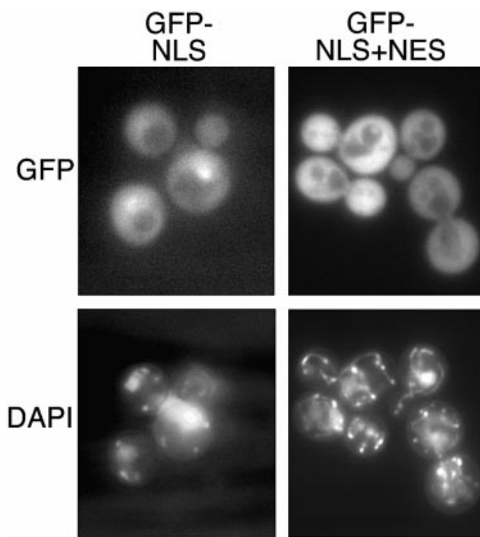

Figure 5. Localization of GFP directed by NLS and NES of Nmd3p. The putative NLS of Nmd3p (aa 387–435) and the NLS plus the NES (aa 387–518) were fused to GFP and introduced into wild-type cells (CH1305). The localization of the GFP fusion proteins was determined by direct fluorescence (GFP), and DNA was stained with DAPI in living cells. The multiple small DAPI-stained spots are mitochondrial DNA.

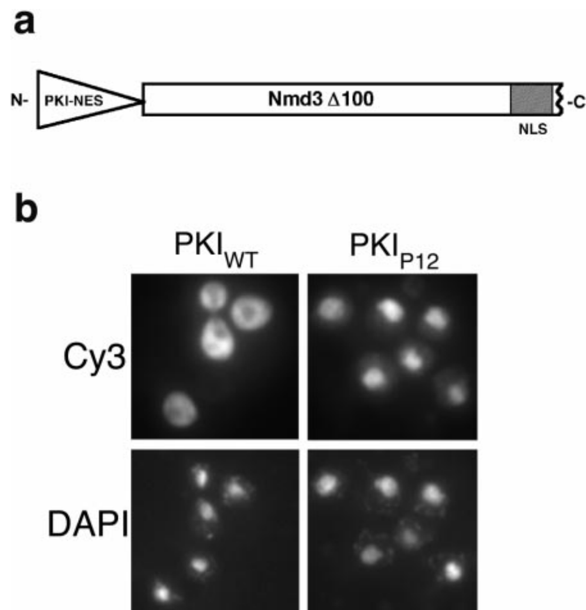

Figure 6. The addition of a functional NES to Nmd3Δ100 relocalizes the protein to the cytoplasm. (a) A cartoon is shown of the addition of a heterologous NES to the NH_2 terminus of Nmd3Δ100. (b) The fusion proteins were expressed from centromeric plasmids in the wild-type strain (CH1305). The localization of the proteins was determined by indirect immunofluorescence (Cy3), and DNA was stained with DAPI.

(data not shown). We have shown previously that this drop in 60S subunit levels was due to the rapid turnover of nascent subunits and not the result of instability of mature subunits (Ho and Johnson, 1999). In contrast, cells containing PKI_{WT}–Nmd3Δ100 supported 60S biogenesis (Fig. 7 b, PKI_{WT}), which is indicated by the increased ratio of 60S to 40S and the absence of halfmers compared with vector alone. The elevated level of free 40S was the consequence of depressed 60S levels and is typical of mutants with defects in 60S biogenesis. Although 60S subunit levels were slightly lower in cells expressing PKI_{WT}–Nmd3Δ100 compared with wild-type, this polysome profile was stable over time at nonpermissive temperature (Fig. 7; and data not shown). Cells containing PKI_{WT}–Nmd3Δ100 showed only a slight growth defect compared with wild-type cells (data not shown), which is consistent with a slightly lower 60S levels in the PKI_{WT}–Nmd3Δ100–containing cells compared with wild-type. The simultaneous restoration of 60S subunit biogenesis and cytoplasmic relocalization of Nmd3p, by the addition of a functional NES to Nmd3 Δ100, strongly suggests that Nmd3p mediates export by providing an NES for nascent 60S subunits. Nmd3Δ100 did assemble onto nascent 60S subunits, since we were able to coimmunoprecipitate these subunits with Nmd3Δ100. However, the addition of a functional NES was required to restore function to the truncated protein, indicating that the primary deficiency of the mutant Nmd3Δ100 protein was the lack of an export signal.

Nmd3p Export Requires Crm1p

Within the COOH-terminal 50 aa of Nmd3p, aa 491–500 (INIDELLDEL) are highly conserved and are predicted to form an amphipathic helix with isoleucine and leucine predominantly on one face. Such a structure is characteristic of a leucine-rich NES (Rittinger et al., 1999), the ligand for the export receptor Crm1p (Fornerod et al., 1997; Stade et al., 1997). This prompted us to examine the dependence of Nmd3p shuttling on Crm1p. We first expressed c-myc–tagged full-length Nmd3p or a GFP-reporter protein fused to the NLS and NES of Nmd3p in the temperature-sensitive *crm1(xpo1-1)* mutant. We were unable to detect nuclear accumulation of these proteins in this strain at nonpermissive temperature (data not shown).

Since temperature shifts have global effects on cell growth and give rise to transient inhibition of ribosome biogenesis (Warner, 1999), we decided to examine Nmd3p localization under conditions in which Crm1p was specifically inhibited. Leptomycin B is an antibiotic inhibitor of Crm1p in most eukaryotic cells (Nishi et al., 1994; Kudo et al., 1998). Although wild-type *S. cerevisiae* is not sensitive to leptomycin B, a single aa change within *S. cerevisiae* Crm1p (threonine 359 to cysteine) renders cells sensitive to leptomycin B (Neville and Rosbash, 1999). We introduced plasmids expressing c-myc–tagged full-length Nmd3p or GFP containing the NLS and NES of Nmd3p into leptomycin-sensitive cells. Cultures of these cells were treated with leptomycin B, and the localization of Nmd3p or GFP was monitored by fluorescence microscopy. The addition of leptomycin B led to the accumulation of Nmd3p within the nucleus in the majority of cells of the leptomycin B–sensitive strain (Fig. 8 b). Nmd3p remained cytoplasmic in wild-type leptomycin B–resistant cells (Fig. 8 a) and in the leptomycin B–sensitive strain in the absence of antibiotic (Fig. 8 c). The nuclear retention of Nmd3p was observed within 15 min of the addition of leptomycin B and persisted for more than 1 h (data not shown). Similar results were obtained with the GFP+NLS+NES reporter (Fig. 9). Since this GFP reporter con-

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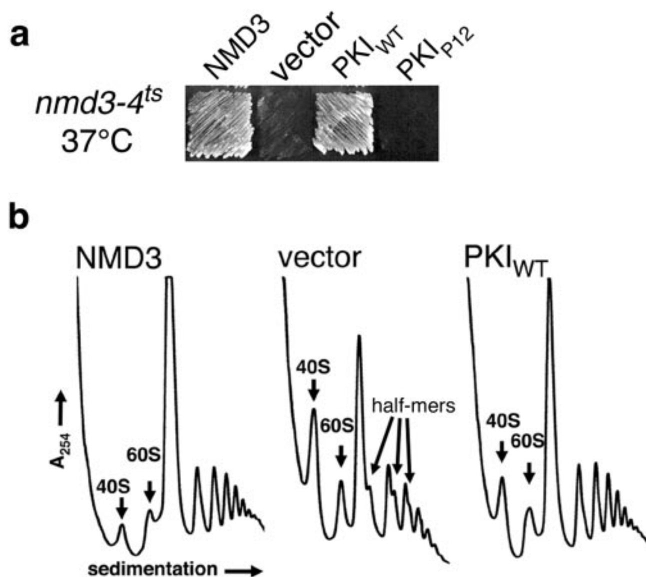

Figure 7. Rescue of Nmd3Δ100 function with a heterologous NES. (a) Plasmids expressing wild-type Nmd3p (NMD3), no protein (vector), and Nmd3Δ100 containing the PKI_{WT} or mutant PKI_{P12} NES were introduced into the temperature-sensitive *nmd3-4* mutant. Transformants were patched onto selective medium and grown for 2 d at 37°C. Similar results were obtained in an *nmd3::TRP1* disruption strain (data not shown). The dominant-negative effect of Nmd3Δ100 containing the mutant NES (PKI_{P12}) severely impaired cell growth even at permissive temperature, leading to a phenotype more severe than that of *nmd3-4* cells containing an empty vector. (b) Sucrose gradient analysis was performed. Cultures of the transformants shown in panel a were grown at permissive temperature, shifted to 37°C for 3 h, and the status of ribosomal subunits and polysomes was analyzed by sucrose gradient sedimentation, as described previously (Ho et al., 2000). Nmd3Δ100 containing the mutant NES (PKI_{P12}) was not analyzed due to its severe growth defect. The positions of free 40S, free 60S, and halfmers (polysomes containing an unjoined 40S subunit at the initiation site resulting from the deficit of free 60S subunits) are shown.

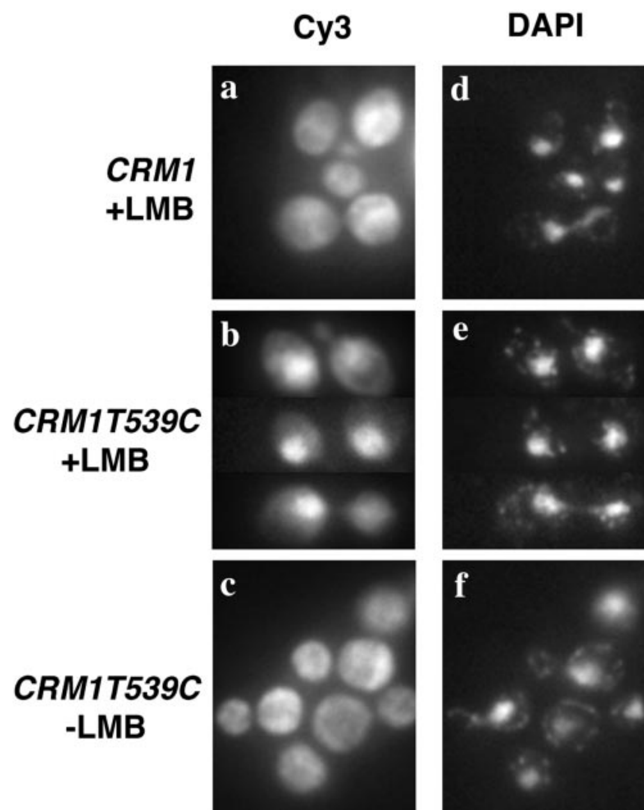

Figure 8. Nmd3p localization is sensitive to the Crm1p inhibitor leptomycin B. (a and d) The wild-type strain MNY7 (*CRM1*) and (b, c, e, and f) the leptomycin-sensitive strain MNY8 (*CRM1T539C*) were transformed with plasmid pAJ412 expressing c-myc–tagged Nmd3p. Cells were grown to early log phase and treated (a, b, d, and e) with (+LMB) leptomycin B (0.1 μg/ml) or (c and f) as controls without (−LMB) leptomycin B and incubated for 15 min. Cells were then fixed and prepared for indirect immunofluorescence. Cy3, rhodamine channel for c-myc–tagged Nmd3p; DAPI, UV channel for DNA. The localization of Nmd3p in *CRM1* cells, in the absence of leptomycin B, was similar to *CRM1* cells in the presence of leptomycin B (data not shown).

tained only the shuttling signals of Nmd3p, the nuclear retention of this protein most likely reflects a direct interaction between the NES of Nmd3p and Crm1p and not an indirect effect mediated by another Crm1p-dependent protein. Thus, inhibition of Crm1p by the addition of leptomycin B, but not by a temperature shift, resulted in nuclear accumulation of Nmd3p. These results suggest that Crm1p is the export receptor for Nmd3p.

If Nmd3p is a Crm1p-dependent adapter for 60S subunit export, 60S subunit export itself should be dependent on Crm1p. 60S subunit export has been reported to be insensitive to temperature shifts in the temperature-sensitive *crm1(xpo1-1)* mutant (Hurt et al., 1999). However, Nmd3p is also not retained in the nucleus under these conditions, though it is trapped in the nucleus by treatment with Crm1p inhibitor leptomycin B. Consequently, we examined the localization of 60S subunits in the presence of leptomycin B. In this experiment we simultaneously treated cells with leptomycin B and induced the expression of L25–GFP to monitor the localization of nascent-60S subunits. L25–GFP is incorporated into nascent subunits (see above), giving rise to functional 60S subunits (Hurt et al., 1999). After 4 h of treatment, we observed a strong nuclear accumulation of L25–GFP in leptomycin-sensitive cells treated with leptomycin B (Fig. 10, *CRM1T539C*). In this experiment, we could detect a signal for L25–GFP in the nucleus within 2 h (data not shown), though the signal was considerably more intense after 4 h. In the leptomycin-insensitive strain, L25–GFP accumulated in the cytoplasm and was not evident in the nucleus (Fig. 10, *CRM1*). L25–GFP accumulated in the cytoplasm in both strains in the absence of leptomycin B (data not shown). Thus, like Nmd3p export, the export of 60S subunits was blocked in a leptomycin B–sensitive manner. The nuclear retention of L25–GFP in a Crm1p-dependent manner suggests that 60S subunit export itself is mediated by Crm1p. Taken together, these results provide compelling evidence that Nmd3p acts as a Crm1p-dependent adapter for the export of the 60S ribosomal subunit.

Discussion

Our delineation of an Nmd3p- and Crm1p-dependent export pathway for the 60S ribosomal subunit is the first re-

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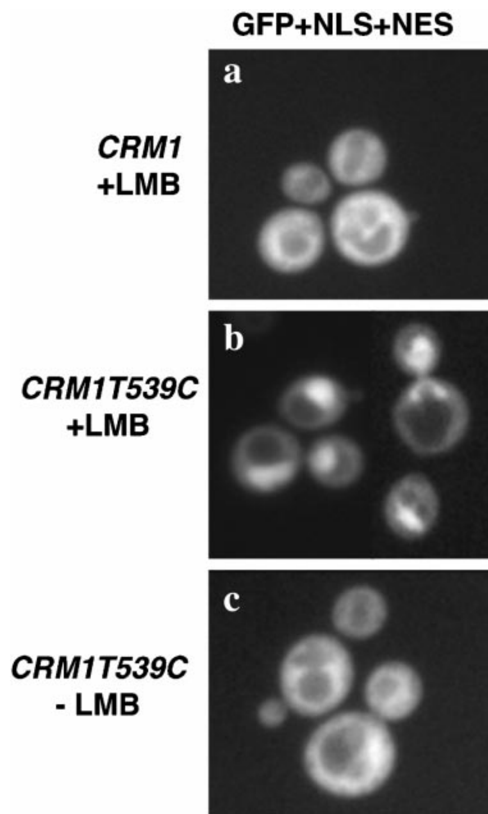

Figure 9. GFP containing the NLS and NES of Nmd3p is restricted to the nucleus, in the presence of leptomycin B. (a) MNY7 (*CRM1*) and the (b and c) leptomycin-sensitive strain MNY8 (*CRM1T539C*) cells were transformed with plasmid pAJ629 expressing galactose-inducible GFP fused to the NLS plus NES of Nmd3p (aa 387–518). Cells were grown to early log phase and galactose was added to a final concentration of 1% to induce GFP expression. After 1 h of induction, leptomycin B was added to 0.1 μg/ml (+LMB). (c) For controls, leptomycin B was omitted (−LMB). GFP was visualized by direct fluorescence microscopy 1 h after the addition of leptomycin B. GFP localization to the nucleus was evident within 15 min of the addition of leptomycin (data not shown).

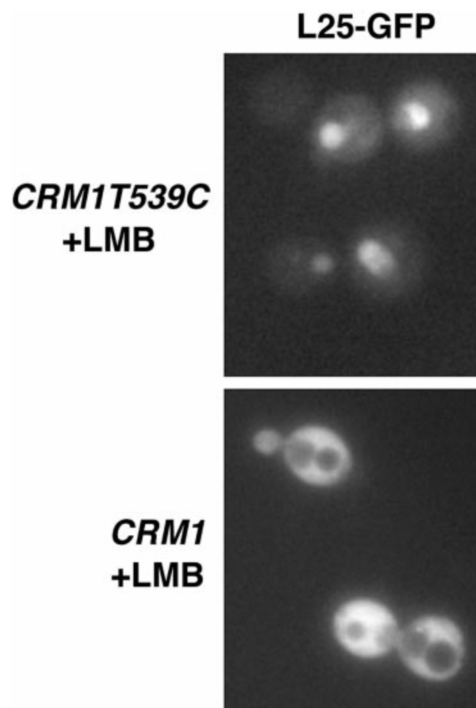

Figure 10. L25–GFP is retained in the nucleus in the presence of leptomycin B. (a) The leptomycin-sensitive strain MNY8 (*CRM1T539C*) and (b) the wild-type strain MNY7 (*CRM1*) were transformed with plasmid pAJ369, which expresses L25–GFP. Overnight cultures grown in medium lacking uracil, with raffinose as the carbon source, were diluted into the same medium and grown for 4 h at 30°C. Leptomycin B and galactose were then added to 0.1 μg/ml and 1%, respectively. After 4 h of incubation at 30°C, GFP fluorescence was visualized. In the absence of added leptomycin B, L25–GFP was cytoplasmic in both strains (data not shown).

port of a nuclear export pathway for ribosomal subunits. Because the NES of Nmd3p is essential for 60S subunit biogenesis and export, Nmd3p appears to be the principal protein providing the export signal for the large ribosomal subunit. Although Nmd3p may have an additional role on the 60S subunit (see below), the ability to modulate 60S subunit export by the presence or absence of an NES on Nmd3p clearly demonstrates that one essential function of Nmd3p is to provide the NES for 60S export. Thus, Nmd3p acts as an adapter protein to bridge the interaction between the 60S subunit and its export receptors. Furthermore, the demonstration that Crm1p is a receptor for Nmd3p to mediate 60S subunit transport is the first evidence that Crm1p is involved in ribosomal subunit export.

Nmd3p is a highly conserved protein. Similar proteins are found throughout eukaryotes and all of the eukaryotic proteins show a high degree of conservation of the shuttling signals that we have identified within Nmd3p. We have recently cloned the human homologue, CGI-07, and found that it complements a temperature-sensitive *nmd3* mutant, suggesting conservation of function of Nmd3p throughout eukaryotes (Johnson, A., unpublished results). Interestingly, related proteins are predicted in archaebacteria as well. However, these archaebacterial proteins lack the shuttling sequences that we have defined in Nmd3p. Thus, eukaryotic Nmd3 proteins appear to have evolved by the addition of an NLS and NES, thereby adapting an archaea-like protein for nuclear shuttling.

Since the COOH-terminal 50 aa of Nmd3p (aa 469–518) is necessary for nuclear export, this domain of the protein likely contains an NES. Within this region, aa 491–500 (INIDELLDEL) are highly conserved and are predicted to form an amphipathic helix with isoleucine and leucine predominantly on one face, similar to a leucine-rich NES (Rittinger et al., 1999). Because export of Nmd3p depends on Crm1p, the receptor for leucine-rich NES–containing proteins, we tentatively conclude that aa 491–500 comprise the NES of Nmd3p. Experiments to determine the minimal NES of Nmd3p are underway. We note that Nmd3Δ100 displayed a stronger dominant-negative phenotype than Nmd3Δ50. The larger deletion in Nmd3Δ100 encompassed aa 419–468, which contains an additional highly conserved domain. Preliminary results indicate that this region is not necessary for function, but may act additively with aa 469–518 (Johnson, A., unpublished results). This region could encode a second, but weaker NES, or a signal for intranuclear localization. Consequently, it is pos-

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sible that there is redundancy in the export signal and possibly in the export pathway. Determination of the intranuclear localization of mutant Nmd3 proteins deleted for these various signals should elucidate their respective contribution to Nmd3p localization.

Leptomycin B is an antibiotic specific for Crm1p in nearly all eukaryotic cells (Nishi et al., 1994; Kudo et al., 1999). Wild-type *S. cerevisiae* cells are resistant to leptomycin B. However, a single aa change within Crm1p renders *S. cerevisiae* sensitive to the antibiotic (Neville and Rosbash, 1999). Since Nmd3p export was inhibited by leptomycin B, we conclude that Crm1p is the receptor for Nmd3p. In preliminary in vitro experiments, we have also observed Ran binding in the presence of both Crm1p and Nmd3p (Kallstrom, G., and A. Johnson, unpublished results), which suggests the cooperative interaction of Ran and Crm1p in the formation of an export complex (Fornerod et al., 1997; Kutay et al., 1998). Furthermore, we showed that Crm1p is needed for efficient 60S subunit export. This is contrary to a previous report in which temperature-sensitive *crm1(xpo1-1)* mutant cells did not inhibit 60S subunit export when shifted to restrictive temperature (Hurt et al., 1999). We also found that Nmd3p did not accumulate in the nucleus in *crm1(xpo1-1)* cells at restrictive temperature. Preliminary results suggest that this failure to observe Nmd3p accumulation in the nucleus was not due to the inhibition of Nmd3p import at restrictive temperature (Ho, J., and A. Johnson, unpublished results). The transient inhibition of ribosome biogenesis due to temperature shifts (Warner, 1999) likely complicates the use of *crm1(xpo1-1)* mutants for examining effects on ribosome export. It is also possible that an alternative and Crm1p-independent pathway acts at elevated temperature to bypass the Crm1p-dependent pathway. Nevertheless, we did observe a strong nuclear accumulation of Nmd3p and 60S subunits in leptomycin B–sensitive cells when treated with leptomycin B. Thus, Crm1p is an export receptor for Nmd3p to mediate 60S subunit export.

The shuttling of Nmd3p in and out of the nucleus depends on the recognition of import and export signals by receptor proteins. When Nmd3p binds to 60S subunits in the nucleus, its NES must be displayed for recognition by Crm1p. Nmd3p also binds mature 60S subunits in the cytoplasm (Ho and Johnson, 1999; Ho et al., 2000). Consequently, the NLS of Nmd3p bound to 60S subunits in the cytoplasm must be masked to prevent retrograde transport of mature subunits to the nucleus. A similar proposal has been made for ribosomal proteins (Rout et al., 1997). Because Nmd3p is predominantly cytoplasmic, where it binds mature free 60S subunits (Ho et al., 2000), the ratio of Nmd3p to free 60S subunits in the cytoplasm may determine the availability of Nmd3p for shuttling into the nucleus.

Does Inhibition of 60S Subunit Export Affect Other Transport Pathways?

In a screen for high-copy suppressors of the growth defect of an *nmd3-1* mutant (Ho and Johnson, 1999) we identified *MEX67*, encoding an mRNA transport factor (Segref et al., 1997; Hurt et al., 2000), and *PAB1*, encoding poly(A) binding protein (Kallstrom, G., and A. Johnson, unpublished results). In addition, *mex67-5* and *nmd3-1* mutations were synthetic lethal (Kallstrom, G., and A. Johnson, unpublished results), however, *NMD3* was not a high-copy suppressor of *mex67-5*. Although high-copy *MEX67* and *PAB1* partially suppressed the growth defect of *nmd3-1* cells, they did not reverse the 60S subunit deficit. Consequently, *MEX67* and *PAB1* are unlikely to be directly involved in 60S export. It is possible that inhibition of 60S export indirectly affects export of mRNA leading to a condition in which mRNA is partially limiting in cells. A link between mRNA transport and the nucleolus, the site of ribosome biogenesis, has been suggested previously (Schneiter et al., 1995). It is possible that overexpression of *MEX67* partially bypasses this block, whereas overexpression of *PAB1* may stabilize mRNAs (Caponigro and Parker, 1995) under conditions in which mRNA is limiting in the cell. Further work is needed to determine the basis of these genetic interactions.

Is There a More Fundamental Function for Nmd3p?

Eukaryotes appear to have adapted an archaeal Nmd3p-like protein for transporting the 60S subunit across the nuclear envelope. The presence of Nmd3p-like proteins in archaebacteria, which lack nuclei, suggests that Nmd3p has an additional function more ancient than nuclear export. Such a role could be in a biogenesis step of the large ribosomal subunit that is distinct from, but perhaps coupled to, export of the subunit. The requirement of Nmd3p for an ultimate maturation step, before export, could provide a mechanism of control of 60S export. Therefore, Nmd3p could provide a quality control mechanism for ribosomal subunit biogenesis analogous to the role of nuclear aminoacylation of tRNAs required for tRNA export (Lund and Dahlberg, 1998). We note that truncated Nmd3p, lacking an export signal, binds to nuclear 60S subunits, which are sufficiently stable to accumulate in the nucleus. However, a temperature-sensitive *nmd3* mutant does not allow such nuclear accumulation of nascent 60S subunits due to their severe instability. Thus, Nmd3p also provides a function in subunit biogenesis that is necessary for stabilization of the nascent 60S subunit. We suggest that eukaryotes have adapted a ribosome biogenesis factor for transport of the large ribosomal subunit.

We thank Dr. Clarence Chan for the GFP vectors pTD125 and pTS395, Dr. Michael Rosbash for the strains MNY7 and MNY8, and Dr. Juan Ballesta for the anti-L12 antibody. We are grateful to Dr. Minoru Yoshida for generously providing leptomycin B. We thank Justin Brown, Dr. Clarence Chan, and Dr. James Dahlberg for comments on the manuscript.

This work was supported by National Institutes of Health grant GM53655 to A. Johnson.

Submitted: 8 September 2000
Revised: 10 October 2000
Accepted: 13 October 2000

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