The NUG1 GTPase Reveals an N-terminal RNA-binding Domain That Is Essential for Association with 60 S Pre-ribosomal Particles* [S]

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The putative yeast GTPase Nug1, which is associated with several pre-60 S particles in the nucleolus and nucleoplasm, consists of an N-terminal domain, which is found only in eukaryotic orthologues, and middle and C-terminal domains that are conserved throughout eukaryotes, bacteria, and archaea. Here, we analyzed the role of the eukaryote-specific Nug1 N-domain (Nug1-N). We show that the essential Nug1-N is sufficient and necessary for nucle(o)lar targeting and association with pre-60 S particles. Nug1-N exhibits RNA binding activity and is genetically linked in an allele-specific way to the pre-60 S factors Noc2, Noc3, and Dbp10. In contrast, the middle domain, which exhibits a circularly permuted GTPase fold and an intrinsic GTP hydrolysis activity in vitro, is not essential for cell growth. The conserved Nug1 C-domain, which has a yet uncharacterized fold, is also essential for ribosome biogenesis. Our findings suggest that Nug1 associates with pre-60 S subunits via its essential N-terminal RNA-binding domain and exerts a non-essential regulative role in pre-60 S subunit biogenesis via its central GTPase domain.

Ribosome synthesis in eukaryotic cells is a strictly coordinated multistep process. It requires transcription by the three RNA polymerases to generate the pre-rRNAs (by polymerase I and polymerase III) and mRNAs (by polymerase II) that encode the ribosomal proteins and the non-ribosomal factors involved in ribosome biogenesis. Concomitant to transcription, early trans-acting factors together with small nuclear ribonucleoparticles and ribosomal proteins are recruited to the 35 S pre-rRNA to form the 90 S pre-ribosome/small subunit processome (1, 2). Within this early 90 S pre-ribosome intermediate, various RNA modification/processing events occur, including methylation, pseudouridylation, and cleavage of pre-rRNA at sites A₀ and A₁. Subsequently, cleavage at site A₁ leads to a splitting of the 90 S precursor particle yielding the earliest pre-40 S and pre-60 S subunits (reviewed in Refs. 3 and 4). Depending on the growth state, the latter cleavage can occur co-translationally or post-trancriptionally in yeast (5). Upon further maturation, nearly all 90 S factors dissociate, and a small number of new factors are recruited (6). The derived nuclear pre-40 S particle contains Rps proteins, 20 S pre-rRNA, and a few non-ribosomal factors. Lastly, the pre-40 S particle is exported to cytoplasm, where final maturation of the 40 S subunit takes place.

In contrast, the early nucleolar pre-60 S particles contain a huge number of factors (~40 – 50), among which are methyltransferases, RNA helicases, ATPases, and GTPases (7). Subsequent maturation steps include processing of the 27 S rRNA into 25 S and 5.8 S rRNA, incorporation of 5 S rRNA and transport of the pre-60 S subunit to the nucleoplasm. During these processes, the composition of the particles is simplified until export competence is achieved by recruitment of export factors including Nmd3 and Mtr2 and possibly others (7). It is assumed that the heterotrimeric Nmd3-Xpo1-RanGTP complex, which assembles onto the transport-competent pre-60 S subunit, mediates export through the nuclear pore complex into the cytoplasm. Subsequently, the late non-ribosomal factors dissociate from the 60 S subunit by mechanisms, which also involve GTPases (8).

Significant insight into the formation and transport of pre-ribosomal particles has been achieved over the past several years (for review, see Refs. 9 – 11). Due to extensive proteomic analyses of pre-ribosomal particles, the vast majority of non-ribosomal factors involved in ribosome synthesis have been identified, and their approximate site of action is beginning to be understood. Moreover, the structural organization of a few pre-ribosomal particles has been determined by electron microscopy (12, 13). Notably, structural rearrangement of a late pre-40 S particle was followed during in vitro maturation and was found to be dependent upon phosphorylation and dephosphorylation events (13). Regulation of pre-60 S subunit biogenesis appears to be strongly dependent on the function of several putative GTPases in yeast, including Nug1,³ Nug2/Nog2, and Lsg1/Kre35 (8, 14 – 18), all belonging to a novel G-family (called YawG) that are earmarked by a permutated order of GTP motifs within the GTP-binding domain (19). Here, we studied one member of these putative GTPases, Nug1. We found that

³ The abbreviations used are: Nug, nuclear/nucleolar GTPase; GST, glutathione S-transferase; RbgA, ribosome biogenesis GTPaseA; RsgA, ribosome small subunit-dependent GTPaseA; Rps, ribosomal protein small subunit; TAP, tandem affinity purification; EGFP, enhanced green fluorescent protein; mRFP, monomeric red fluorescent protein; TEV, tobacco etch virus; SL, synthetic lethal; 3–AT, 3–amino triazole; SDC, synthetic dextrose complete-medium; FOA, 5–fluoroorotic acid; HE-EGFP, high efficiency EGFP filter.
The GTPase Nug1 Is an RNA-binding Protein

The essential N-terminal Nug1 domain targets Nug1 to the nucleolus/nucleus, mediates association with pre-60 S particles, and exhibits RNA binding activity. In contrast, the middle part of Nug1, which comprises the GTPase fold, is not essential for cell growth and thus may fulfill a redundant role with other GTPases involved in ribosome biogenesis.

EXPERIMENTAL PROCEDURES

Plasmids, Strains, DNA Recombinant Work, and Microbiological Techniques—Yeast strains and plasmids used in this study are listed in supplemental Tables 1 and 2. Double knockout strains were generated as described previously (20). DNA recombinant work was done according to (21) using Escherichia coli strain DH5α.

Live Cell Imaging—Prior to imaging, cells were grown in SDC-leu liquid medium (30 °C) until logarithmic phase. Fluorescence microscopy was performed using an Imager Z1 (Carl Zeiss) with a ×100 NA 1.4 Plan-Apo-Chromat Oil immersion lens (Carl Zeiss) and DICIII, HE-EGFP, or HE-Cy3 filter set, respectively. Pictures were acquired with an AxioCamMRm camera (Carl Zeiss) and software AxioVision 4.3 (Carl Zeiss) at resolution 1388 × 1040 (Binning 1 × 1, gain factor 1). Pictures were exported as jpg files and processed in PhotoShop 7.0 for gray levels.

GTP Hydrolysis Assays—Recombinant GST-TEV-Nug1 or GST-TEV-Nug1–4G mutant was expressed from E. coli Star+, purified via GST affinity purification, and eluted by incubation with TEV protease. GST-Nug1–4G is a mutant of Nug1, in which the G1 consensus motif GKS was mutated to 4 glycines (GGGG). Eluted proteins were further purified by ion exchange chromatography (MonoS/GE Healthcare) and gel filtration (SuperdexTM 200/GE Healthcare) and concentrated using ULTRAFREE 0.5 with Biomax-10K NMWL membrane (Millipore). Cell lysis buffer contained 100 mM KOAc, 400 mM NH4Cl, 100 mM NaCl, 5 mM MgCl2, and 20 mM HEPES, pH 7.5. Subsequent purification steps were performed in the same buffer with 200 mM NH4Cl. For the GTPase assay, the purified protein was incubated with [γ-32P]GTP at 30 °C. To stop the reaction, SDS was added. The cleaved radioactive [γ-32P]phosphate was separated by thin layer chromatography and visualized by autoradiography.

Kinetic analysis of Nug1-mediated GTP hydrolysis was performed using a colorimetric assay according to Ref. 22. Purified Nug1 (80 μl) was mixed with 20 μl of various concentrations GTP to obtain 16–30 μM Nug1 and 0.4, 1, 2, 4, or 10 mM GTP and incubated at 30 °C. Aliquots (15 μl) were taken in 45-min intervals, and the reaction was stopped by freezing in liquid N2. The amount of hydrolyzed phosphate was determined by incubation with 885 μl of malachite green solution (0.3 g of malachite green, 2 g of ammonium molybdate, 0.5 g of Triton in 1 liter of 1 N HCl). The colorimetric reaction was terminated after 3 min by adding 100 μl of sodium citrate (34 g/100 ml). Finally, A400 was measured after a 30–90-min incubation. The catalytic constant was derived from $k_{cat} = v_{max}/c_{Nug1}$. $v_{max}$ was calculated using a Lineweaver-Burk plot, and $c_{Nug1}$ was determined by $A280$ in a NanoDrop® 1000 spectrophotometer.

RNA- Electrophoresis Mobility Shift Assay—Nug1-N was incubated with RNase during incubation with nickel-nitrilotriacetic acid resin (Qiagen). Subsequently, Nug1-N was eluted by imidazole and applied to a gel filtration column containing SuperdexTM-200 (Amersham Biosciences). All purification steps were performed in buffer containing 50 mM KOAc, 100 mM NH4Cl, 50 mM NaCl, 10 mM MgOAc2, and 20 mM HEPES, pH 7.0. In vitro transcription of 5 S RNA was performed using linearized plasmid DNA carrying the 5 S rRNA gene under the control of T7 promoter (pET9D-5 S-RNA). Total yeast tRNA was purchased from Sigma. RNA binding assays were performed at room temperature (23 °C) in protein purification buffer. Samples were analyzed by loading on 8% polyacrylamide gels (0.5 × Tris-borate-EDTA) containing ethidium bromide to visualize RNA under UV light.

RESULTS

Domain Analysis of the Nug1 Protein—Inspection of primary amino acid sequence suggests that Nug1 consists of three domains (Fig. 1 and supplemental figure Fig. S1). An N-terminal domain (residues 1–154), which is conserved within the Nug1 orthologues (nucleostemin family), a middle domain (residues 155–344), which has a highly conserved GTPase fold and a conserved C-terminal domain (residues 345–520) lacking known motifs. The protein fold of the latter two domains most likely resembles the prokaryotic RbgA fold, whose x-ray structure is known (see “Discussion”). To determine whether all three domains of Nug1 are essential for cell growth, a deletion analysis was performed (Fig. 1A). In the case of the N-domain, removal of the first 37 amino acids (nug1-ΔN1) exhibited a slightly reduced growth rate, whereas deletion of the first 100 amino acids or the entire N-domain caused a lethal phenotype (nug1-ΔN; Fig. 1A). In the case of the C-terminal domain, deletion of the last 52 non-conserved residues did not affect the growth rate, but further (last 116 amino acids) or complete deletion of the conserved C-domain (last 176 amino acids; nug1-ΔC) resulted in lethality (data not shown and Fig. 1A). Unexpectedly, deletion of the M-domain containing the GTPase fold resulted only in a slightly reduced growth rate (nug1-ΔM; Fig. 1A). In contrast, deletion of the M-domain in the homologous putative GTPase Nug2 (Fig. 1B) or point mutations in the GTPase motifs caused lethality (15). These results suggest that the putative Nug1 GTPase has essential N- and C-domains, which enclose the central but non-essential putative GTPase domain.

Purified Nug1 Exhibits GTPase Activity—The finding that the Nug1 middle domain with a predicted GTPase fold is not essential for growth prompted us to test for Nug1 GTPase activity. Thus, we expressed GST-tagged Nug1 in E. coli and purified it via GSH affinity, ionic exchange, and gel filtration chromatography (Fig. 2A). As a negative control, we purified in the...
same manner GST-Nug1–4G that is a mutant form of Nug1, containing an inactive Walker A motif (see “Experimental Procedures”). This Nug1–4G construct was expected to be inactive in GTP hydrolysis.

The purified GST-tagged Nug1 and Nug1–4G proteins were tested in vitro for GTP hydrolysis activity by incubation with γ-32P-labeled GTP. The release of [γ-32P]phosphate was followed by thin layer chromatography in combination with autoradiography (Fig. 2B). This analysis revealed that purified recombinant Nug1 exhibits a distinct GTPase activity (Fig. 2B), which is largely abolished in the Nug1–4G mutant. Thus, it is likely that Nug1 has GTP hydrolysis activity in vivo.

For further characterization of the Nug1 GTPase activity, we determined the enzymatic parameters using a colorimetric assay (22). Purified Nug1 was incubated with various concentrations (0.2–10 mM) of GTP, and the amount of generated PO4 was determined (see “Experimental Procedures”). Lineweaver-Burk plot analysis revealed a $K_m$ of 0.2 mM $\pm$ 0.1 mM and $k_{cat}$ of 0.11 min$^{-1}$ $\pm$ 0.01 min$^{-1}$ for the GTP hydrolysis reaction (“Experimental Procedures”). Notably, for E. coli RsgA (a Nug1 homolog), similar kinetic parameters for the intrinsic GTPase activity were reported (26, 27). We conclude from our studies that purified Nug1 has an intrinsic GTP hydrolysis activity (see “Discussion”).

**Trans-complementation by the Various Nug1 Domains**—In the course of our studies, we found that N-terminal truncations of Nug1 could be rescued by co-expression of an intact N-domain. Therefore, we tested whether the essential Nug1 domains, when separated from each other but co-expressed in the same cell, would allow for trans-complementation. Co-expression of the split N-domain together with the Nug1-ΔN construct revealed efficient complementation of the otherwise lethal nug1 null mutant (Fig. 1C). Moreover, co-expression of the C-domain together with the Nug1-ΔC construct could complement the nug1 null mutant (Fig. 1C). This suggests that the various Nug1 domains perform unique but overlapping functions.

**The Nug1-N Domain Exhibits Nucleolus Tagging and Pre-ribosome Binding Properties**—To determine how the three Nug1 domains contribute to nuclear and/or nucleolar targeting and association with pre-60 S particles, we tagged the individual domains or various combinations of them (ΔN, ΔM, ΔC) with either EGFP or TAP and analyzed their subcellular location and association with pre-60 S particles. The EGFP-tagged Nug1-N domain, as well as the Nug1-ΔC and Nug1-ΔM constructs, localized to the nucleolus/nucleoplasm with a distribution that is similar to that of the endogenous Nug1 (Fig. 3). The nucleolar localization of these constructs was confirmed by colocalization with the nucleolar marker Nop1 tagged with mRFP. Interestingly, expression of frag-
The GTPase Nug1 Is an RNA-binding Protein

The N-terminal Domain of Nug1 Interacts with RNA—The observation that the N-domain of Nug1 mediates recruitment to the pre-60 S subunit suggested that it binds to the particle through either a protein or an RNA interaction. Notably, we observed that recombinant Nug1 purified from E. coli co-enriched RNA.4 Therefore, we sought to test whether Nug1 via its

4 J. Bassler and E. Hurt, unpublished results.

37 amino acids from Nug1 (Nug1-ΔN1-EGFP) no longer mediated nucleolar accumulation but was still able to target to the nucleus. Moreover, a fraction of Nug1-ΔN1-EGFP was also detected in the cytoplasm (Fig. 3). In conclusion, the N-terminal domain of Nug1 exhibits a basic sequence that is sufficient and necessary for nucleolar accumulation of Nug1.

To correlate nucleolar location of Nug1-N with binding to 60 S pre-ribosomes, we performed sucrose gradient centrifugation to analyze the association of wild-type Nug1 and its various truncation constructs with pre-60 S subunits (Fig. 4). The A260 nm profile of these gradients shows the sedimentation of 40 S and 60 S subunits, 80 S ribosomes, and polysomes (Fig. 4, upper panels). The sedimentation of the TAP-tagged wild-type and mutant Nug1 proteins was determined by Western analysis (Fig. 4, lower panels).

Interestingly, wild-type Nug1 and the N-terminal constructs Nug1-N and Nug1-N1 are associated with high molecular weight complexes, presumably pre-60 S particles. In contrast, constructs lacking the N-terminal domain (Nug1-ΔN) or part of it (Nug1-ΔN1) did not associate with the high molecular particles and were mainly detected in the fractions of the gradient, containing the soluble proteins (Fig. 4). Notably, Nug1-N1 revealed a broad distribution with large complexes (in the range of pre-60 S particles). Notably, if endogenous Nug1 was absent, the viable Nug1-ΔN1 (38–520) was also weakly associated with large structures. These data indicate that the entire N-domain is necessary and sufficient for association with large assemblies, which likely correspond to pre-60 S subunits.

To show a direct association with pre-60 S particles, we performed tandem affinity purification of various Nug1 constructs. Significantly, when TAP-tagged Nug1-N was affinity-purified, it co-precipitated pre-60 S subunits that contained a set of non-ribosomal factors, the most prominent ones being Dbp10, Erb1, Nop2, Nop7, Noc3, Nug1, Has1, and Nug2 (Fig. 5). The same pre-60 S factors were also co-enriched when full-length Nug1-TAP was affinity-purified (Fig. 5). Purification of the Nug1-N1 construct, which has a shortened N-domain, caused substantial loss of these pre-60 S factors, but association with 60 S subunits was not abolished. Thus, Nug1-N1 may exhibit a relatively unspecific binding to 60 S subunits. Consistent with this interpretation, Nug1-N1 was also detected in fractions corresponding to polysomes (Fig. 4). In contrast, affinity purification of TAP-tagged Nug1-ΔN or Nug1-ΔN1 lacking the entire N-domain or part of it, respectively, revealed a complete loss of both pre-60 S factors and ribosomal Rpl proteins (Fig. 5). Taken together, the data showed that the N-domain of Nug1 confers nucleolar location and association with pre-60 S particles that exhibit a typical set of pre-60 S factors. However, the middle and C-domain of Nug1 (Nug1-ΔN) are not significantly associated with pre-60 S particles under the biochemical conditions tested.

The N-terminal Domain of Nug1 Interacts with RNA—The observation that the N-domain of Nug1 mediates recruitment to the pre-60 S subunit suggested that it binds to the particle through either a protein or an RNA interaction. Notably, we observed that recombinant Nug1 purified from E. coli co-enriched RNA.4 Therefore, we sought to test whether Nug1 via its

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ments that lacked the N-domain (e.g. Nug1-ΔN) revealed a predominantly cytoplasmic location with a weak nuclear signal (Fig. 3). Altogether the data suggest that the N-domain of Nug1 when attached to a reporter protein can mediate nucleolar localization.

To further delimit the nucleolar targeting signal, the first 39 amino acids of the N-domain, which is rich in positively charged amino acids (MRVKKRQSRRTSTKLKEGIKKKASKAKHRKKEKKMAKDKDV; pI = 11.76), were fused to EGFP. In contrast, the second half of the N-domain is acidic (pI = 4.37). As seem in Fig. 3, the N-terminal basic sequence of Nug1 (Nug1-ΔN1) is sufficient to target the attached EGFP reporter to the nucleus/nucleolus. Conversely, a construct lacking the first

FIGURE 3. Subcellular localization of Nug1 domain constructs. The indicated Nug1 constructs were C-terminally fused to EGFP and analyzed for co-localization with mRFP-NOP1 in exponentially growing yeast cells (Nug1 shuffle strain) by fluorescence microscopy (see “Experimental Procedures”). Representative pictures of yeast cells show the signals for EGFP (Nug1), mRFP (Nop1), an overlay of EGFP with mRFP (merge), and differential interference contrast (DIC).

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N-domain can bind to RNA in general. As a first approach, we employed the yeast three-hybrid system to test for a possible interaction of Nug1 with RNA (23,28) (Fig. 6A). In the past, several specific RNA-protein interactions have been demonstrated in the three-hybrid system including interaction between ribosomal protein Rps14 and helix 23 of 18 S rRNA (29). We could validate interaction of Rps14 with helix 23 rRNA in our three-hybrid analysis, but Nug1 did not interact with helix 23 rRNA (Fig. 6B). Notably, Nug1 revealed a strong three-hybrid interaction with 5 S rRNA (Fig. 6B). However, no three-hybrid interaction was observed between Nug1 and 5.8 S rRNA or reverse 5 S rRNA. Additional controls revealed that Rps14 did not interact with 5 S, 5.8 S, and reverse 5 S rRNA (Fig. 6B).

Since the N-domain of Nug1 mediates binding to pre-60 S subunits, we tested whether Nug1-N interacts with 5 S rRNA in the three-hybrid assay. As shown in Fig. 6C, the Nug1-N construct, but not Nug1-ΔN, exhibited a three-hybrid interaction with 5 S rRNA.

To confirm that Nug1 binds directly to RNA via its N-domain, we performed RNA electrophoresis mobility shift assays. First, the Nug1 N-terminal domain was expressed in E. coli as His<sub>N</sub>-Nug1-N and purified to homogeneity by nickel-nitrilotri-
acetic acid affinity purification and subsequent gel filtration on Superdex™200 (Fig. 7A). As an RNA substrate, we used in vitro synthesized 5 S rRNA and commercially available yeast tRNA. We observed that 5 S rRNA, but also tRNA, were efficiently retarded in their migration on the native polyacrylamide gel (band shift) when increasing amounts of the Nug1 N-domain were added (Fig. 7B). The formation of Nug1-N/5 S rRNA complexes was still observed in buffers that contained high salt (e.g. 500 mM NaCl). Since nug1-ΔN1 or nug1-1 mutants are not genetically linked to rpl5 or 5 S rRNA mutants (data not shown), we assume that in vitro binding of Nug1 to 5 S rRNA and tRNA reflects a general RNA binding activity of the Nug1 N-domain. However, it remains to be shown whether in vivo Nug1 binds to 5 S rRNA or to another rRNA species within the pre-60 S subunit (see "Discussion").

**NUG1 via Its N-terminal Domain Genetically Interacts with pre-60 S Factors NOC2, NOC3, DBP10, and BUD20**—To characterize the functional interaction of the Nug1 N-domain with components of the pre-60 S subunit in vivo, we performed a synthetic lethal (SL) screen with the nug1-ΔN1 allele using a red-white colony-sectoring assay (14). From this SL screen, we could isolate synthetic lethal mutants SL46, SL78, and SL105 that were complemented by DBP10, NOC3, and BUD20, respectively. Noc3 and Dbp10 are known pre-60 S factors that are essential for 60 S subunit biogenesis (30, 31) and co-purify

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**FIGURE 6.** The Nug1-N domain interacts with 5 S rRNA in the three-hybrid assay. A, schematic drawing of the principle of the three-hybrid assay. LexA-MS2 fusion protein is targeted via LexA to LexA operator (LexA OP). MS2 binds to its cognate RNA, which is fused to the RNA of interest. Protein of interest to be tested for RNA interaction (e.g. NUG1) is fused to the GAL4 activation domain (GAL4 AD). Interaction of Nug1 with RNA induces expression of the HIS3 reporter gene. B, the L40-coat yeast strain was transformed with pACTII-NUG1 or pACTII-RPS14, respectively, and various p3A-MS2 vectors expressing indicated the RNAs fused to MS2 RNA. Transformants were spotted in 10−1 dilution steps on SDC-leu-trytura (SDC; left panel) and SDC-leu-trytura-plates containing 5 mM 3-AT (SDC-his; right panel). Growth behavior is shown after 4 days of incubation at 30 °C. A similar doubling time could be observed on SDC-his plates up to 15 mM 3-AT (data not shown). C, the L40-coat yeast strain was co-transformed with pACTII-NUG1 (1–520), pACTII-NUG1-ΔN (residues 146–520), or pACTII-NUG1-ΔN (residues 1–145), respectively, and p3A-MS2–5 S rRNA vector expressing 5 S rRNA. Transformants were spotted in 10−1 dilution steps on SDC-leu-trytura-plates containing 5 mM 3-AT (SDC-his). Growth phenotype is shown after 3 days incubation at 30 °C.

**FIGURE 7.** Nug1-N binds directly to RNA. A, recombinant His6-Nug1-N (residues 1–145) was purified via affinity purification and gel filtration. Purified Histagged Nug1-N was analyzed on a 5%–12% polyacrylamide gel and visualized by Coomassie Blue staining. B, 5 S rRNA (upper panel) and tRNA (lower panel) were incubated with no protein (lane 1) and increasing amounts of Histagged Nug1-N (lanes 2–9). The size of the RNA ladder (in nucleotides) is indicated at the left side of the gel. Non-shifted RNA is marked by an arrow, and the formation of RNA-protein complexes is indicated by a dark line on the right.
with the Nug1 pre-ribosomal particle (7, 14). Likewise, the non-essential Bud20 was found in several pre-60 S particles that also contained Nug1 (32, 33). Affinity purification of Bud20-TAP co-enriched a late pre-60 S particle that contained several pre-60 S factors including Nug1 (data not shown).

To confirm the genetic interactions between Nug1-N domain and the various pre-60 S factors, we directly combined mutant alleles of nug1 with mutant alleles of dbp10 and noc3 (see “Experimental Procedures”). Noc3 is part of the nuclear Noc2-Noc3 complex that replaces the nucleolar Noc1 from the Noc1-Noc2 complex during pre-60 S biogenesis (30). Therefore, we tested for a genetic interaction between noc1-1 and noc2-1 alleles with nug1-ΔN1. This analysis revealed that the nug1-ΔN1, but not nug1-1, which carries a mutation in the C-domain (R420P), is synthetically lethal with noc2-1 and noc3-1. Notably, nug1-ΔN1 is not SL with noc1-1 (Fig. 8). Moreover, dbp10-2 is synthetically lethal with nug1-ΔN1 but not with nug1-1. These results correlate with the findings that Noc2-Noc3 and Dbp10 are present in the Nug1 particle, whereas Noc1 is absent (7, 14). In contrast, bud20Δ is genetically linked to several nug1 mutant alleles, which are not restricted to the Nug1 N-terminal domain (Fig. 8 and data not shown). Interestingly, the mtr2-33 allele, which was shown to be SL with the C-terminal mutant nug1-1 (14), is not linked to the nug1-ΔN1 mutation (data not shown). Moreover, other factors present in the Nug1 pre-60 S particle such as Rlp7, Rea1, Nop7, and Nsa3 did not show genetic interactions with the various nug1 mutants (data not shown). Thus, the genetic analyses revealed that Noc2-Noc3 and Dbp10 are specifically linked to the N-domain of Nug1.

**DISCUSSION**

In this study, we showed that the N-terminal domain of Nug1 is essential for the correct nucleolar localization and association with pre-60 S particles. Moreover, we demonstrated that the Nug1-N domain binds directly to RNA. Due to this general binding activity, we suggest that Nug1 binds to the RNA of the pre-60 S subunit. However, additional pre-60 S factors could play a role in targeting Nug1 to its specific site(s) at the pre-60 S particle.

Sequence analysis revealed that Nug1 belongs to the family of YawG GTPases characterized by permutated GTP motifs (19). Significantly, many of these GTPases have been described to function in ribosome biogenesis. It was demonstrated that yeast Nug1, Nug2/Nog2, and Lsg1/Kre35, which all are YawG family members, are essential for 60 S subunit maturation (8, 14–17). Furthermore, another YawG member, Mtg1, was suggested to play a role in assembly of the mitochondrial large ribosomal subunit (34). In *Saccharomyces pombe*, it was demonstrated that the Nug1 homologue Grn1 is involved in 60 S biogenesis (35).

Do these GTPases with permutated GTP motifs have related roles in large subunit biogenesis? Recently, the three-dimensional structures of two members of this family, RsgA (ribosome small subunit-dependent GTPaseA/YjeQ (36)) and RbgA (ribosome biogenesis GTPase/Ylqf (Protein Data Bank accession number 1PUJ)) (35) have been solved. Despite the permutated order, the GTP-binding pocket is highly homologous to those of classical GTPases. Significantly, the prokaryotic GTPases have different N- and C-terminal domains, indicative of different functions in vivo. RsgA binds to the small 30 S subunit, which is mediated by the N terminus (27, 38). This domain has an OB fold (26, 36) that is characteristic for a class of RNA-binding proteins. Moreover, RsgA has an intrinsic GTPase activity (*k*_cat = 0.13 min^{-1} at 23 °C; ref. 38) that is similar to the activity determined for Nug1 (*k*_cat of 0.11 min^{-1} ± 0.01 min^{-1}). Significantly, the intrinsic GTP hydrolysis activity of Nug1 is ~20-100 times higher than the GTP hydrolysis activity of Rbg GTPases. However, Rbg GTPases that are stimulated by GTPase-activating protein have a still ~5–20 times higher GTPase activity than the intrinsic Nug1 GTPase activity (39, 40). Therefore, it remains to be shown whether a GTPase-activating protein exists for Nug1. Interestingly, RsgA GTPase activity could be stimulated 80-fold by the small subunit (27). Thus, binding of Nug1 to pre-60 S subunits could regulate its GTPase activity.

Significantly, the C-domain of the essential RbgA, which is involved in 50 S biogenesis (41), shares homology to the Nug1-C domain (supplemental figure Fig. S1). Moreover, modeling of the Nug1 sequence, based on the structure of RbgA, revealed that the R420P mutation in nug1-1 is in an exposed helix that is part of the C-domain. It is possible that the C-domain recruits pre-60 S factors, which is regulated by the middle GTPase domain.

The human homologue of Nug1 is nucleostemin (supplemental figure Fig. S1). Despite the high sequence homology, nucleostemin was not able to complement a nug1 or nug2 deletion strain. Notably, nucleostemin is highly expressed in stem and cancer cells but is down-regulated in differentiated cells, suggesting that nucleostemin is involved in regulation of cell proliferation and cell cycle progression (42–44). These findings would be consistent with a role of nucleostemin in ribosome biogenesis since dividing cells are highly dependent on newly

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5. R. Kiewel, J. Buglio, and C. D. Lima, personal communication.
6. J. Bassler, unpublished results.
The GTPase Nug1 Is an RNA-binding Protein

...synthesized ribosomal subunits. Similar to our findings with Nug1, it was reported that the nucleolar localization of nucleostemin depends on the first 46 amino acids (42). Moreover, the nucleolar location of nucleostemin was shifted toward the nucleoplasm upon mutation of the G1 motif (37).

Taken together, our studies suggest that Nug1 binds to pre-60 S particles in the nucleolus and accompanies them to the nucleoplasm. Binding of Nug1 to pre-60 S subunits is mediated by an N-terminal RNA-binding domain, which is functionally linked to the Noc2/Noc3 complex and to the RNA helicase Dpb10. Future analysis will reveal the role of the Nug1 GTPase domain and other trans-acting factors in regulating these various 60 S biogenesis steps.

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