Rlp24 activates the AAA-ATPase Drg1 to initiate cytoplasmic pre-60S maturation

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Formation of eukaryotic ribosomes is driven by energy-consuming enzymes. The AAA-ATPase Drg1 is essential for the release of several shuttling proteins from cytoplasmic pre-60S particles and the loading of late joining proteins. However, its exact role in ribosome biogenesis has been unknown. Here we show that the shuttling protein Rlp24 recruited Drg1 to pre-60S particles and stimulated its ATPase activity. ATP hydrolysis in the second AAA domain of Drg1 was required to release shuttling proteins. In vitro, Drg1 specifically and exclusively extracted Rlp24 from purified pre-60S particles. Rlp24 release required ATP and was promoted by the interaction of Drg1 with the nucleoporin Nup116. Subsequent ATP hydrolysis in the first AAA domain dissociated Drg1 from Rlp24, liberating both proteins for consecutive cycles of activity. Our results show that release of Rlp24 by Drg1 defines a key event in large subunit formation that is a prerequisite for progression of cytoplasmic pre-60S maturation.

Introduction

Ribosomes are essential for all living cells by translating the genetic information into the amino acid sequence of proteins. Eukaryotic ribosomes are composed of one 40S and one 60S subunit containing different ribosomal RNA species and ribosomal proteins. The formation of the subunits starts in the nucleolus with the assembly of a long precursor (pre) ribosomal RNA with ribosomal and nonribosomal proteins. This precursor particle gives birth to progenitors of the large and small subunit. Further maturation steps lead to export-competent pre-60S and pre-40S particles, which are transported through the nuclear pore complex (NPC) into the cytoplasm, where the final maturation steps take place (for ribosome biogenesis see Henras et al. [2008] and Kressler et al. [2010]). For the pre-60S particle, these cytoplasmic maturation steps involve formation of the characteristic ribosomal stalk structure, incorporation of the last ribosomal proteins, and release and recycling of shuttling proteins and export factors (Panse and Johnson, 2010).

A key player in cytoplasmic pre-60S maturation in Saccharomyces cerevisiae is the AAA (ATPases associated with diverse cellular activities) protein Drg1 (Pertschy et al., 2007; Lo et al., 2010). Drg1 is a cytoplasmic protein that forms hexamers and exhibits high homology to Cdc48 from yeast (47% sequence identity of the AAA domains) and its mammalian orthologue p97. Similar to these proteins, Drg1 contains an N-terminal domain and two AAA domains, D1 and D2 (see Fig. 3 A; Thorsness et al., 1993; Zalalskiy et al., 2002). The AAA domains consist of a conserved stretch of ~230 amino acid residues with Walker A and B motifs. AAA proteins use the energy of ATP hydrolysis to generate mechanical force that acts on specific substrates and results in ATP-dependent remodeling of proteins or macromolecular complexes (for short overviews on general aspects of AAA proteins see Lupas and Martin [2002], Hanson and Whiteheart [2005], and White and Lauring [2007]). The D1 domain of p97 is required for oligomerization, whereas D2 is regarded as the main catalytic site, which generates tension by means of ATP hydrolysis that is finally transmitted to the substrate proteins (DeLaBarre and Brunger, 2005; Pye et al., 2006; Briggs et al., 2008). The N domains of p97 and Cdc48 serve as interaction platforms for adaptor proteins that target the proteins into different cellular pathways (Dreveny et al., 2004; Yeung et al., 2008). Based on the sequence homology,
a similar structural and functional organization was proposed for the N, D1, and D2 domains of Drg1 (Kressler et al., 2012).

Functional inactivation of Drg1 is lethal. It leads to a failure to release shuttling proteins from pre-60S particles in the cytoplasm and prevents association of late joining maturation factors and ribosomal proteins. This results in a block in the transition to mature 60S subunits (Pertschy et al., 2007). Consequently, aberrant pre-60S particles accumulate in the cytoplasm of temperature-sensitive <i>drg1</i> mutants that contain accumulated shuttling proteins and lack late joining cytoplasmic maturation factors. The accumulation of several shuttling proteins in the <i>drg1-ts</i> mutant raises the question of whether the release of these proteins occurs in a concerted action or in a strictly ordered process with Drg1 catalyzing the first reaction. However, the direct release substrate and the exact function of Drg1 in this process were hitherto unknown.

We show here that Drg1 is recruited to pre-60S particles by the shuttling factor Rlp24. Binding to Rlp24 causes enhanced ATP hydrolysis of Drg1, which is used to specifically extract Rlp24 from pre-60S particles but none of the other shuttling proteins. Besides the requirement for ATP, we show that the nucleoporin Nup116 is necessary for the release reaction in vitro, suggesting a close cooperation between nuclear export and cytoplasmic maturation of pre-60S particles in vivo.

**Results**

**Rlp24 interacts with Drg1 and stimulates its ATPase activity**

Drg1 binds to cytoplasmic pre-60S particles and is required for the release of Nog1, Rlp24, Arxl, and Tif6 (Pertschy et al., 2007). To identify additional factors that require Drg1 for their release we performed a detailed analysis of the composition of pre-60S particles in the temperature-sensitive <i>drg1-18</i> mutant. As shown in Figs. 1 A and S1, functional Drg1 is necessary for the release of the shuttling proteins Nog1, Rlp24, Tif6, and Mrt4 and the export factors Mex67/Mtr2 as well as for joining of the late cytoplasmic factors Rei1, Sqt1, and Yvh1. As joining of Yvh1 and release of Mrt4 have been shown to be required for assembly of the characteristic ribosomal stalk of 60S subunits, we conclude that in <i>drg1-18</i> mutants this structure cannot be formed. This suggestion is further supported by cytoplasmic accumulation of Mrt4-YFP in the <i>drg1-18</i> mutant (Lo et al., 2010).

Up to now, no direct substrate of Drg1 has been identified, although it is likely that Drg1 uses ATP hydrolysis to actively strip one or more nonribosomal proteins from the pre-60S particle or to load a late-acting factor. In vivo, ATP hydrolysis in the D2 AAA domain of Drg1 is strictly required for the release of shuttling proteins and export factors (Fig. 3 D; Pertschy et al., 2007). However, the in vitro ATP hydrolysis rate of Drg1 is low (Zakalskiy et al., 2002). We therefore reasoned that interaction with a substrate protein or cofactor might stimulate the ATPase activity of the AAA protein. To test this hypothesis, we purified proteins that we considered potential binding partners of Drg1 and investigated their influence on its ATPase activity. Particularly, proteins were chosen that showed altered levels on late pre-60S particles from the <i>drg1-18</i> mutant. This set of proteins included the export factors Mex67/Mtr2 and Arxl, the shuttling proteins Rlp24, Nog1, and Alb1, the late cytoplasmic factors Sqt1 and Yvh1, as well as the late binding ribosomal proteins Rpl10 and Rpp0. Factors that are known to be released or to bind at a later stage of maturation (e.g., Tif6 and Lsg1) were not taken into consideration in this experiment. Drg1 was incubated with the purified proteins and the ATPase activity was determined (see Materials and methods for details). As shown in Fig. 1 B, Rlp24 stimulated the ATPase activity of Drg1, whereas none of the other proteins had an influence on ATP hydrolysis by Drg1. ATP was not hydrolyzed when Drg1 was omitted and only Rlp24 was present in the assay (unpublished data). Rlp24 is a shuttling pre-60S maturation factor that accompanies the preribosomal particle into the cytoplasm (Saveau et al., 2003), where it is released in a Drg1-dependent manner (Fig. 1 A; Pertschy et al., 2007). Rlp24 exhibits significant sequence identity with the ribosomal protein L24 (Saveau et al., 2003). The most evident difference between Rlp24 and L24 is the presence of a 53-residues-long C-terminal extension rich in amino acids with acidic side chains that is specific to the nonribosomal protein Rlp24 (Fig. 1 C). Both proteins are thought to recognize the same binding site on (pre-)60S particles (Saveau et al., 2003). Consistent with this view, inactivation of Drg1 results in decreases of L24 on pre-60S particles, whereas Rlp24 increases (Fig. 1 D).

Stimulation of the ATPase activity of Drg1 by Rlp24 necessitates a direct interaction of the two proteins. Indeed, pull-down experiments demonstrated in vitro interaction of Drg1 with purified GST-Rlp24. The amount of copurified Drg1 was increased by the presence of ATP or the nonhydrolyzable analogue AMP-PNP (Fig. 1 E). Thus, ATP binding, but not hydrolysis, is important for the interaction of Drg1 with Rlp24. Because nucleotide binding is known to trigger oligomerization of Drg1 (Zakalskiy et al., 2002), we propose that only hexameric Drg1 binds to Rlp24. The binding detected in the absence of nucleotide is likely caused by the presence of ~30% hexameric Drg1 in the protein preparations (Fig. S2). Indeed, the protein from the <i>drg1-18</i> mutant, which is unable to form hexamers (Zakalskiy et al., 2002), does not bind GST-Rlp24 in vitro (Fig. 2 A) and fails to be activated by Rlp24 (Fig. 2 C). Notably, Drg1-18 does not associate with pre-60S particles (Fig. 1 A; Pertschy et al., 2007), suggesting that the interaction with Rlp24 is necessary to recruit Drg1 to the pre-60S particle. Consistent with a role of Rlp24 in recruitment of Drg1, deletion of the last 53 amino acid residues of Rlp24 prevents interaction of Drg1 with preribosomal particles in vivo (Lo et al., 2010). To examine the contribution of this C-terminal domain to in vitro interaction with Drg1, a C-terminally truncated Rlp24 variant was used for GST pull-down experiments. As shown in Fig. 2 A, GST-Rlp24ΔC still bound Drg1, albeit binding was reduced compared with full-length Rlp24. However, Rlp24ΔC did not activate ATP hydrolysis of Drg1 (Fig. 2 C). Furthermore, L24, which lacks this C-terminal extension, did not stimulate the ATPase activity of Drg1. In contrast, the C-terminal 53 amino acid residues of Rlp24 (designated Rlp24C) are sufficient to bind Drg1 and to stimulate its ATPase activity.
Rlp24C variant and the Rlp24C domain, which were used in subsequent experiments are also indicated. (D) Inactivation of Drg1 results in decreased levels of L24 on pre-60S particles. Late logarithmic phase wild-type and temperature-sensitive drg1-18 cells expressing chromosomally HA-tagged L24A (YGL031C) were incubated at 37°C for 1 h. Afterward, pre-60S particles were isolated by protein A affinity purification and TEV elution using Arx1-TAP as bait protein. Purified particles were analyzed for the presence of L24 using an HRP-conjugated goat anti-HA antibody (Roche). Western blots with polyclonal antibodies directed against Rlp24, Arx1, or the ribosomal protein L16 using a secondary HRP-conjugated goat anti-rabbit antibody served as controls. (E) In vitro binding of Drg1 to Rlp24 is enhanced in the presence of nucleotide. GST-Rlp24 immobilized on glutathione beads was incubated with Drg1 in the presence of 1 mM ATP or 1 mM of the nonhydrolyzable analogue AMP-PNP (PNP) at room temperature for 2 h. As a control for nonspecific binding, the GST tag bound to the beads was incubated with Drg1. After extensive washing, GST-tagged Rlp24 was eluted using free glutathione and eluates were investigated by SDS-PAGE and Coomassie staining. Std, protein standard; load, an aliquot of purified Drg1 used for the binding assay was loaded.

(Fig. 2 B and C). These results suggest that Drg1 interacts with two domains of Rlp24, the C-terminal domain that stimulates the ATPase activity of Drg1 and a second region that does not have any stimulatory effect on ATP hydrolysis.

Although heterologously expressed full-length GST-Rlp24 and 6HisRlp24 were integrated into Escherichia coli 50S ribosomal subunits, the fusion protein containing only the last 53 amino acid residues of Rlp24 did not copurify E. coli ribosomes (Fig. 2 B) and was therefore used for further enzymatic characterization. As shown in Fig. 2 D, in the presence of 1 mM ATP, Rlp24C stimulated the ATPase activity of Drg1 to a Vmax of 22.4 (±0.5) µmol ATP h⁻¹ mg⁻¹. The curve could be fitted best to a sigmoid function with a Hill factor of 0.96 (±0.05). The required concentration for half-maximal activity was calculated to be 28.3 (±0.6) nM Rlp24C (mean and SD of three biological replicates). However, when Rlp24 was present in the assay, sigmoid kinetics were observed (Fig. 2 E). With saturating concentrations of 400 nM Rlp24 a Hill coefficient of 2.4 (±0.17) was determined, indicating pronounced positive cooperativity of the Drg1 subunits in response to the nucleotide. The half-maximal activation (EC50) was achieved with 0.8 mM ATP and Vmax was calculated to 28.7 (±0.7) µmol ATP h⁻¹ mg⁻¹. These data show that binding of Rlp24 to Drg1 induces increased cooperativity of the individual subunits of Drg1 and increases both Vmax and EC50 to fuel ATP hydrolysis.

The D2 AAA domain is required for shuttling protein release
To investigate whether the increased ATPase activity arises from the first or second ATPase domain, we tested two mutant variants of Drg1. These variants contain exchanges of the conserved glutamate residues in Walker motif B of the first (Drg1EQ1) and second ATPase domain (Drg1EQ2) to glutamine (Fig. 3 A). As the carboxylic group of the glutamate activates a catalytic water molecule for nucleophilic attack on the γ phosphate, these exchanges render the respective domains nonfunctional in ATP hydrolysis (Hanson and Whiteheart, 2005). As shown in Fig. 3 B, in the absence of Rlp24, Drg1EQ1 showed lower ATPase activity, whereas Drg1EQ2 showed higher ATPase activity than
the wild-type protein. In this respect Drg1 resembles the AAA-ATPases Hsp104 and ClpB, which also show higher ATPase activity of one AAA domain upon inactivation of the other AAA domain (Watanabe et al., 2002; Mogk et al., 2003; Doyle et al., 2007; Schaupp et al., 2007). The higher ATPase activity of the EQ2 mutant suggests a tight coordination of ATP hydrolysis in the two ATPase domains of Drg1. In the presence of Rlp24C, increased activity was measured for wild-type, Drg1EQ1, and Drg1EQ2, demonstrating that the interaction with Rlp24 activates ATP hydrolysis in both AAA domains.

To investigate the contribution of ATP hydrolysis in the D1 and D2 domains to the in vivo function of Drg1, we analyzed the growth behavior of strains expressing only Drg1EQ1 or Drg1EQ2. The strain expressing Drg1EQ1 showed no obvious growth phenotype, whereas the strain expressing Drg1EQ2 was nonviable (unpublished data). Furthermore, overexpression of Drg1EQ2 from the strong CUP1 promoter resulted in a dominant-negative growth phenotype, whereas Drg1EQ1 overexpression did not affect growth (Fig. 3 C). To correlate these results with the effect of Drg1 on the composition of pre-60S particles, we analyzed Arx1-TAP containing particles from strains expressing Drg1EQ1 or Drg1EQ2 variants by Western blotting. As the EQ2 exchange is nonfunctional, this variant was overexpressed in the wild-type background. To allow distinguishing Drg1EQ2 from endogenous wild-type protein, the mutant variant was fused to GST. Although very little GST-Drg1EQ2 fusion protein was present in the crude extract of overexpressing cells compared with overexpressed wild-type protein, it was detected in preribosomal particles, suggesting a failure to release Drg1EQ2 (Fig. 3 D). The overexpression of the Drg1EQ2 protein resulted in accumulation of shuttling proteins and export factors similar as observed for the temperature-sensitive
suggesting that a failure to hydrolyze ATP in the second ATPase only slightly higher response units (RU) than the wild type, deficient variants in this analysis. The Drg1EQ2 variant reached action with Rlp24 was addressed by including the ATP hydrolysis-subsquent experiments.

responding nucleotides were added to the running buffer in all 1 mM ATP or AMP-PNP (Fig. S3 B). Therefore, the corre- nucleotide from Drg1 and could be prevented by addition of

dissociation is caused by washing out the nucleotide during the ex-

The stronger interaction under conditions where nucleo-
tide hydrolysis is blocked could indicate a higher affinity of Drg1 to Rlp24 when ATP is bound in D1. Alternatively, ATP hydrolysis in D1 could trigger dissociation of Drg1 from Rlp24. To distinguish between these possibilities, kinetic SPR analyses of the Drg1–Rlp24 interaction were performed. See Fig. S3 C for a representative experiment. The increase of dRU/dt for the wild-type protein and the mutant variant could not be fitted to a

drg1-18 mutant (Fig. 1 A). Therefore, the ATP hydrolysis defi-
ciency in D2, although supporting binding to pre-60S particles, renders the protein nonfunctional for its physiological activity. In contrast, strains expressing Drg1EQ1 did not show an altered composition of pre-60S particles (Fig. 3 E).

ATP hydrolysis in D1 dissociates Drg1 from Rlp24

To further characterize the interaction of Drg1 with Rlp24 we used the surface plasmon resonance (SPR) technology. SPR allows real-time monitoring of the interaction between two proteins after immobilization of one binding partner on a solid surface. GST-Rlp24C fusion protein was covalently linked toGST-Rlp24C fusion protein was covalently linked to

GST-Drg1EQ1 variant gained about fourfold higher RU values than the wild-type and EQ2 proteins (Fig. 4 A). Thus, inhibition of ATP hydrolysis in D1 enhances the interaction between Rlp24 and Drg1. Scatchard blot analysis showed that the equilibrium dissociation constant $K_d$ was significantly lower for the EQ1 mutant compared with the wild-type protein (Fig. 4 B). To further dissect the role of ATP hydrolysis for the Drg1–Rlp24 interaction, we monitored binding of the wild-type protein and the EQ1 variant in the presence of AMP-PNP. As shown in Fig. 4 C, the wild-type protein exhibited a longer initial phase and a steeper binding curve in the presence of the nonhydrolyzable analogue, resulting in higher RU values compared with ATP. This result suggests that nucleotide hydrolysis during the experiment significantly reduces the amount of bound Drg1. In contrast, the measured RU values for the Drg1EQ1 variant were lower in the presence of AMP-PNP compared with those in the presence of ATP (Fig. 4 C). Therefore, the ability to hydrolyze ATP in the D1 domain is a major determinant for interaction with Rlp24.

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presence of ATP stimulated binding of the wild-type protein to
GST-Rlp24C. However, significantly increased Drg1 levels were
observed when AMP-PNP was used instead of ATP. In this
respect, the C-terminal domain behaves differently from the
full-length Rlp24, which shows similar binding in the presence
of either nucleotide (compare with Fig. 1 D). The Drg1EQ1
variant showed even stronger binding to GST-Rlp24C than
the wild-type protein in the presence of nucleotide and AMP-
PNP did not further enhance binding, confirming the results
from the SPR analysis.

Drg1-mediated release of Rlp24 from pre-
60S particles is promoted by nucleoporins

To confirm these results using an independent approach,
in vitro binding studies of wild-type Drg1 protein and Drg1EQ1
with GST-Rlp24C were performed. As shown in Fig. 4 D, the
presence of ATP stimulated binding of the wild-type protein to
GST-Rlp24C. However, significantly increased Drg1 levels were
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**Drg1-mediated release of Rlp24 from pre-
60S particles is promoted by nucleoporins**

The increased ATPase activity of Drg1 upon binding to Rlp24
raised the question of whether ATP hydrolysis triggers the
direct release of Rlp24 or is used to liberate one or more other
shuttling proteins from pre-60S particles. To address this issue, we aimed to develop an in vitro release assay. For this purpose, pre-60S particles were purified from the \textit{drg1-18} mutant. These particles lack Drg1 but contain increased levels of shuttling proteins (Fig. 1 A). The purified particles were incubated with purified Drg1 and ATP. However, no release of shuttling proteins was observed (see Fig. 6 A, third lane). We therefore speculated that an additional factor might be required for the release reaction that was missing in our in vitro reaction mixture. To identify such a cofactor of Drg1, we performed a genomic two-hybrid screen (James et al., 1996). Because the N-terminal domain is the main interaction surface of AAA-ATPases, we specifically used this region of the protein for screening. The two-hybrid screen resulted in the isolation of 13 interacting clones from 8 \times 10^6 transformants. DNA sequence analysis of interacting clones identified fragments of the genes \textit{NUP42} (seven isolates representing three different clones), \textit{NUP100} (three isolates representing three different clones), \textit{NUP116} (two clones), and \textit{NUP159} (one clone). These genes encode FG repeat–containing nucleoporins present in the Nup82 subcomplex and are preferentially or exclusively located at the cytosolic surface of the NPC (Alber et al., 2007). Interaction occurred between the N-terminal domain of Drg1 and the FG repeat portion of the nucleoporins and was strongest for Nup116 (Fig. 5 A). Direct interaction between Drg1 and nucleoporins was confirmed by GST pull-down assays (Fig. 5 B). Further mapping of the Drg1 binding sites of Nup116 showed strongest binding for a fragment ranging from the N terminus of Nup116 to residue 172, with only minor contributions of the first 91 amino acid residues to this interaction (Fig. 5 C). To investigate whether this interaction is important in vivo, a mutant of Nup116 containing a deletion of amino acid residues 110–166 was tested for genetic interaction with \textit{drg1-ts} (James et al., 2007). Interaction occurred between the N-terminal domain of Drg1 and the FG repeat portion of the nucleoporins and was strongest for Nup116 as well as full deletion of the nucleoporin resulted in increased levels of Rlp24 on purified pre-60S particles. As a consequence, other shuttling factors like Nog1 are also accumulating (Fig. 6 B and not depicted). The increased levels of Drg1 on the particles from the mutants suggest that Nup116 is required for full activity and subsequent dissociation of Drg1 in vivo. We conclude that by means of ATP hydrolysis Drg1 specifically releases Rlp24 from pre-60S particles and that this release reaction is assisted by nucleoporins.

### Table 1. Kinetic constants of the interaction of Drg1 and Drg1EQ1 with immobilized Rlp24C (two-state reaction)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nucleotide</th>
<th>( k_{m1} )</th>
<th>( k_{m2} )</th>
<th>( k_{a1} )</th>
<th>( k_{a2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drg1</td>
<td>ATP</td>
<td>5.4 ± 0.9 \times 10^6</td>
<td>1.3 ± 0.1 \times 10^1</td>
<td>2.8 ± 0.5 \times 10^2</td>
<td>2.5 ± 0.5 \times 10^3</td>
</tr>
<tr>
<td>Drg1</td>
<td>AMP-PNP</td>
<td>1.1 ± 0.1 \times 10^5</td>
<td>6.0 ± 0.9 \times 10^2</td>
<td>1.5 ± 0.2 \times 10^2</td>
<td>6.9 ± 0.5 \times 10^4</td>
</tr>
<tr>
<td>Drg1EQ1</td>
<td>ATP</td>
<td>5.8 ± 0.4 \times 10^6</td>
<td>5.1 ± 1.5 \times 10^3</td>
<td>7.2 ± 1.2 \times 10^3</td>
<td>9.8 ± 0.2 \times 10^{14}</td>
</tr>
<tr>
<td>Drg1EQ1</td>
<td>AMP-PNP</td>
<td>5.4 ± 0.7 \times 10^6</td>
<td>3.5 ± 1.1 \times 10^3</td>
<td>1.1 ± 0.2 \times 10^2</td>
<td>8.4 ± 7.4 \times 10^{14}</td>
</tr>
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Mean values determined from five independent injections over at least three different CM5-chips.

The AAA-ATPase Drg1 functions in the initial steps of cytoplasmic pre-60S maturation where it is required for the release of several shuttling proteins including Mex67, Tif6, Nog1, and Rlp24 (Fig. 1; Pertschky et al., 2007). Here we show that the direct binding target and release substrate of Drg1 on preribosomal particles is the shuttling protein Rlp24. This protein joins the pre-60S particle in the nucleolus and accompanies it into the cytoplasm. After its release from the particle, it is substituted by the ribosomal protein L24 (Saveau et al., 2003). In vitro, Drg1 interacts with two independent binding sites within Rlp24. One lies in the part of Rlp24 that is related to the ribosomal protein L24, whereas the second site comprises the 53-residues-long Rlp24-specific C-terminal domain. Because this domain is unique for the shuttling protein, it could provide some degree of specificity to target Drg1 to the immature 60S subunit. Although each individual domain is sufficient for interaction with Drg1, the strongest binding was achieved with full-length Rlp24 (Fig. 2 B). Direct interaction between Drg1 and Rlp24 suggests that Rlp24 is the attachment site for Drg1 on pre-60S particles. Consistently, Rlp24, and particularly its C-terminal domain, is required for association of Drg1 with pre-60S particles in vivo (Lo et al., 2010).

Binding of Rlp24 stimulates the ATPase activity of Drg1. Intriguingly, this stimulatory effect is exclusively conferred by the C-terminal domain of Rlp24. Hence, Rlp24 not only acts in recruitment of Drg1 but also functions as an activator that stimulates ATP hydrolysis by the AAA-ATPase. Full activation of Drg1 is reached at a stoichiometry of one molecule of Rlp24 per hexamer Drg1, which presumably reflects the cellular situation where hexameric Drg1 encounters pre-60S particles containing the importance of ATP hydrolysis in the release reaction. In contrast to Rlp24, other tested shuttling proteins like Nog1, Mex67, and Nmd3 remained bound to the pre-60S particles. The influence of Nup116 on the release of Rlp24 was also tested in vivo. As shown in Fig. 6 B, deletion of the Drg1 binding site on Nup116 as well as full deletion of the nucleoporin resulted in increased levels of Rlp24 on purified pre-60S particles. As a consequence, other shuttling factors like Nog1 are also accumulating (Fig. 6 B and not depicted). The increased levels of Drg1 on the particles from the mutants suggest that Nup116 is required for full activity and subsequent dissociation of Drg1 in vivo. We conclude that by means of ATP hydrolysis Drg1 specifically releases Rlp24 from pre-60S particles and that this release reaction is assisted by nucleoporins.

### Discussion

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one Rlp24 molecule. The interaction with Rlp24 triggers a structural change in Drg1 that leads to positive cooperativity of the subunits and an increased ATP hydrolysis rate. In the presence of saturating concentrations of Rlp24 this rate showed a strong dependency on the nucleotide concentration. In contrast, in the absence of Rlp24, ATP hydrolysis showed only little increase when the nucleotide concentration was raised. We therefore propose that the ATPase domains of Drg1 adopt a repressed conformation, which is converted into an active form by binding of Rlp24. The transition into the active conformation could, for example, increase accessibility of the nucleotide binding pocket and thus facilitate exchange of the spent nucleotide.

The results obtained with the ATP hydrolysis–deficient mutants show that the interaction of Rlp24 with Drg1 stimulates ATP hydrolysis in both the D1 and D2 AAA domains. What is the contribution of the two ATPase domains of Drg1 to Rlp24 release? Only ATP hydrolysis in D2 is essential for viability and is required for the release of shuttling proteins including Rlp24 in vivo (Fig. 3 D). Consequently, D2 provides the energy for extracting Rlp24 from the preribosome. In contrast, inactivation of ATP hydrolysis in D1 does not affect growth and has only minor impact on the release of shuttling proteins from the pre-60S particle (Fig. 3 E). Notably, however, Drg1EQ1 forms more stable hexamers than the wild-type protein and shows very slow dissociation rates from Rlp24. We therefore speculate that ATP hydrolysis in D1 is necessary for disassembly of Drg1 into monomers and, as a consequence, dissociation of Rlp24 from Drg1. Considering that Rlp24 does not interact with monomeric Drg1 variants (i.e., Drg1-18), it is tempting to speculate that one Rlp24 molecule requires at least two Drg1 protomers for efficient binding. Hence, disassembly into monomers might be used as a mechanism to liberate Drg1 from Rlp24.

In summary, binding of Rlp24 to Drg1 not only triggers the extraction of Rlp24 from the pre-60S particle by ATP hydrolysis in D1 but also the disassembly of Drg1 into monomers and dissociation of Drg1 from Rlp24 by ATP hydrolysis in D2 (Fig. 7). The switch between monomeric and oligomeric states within its ATPase cycle is a remarkable feature that distinguishes Drg1 from its closest relatives, Cdc48, and its mammalian orthologue, p97. The latter forms stable oligomers...
Intriguingly, another direct interaction partner of Drg1, the nucleoporin Nup116, is needed for Drg1 to release Rlp24. Apart from Nup116 we also found interactions with other cytoplasmically exposed FG repeat nucleoporins, suggesting a certain redundancy of the interaction with Drg1. As the interaction with Nup116 was significantly stronger than with any of the other tested nucleoporins, Nup116 is most likely the main nucleoporin binding partner of Drg1. Because Nup116 does not affect the ATPase activity of Drg1, the nucleoporin has to serve a different function. The interaction of Nup116 with the N domain of Drg1 is reminiscent of binding of adapter proteins to the N domain of p97. These adapter proteins are thought to target the ATPase into distinct cellular pathways (Wang et al., 2004). However, the strict requirement for Nup116 in the in vitro release assay suggests a more active role of the nucleoporin in the release reaction and not a sole targeting function. For example, Nup116 could allow conversion of an otherwise nonproductive futile ATP hydrolysis cycle into successful extraction of Rlp24. Nevertheless, the interaction of Drg1 with nuclear pore proteins has to be rather transient, as GFP-Drg1 fusions do not exhibit nuclear rim staining (Pertschy et al., 2007; unpublished data). Regardless of the exact function of Nup116 in the release of Rlp24 by Drg1, its requirement suggests that export of the preribosome and the initiation of cytoplasmic pre-60S maturation are tightly coupled events. This coordination could ensure that the cytoplasmic maturation cascade starts as soon as the particle appears at the cytoplasmic side of the NPC complex.

In vivo, Drg1 is necessary for the release of several shuttling proteins from the pre-60S particle; however, in our in vitro assay, Drg1 specifically extracted Rlp24 but none of the other tested nonribosomal proteins (like Nog1 or Mex67). This suggests that Rlp24 is released before other shuttling proteins and export factors can leave the particle. Moreover, these factors do not dissociate spontaneously once Rlp24 has been released, but require additional, active stripping processes. The identification of factors catalyzing these processes will be a major task for future studies. By gradually supplying the assay with additional factors, the in vitro system we developed will provide an excellent tool to reconstitute cytoplasmic maturation steps in vitro and to finally obtain a detailed lineup of all cytoplasmic pre-60S maturation steps.
Materials and methods

Yeast strains and growth conditions
The yeast and bacterial strains used in the present study are listed in Table S1. All plasmids used are listed in Table S2. Chromosomal deletions or gene fusions were generated by homologous recombination using PCR products to transform the respective yeast strain as described previously (Longtine et al., 1998). For generation of deletion strains, PCR products were generated with the plasmids pFA6a-HIS3 or as templates and 47- to 50-nucleotide-long gene-specific primers hybridizing to the regions immediately upstream and downstream of the open reading frames. For generating gene fusions, the plasmids pFA6a-TAP(Tryc1), pFA6a 3HA-kanMX, and pDH5 (Yeast Resource Center, University of Washington) were used as templates. Correct integration was confirmed by colony PCR using one gene-specific primer and one primer specific to the selection cassette. Strains were grown at different temperatures (25, 30, or 37°C) either in YPD complex medium or for plasmid maintenance, in synthetic dextrose (SDC) or galactose medium supplemented with the appropriate amino acids.

Two-hybrid screen
For the yeast two-hybrid screen, the N domain (codon 1–255) of Drg1 was cloned in frame with the Gal4 DNA-binding domain in pGBDU-C2 (URA3 selection marker) after introduction of EcoRI (forward primer) and BamHI (reverse primer) sites. The plasmid was transfected into the reporter strain PJ69-4A (James et al., 1996). The resulting strain was transformed with a two-hybrid library derived from a mixture of pGAD-C1, -C2, and -C3 libraries (LEU2 selection marker) containing short yeast genomic DNA fragments (James et al., 1996). The library plasmids were isolated from transformants positive for all three reporter genes (HIS3, ADE2, and LacZ) after growth in S-FOA media lacking leucine, and then amplified in E. coli and characterized by DNA sequencing. For further characterization, plasmids were retransformed into PJ69-4A carrying pGAD-C1 with full-length DRG1, the N domain, or the two AAA domains (codon 235 to termination codon).

Tandem affinity purification (TAP)
Complexes were purified according to the standard TAP protocol (Rigaut et al., 1999; Puig et al., 2001) starting from 4 liters of yeast culture. Cells were grown to an OD$_{600}$ of 2.5, harvested by centrifugation, and resuspended in lysis buffer (20 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 2.5 mM Mg(OAc)$_2$, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and HP protease inhibitor cocktail [Serva]), and broken by sonification. After removal of cell debris by centrifugation, the supernatant was incubated with glutathione agarose beads (Sigma-Aldrich) at 4°C for 60 min. After washing of the beads three times with lysis buffer and once with binding buffer, proteins were eluted with binding buffer containing 300 mM imidazole. To remove imidazole, buffer exchange was performed using Zeba spin desalting columns 7K (Thermo Scientific) according to the manual. GST fusions and truncated versions of Rlp24 were generated by cloning into pGEX6P-1. Expression and crude extract preparation for purification of GST-Rlp24 variants and FG repeat containing fragments of nuclear pore proteins Nup42, Nup116, and Nup159 (Allen et al., 2001) was performed as described for the 6His-tagged proteins. Crude extracts were incubated with glutathione agarose beads (Sigma-Aldrich) at 4°C for 90 min and after extensive washing with lysis buffer, beads bound proteins were used for GST pull-down assays. Fragments of Nup116 were PCR amplified with primers containing EcoRI (forward primer) and SalI (reverse primer) sites and cloned into pGEX6P-1 and pET28a and purified as described earlier in this paragraph. All expression plasmids are listed in Table S2.

Protein–protein interaction
Interaction assays of purified Drg1 and Arx1 proteins with FG repeat containing nuclear pore proteins were performed in binding buffer using 30 µg of purified Drg1 or Arx1. Interaction assays of Rlp24 with Drg1 variants were performed similarly with 30 or 60 µg of purified Drg1. The GST-tagged bait proteins (Nups and Rlp24) were bound to GST beads and incubated with 30 or 60 µg of purified Drg1. The GST-Rlp24 variants and FG repeat containing nuclear pore proteins were performed in binding buffer using 30 µg of purified GST-Rlp24 variants and FG repeat containing nuclear pore proteins were performed in binding buffer using 30 µg of purified Rlp24.

Western blotting
Unless otherwise stated, all antisera used in this study were raised in rabbits with GST- or 6His-tagged fusion proteins purified from E. coli as antigen. Antisera directed against Nog1, Rlp24, Arx1, and Rlp1 were purchased from M. Fromont-Racine (Institut Pasteur, Paris). The anti-Rpl16 antiserum was a gift from S. Rospert (University of Freiburg, Freiburg, Germany). The Sqt1 and Rpl10, Nmd3, and Mex67/Mtr2 antisera were provided by B.L. Trumpower (Dartmouth Medical School, Hanover, NH), A.W. Johnson (University of Texas at Austin, Austin, TX), and E. Hurt (Biochemistry-Zentrum der Universität Heidelberg, Heidelberg, Germany), respectively. The anti-Drg1 antiserum was described previously (Pertsch et al., 2007). Antiserum directed against Rlp25 and Rpp0 were a gift from J.P. Ballesta (Universidad Autónoma de Madrid, Madrid, Spain). Anti-GFP antibody was obtained from Roche and anti-GST antibody was purchased from Sigma-Aldrich. Antiserum against goat against E. coli ribosomal S3 and L5 proteins were provided by O. Vesper (Max F. Perutz Laboratories, Vienna, Austria). Anti-secondary antibodies used anti-rabbit HRP (Roche) and donkey anti-goat HRP (Santa Cruz Biotechnology, Inc.) were used at a 1:15,000 dilution. The peroxidase activity was visualized with the ECL Western chemiluminescence kit (GE Healthcare).

SPR
SPR measurements were performed using a Biacore X system. GST-Rlp24C GST and (as characterized from the flow reference cell) were immobilized on a CMS sensor chip (GE Healthcare) using amine-coupling chemistry. The sensor surface was activated by injection of a 1:1 mixture of 0.1 M N-hydroxysuccinimide and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma-Aldrich). GST-Rlp24C and GST were diluted to 20 µg/ml in 10 mM sodium acetate, pH 5.0, and injected over the activated surface. The remaining noncoated ester groups were blocked with 1 M ethanolamine (Sigma-Aldrich). The amounts of immobilized GST-Rlp24C and GST were
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References


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