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Defining the pathway of cytoplasmic maturation of the 60S ribosomal subunit

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Abstract

In eukaryotic cells the final maturation of ribosomes occurs in the cytoplasm, where trans-acting factors are removed and critical ribosomal proteins are added for functionality. Here, we have carried out a comprehensive analysis of cytoplasmic maturation, ordering the known steps into a coherent pathway. Maturation is initiated by the ATPase Drg1. Downstream, assembly of the ribosome stalk is essential for the release of Tif6. The stalk recruits GTPases during translation. Because the GTPase Efl1, which is required for the release of Tif6, resembles the translation elongation factor eEF2, we suggest that assembly of the stalk recruits Efl1, triggering a step in 60S biogenesis that mimics aspects of translocation. Efl1 could thereby provide a mechanism to functionally check the nascent subunit. Finally, the release of Tif6 is a prerequisite for the release of the nuclear export adapter Nmd3. Establishing this pathway provides an important conceptual framework for understanding ribosome maturation.

Keywords

ribosome; ribosome biogenesis; EFL1; NMD3; TIF6

Introduction

In eukaryotic cells, the ribosomal subunits are assembled in the nucleolus, a subcompartment of the nucleus that is organized around the rDNA transcription units. Pre-ribosomal particles released from the nucleolus must be exported out of the nucleus for final rRNA processing and protein assembly events in the cytoplasm (Fromont-Racine et al., 2003; Johnson, 2009; Johnson et al., 2002; Tschochner and Hurt, 2003; Venema and

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Methods for HeLa cell work are described in Supplemental Material

Tollervey, 1999; Zemp and Kutay, 2007). Furthermore, both subunits are exported with a small complement of non-ribosomal trans-acting factors that must be released in the cytoplasm and shuttled back to the nucleus for subsequent rounds of 60S maturation and export. Some of these factors (Nmd3 and Arx1) facilitate export (Bradatsch et al., 2007; Gadal et al., 2001; Ho et al., 2000; Hung et al., 2008) while others (Tif6) may prevent the premature interaction of the ribosomal subunits (Valenzuela et al., 1982). Recycling these factors requires specialized cytoplasmic proteins that we will refer to as "releasing factors." These include the two ATPases Drg1 and the Hsp70 Ssa1/Ssa2, the two GTPases, Efl1 and Lsg1, the dual specificity phosphatase Yvh1 and additional associated proteins, reviewed in (Kressler et al., 2009; Panse and Johnson; Strunk and Karbstein, 2009; Zemp and Kutay, 2007).

Drg1 belongs to the AAA-ATPase family of proteins (Zakalskiy et al., 2002) that form hexameric ring-like structures and disassemble macromolecular complexes using the energy of ATP hydrolysis (Vale, 2000). ATPase-defective Drg1 cannot release Rlp24 and several additional pre-ribosomal proteins, including Nog1, Tif6 and Arx1 from the nascent subunits. Consequently, these proteins accumulate in the cytoplasm and are prevented from recycling to the nucleus (Pertschy et al., 2007).

The Hsp70 ATPase Ssa1/Ssa2, together with Jjj1 and Rei1, recycles Arx1 and Alb1 (Demoinet et al., 2007; Hung and Johnson, 2006; Lebreton et al., 2006; Meyer et al., 2007). Rei1 is a cytoplasmic C_2H_2 zinc finger protein, whereas Jjj1 is a J domain-containing chaperone belonging to the Hsp40 family whose members all bind Hsp70 ATPases. Jjj1 stimulates the ATPase activity of Hsp70 Ssa1 (Demoinet et al., 2007; Hung and Johnson, 2006; Lebreton et al., 2006; Meyer et al., 2007) that may remodel the nascent 60S subunit to facilitate the release of Arx1. Deletion of either of *REI1* or *JJJ1* blocks Arx1 release from nascent 60S subunits in the cytoplasm (Demoinet et al., 2007; Hung and Johnson, 2006; Lebreton et al., 2006; Meyer et al., 2007), preventing its recycling to the nucleus. Deletion of *ARX1* suppresses the growth defect of *rei1* and *jjj1* mutants, implying that it is the persistence of Arx1 on subunits that is detrimental to these mutants (Hung and Johnson, 2006; Lebreton et al., 2006).

The release of Tif6 from 60S subunits in the cytoplasm requires the GTPase Efl1 (elongation factor-like 1) (Becam et al., 2001; Senger et al., 2001), and Sdo1, the yeast ortholog of human Shwachman-Bodian-Diamond syndrome protein, SBDS (Menne et al., 2007). The mammalian ortholog of Tif6, eIF6, was initially identified as a subunit anti-association factor (Valenzuela et al., 1982). However, Tif6 in yeast is a nuclear shuttling protein required for 60S subunit biogenesis and does not appear to play a role in translation (Basu et al., 2001; Si and Maitra, 1999). Mutations in Tif6 that reduce its affinity for 60S subunits suppress the growth defect of *efl1* and *sdo1* mutants (Becam et al., 2001; Menne et al., 2007; Senger et al., 2001) and restore Tif6 shuttling, indicating that Tif6 is the primary target of Efl1 and Sdo1.

The fourth releasing factor, Lsg1, is a cytoplasmic GTPase required for recycling Nmd3 (Hedges et al., 2005; Kallstrom et al., 2003; West et al., 2005). Release of Nmd3 also depends on the ribosomal protein Rpl10. Overexpression of *NMD3* or mutations in Nmd3 that decrease its affinity for 60S subunits bypass the growth defect of *lsg1* and *rpl10* mutants, indicating that Nmd3 is the primary target of Lsg1 (Hedges et al., 2005).

Recently, we and others have identified Yvh1 as a fifth releasing factor that is required for the release of Mrt4 (Kemmler et al., 2009; Lo et al., 2009). Mrt4 is the nuclear paralog of P0, the ribosomal protein that forms the base of the stalk and supports the binding of two dimers of the acidic stalk proteins, P1 and P2. The bacterial counterpart of P0 is L10, and P1

and P2 correspond to bacterial L7/L12. The stalk is an essential feature of the large ribosomal subunit and is required for the recruitment of translation factors. Failure to release Mrt4 prevents the loading of P0 and thus the assembly of the stalk.

Although we now understand that multiple cytoplasmic maturation events are needed to produce a functional 60S subunit, an understanding of the order of these events has been lacking. Here, we carry out a comprehensive analysis of the order and potential interdependence of these release events. We confirm previous results showing that a failure to release Rlp24 prevents the loading of Rei1 and consequently the release of Arx1. We also show that the release of Arx1 is linked to the release of Tif6. Furthermore, we found that Drg1 is also required for the efficient release of Mrt4, to initiate stalk assembly. The assembly of the stalk precedes the release of Tif6 which itself is required for the final step, the release of the nuclear export adapter Nmd3. Thus, maturation of the pre-60S subunit involves an ordered series of linked steps, each dependent on the previous step, analogous to a biochemical pathway.

Results

The ribosome stalk protein P0 is required for the release of Yvh1

The ribosome stalk is an essential structure that recruits and activates translation factors. In yeast it is composed of P0 and two heterodimers of the small acidic P proteins P1A, P2B and P1B, P2A (Gonzalo and Reboud, 2003). Pre-60S subunits are initially assembled in the nucleolus with Mrt4 in place of P0. We have recently shown that the dual specificity phosphatase Yvh1 is required to release Mrt4 to allow the assembly of the ribosome stalk (Lo et al., 2009). Yvh1 and P0 bind sequentially, raising the possibility that P0 displaces or is required for the removal of Yvh1. To ask if this is the case we followed the localization of Yvh1 when P0 expression was repressed. However, because Yvh1 is a predominantly cytoplasmic protein, its persistence on subunits in the cytoplasm cannot be easily monitored. To deal with this problem, we fused the strong NLS from SV40 large T antigen to Yvh1 to drive it into the nucleus. NLS_{SV40}-Yvh1-GFP fully complements the function of *yvh1*Δ (Lo et al., 2009). If P0 were required for the release of Yvh1 from 60S subunits in the cytoplasm, we would expect a change in the localization of NLS_{SV40}-Yvh1-GFP from the nucleus to the cytoplasm as P0 is depleted. This redistribution would be analogous to what is observed for Mrt4 in the absence of Yvh1 (Kemmler et al., 2009; Lo et al., 2009). NLS_{SV40}-Yvh1-GFP localized in the nucleus in wild type cells (Fig 1A). However, in a strain in which P0 was under control of the glucose-repressible *GAL1* promoter, repression of P0 resulted in a dramatic relocalization of NLS_{SV40}-Yvh1-GFP to the cytoplasm (Fig 1A). If depletion of P0 prevents the release of Yvh1, then free Yvh1 will not be available for recycling Mrt4 (Kemmler et al., 2009; Lo et al., 2009). This is expected to lead to a secondary defect of blocking Mrt4 recycling. Indeed, Mrt4 was also mislocalized from the nucleus to the cytoplasm in P0 depleted cells (Fig 1B and (Rodriguez-Mateos et al., 2009)).

To confirm that the cytoplasmic mislocalized Yvh1 was associated with ribosomes and not free protein unable to be reimported into the nucleus, we monitored its sedimentation in sucrose gradients under conditions of P0 repression. NLS_{SV40}-Yvh1 remained associated with free 60S subunits under repressing conditions (Fig 1C), indicating that indeed the protein was not released from subunits in the absence of P0. We note that the P_{GAL}-P0 strain displays 60S defects even in the presence of galactose (Fig 1C, left panel) suggesting that the GAL promoter does not express optimal levels of P0.

Assembly of the stalk is required for the downstream release of Tif6 and Nmd3

Although Yvh1 is required for the release of Mrt4, Yvh1 could affect the release of other factors as well. We saw no effect of *yvh1*Δ on the localization of the shuttling factors Rlp24 or Arx1 (data not shown). However, we found that both Tif6 and Nmd3 were mislocalized in *yvh1*Δ cells (Fig 2A and Fig 2B). Nmd3 shuttles but is predominantly cytoplasmic at steady state, making it difficult to observe a redistribution of the protein due to a failure to recycle Nmd3. Consequently, we used an Nmd3(AAA) mutant that shows a nuclear bias due to point mutations in its nuclear export signal that reduce its rate of export (Hedges et al., 2006). A similar mislocalization of Tif6 in *yvh1*Δ cells was recently reported (Kemmler et al., 2009). To determine if the absence of Yvh1 or the persistence of Mrt4 blocked the release of Tif6 and Nmd3, we also monitored the localization of Tif6 and Nmd3(AAA) in *mrt4*Δ and *yvh1*Δ *mrt4*Δ cells. We did not observe mislocalization of Tif6 or Nmd3(AAA) in either of these mutants (Fig 2A and Fig 2B), indicating that it is the persistence of Mrt4 on the subunit that prevents the release of Tif6 and Nmd3.

The presence of Mrt4 on the pre-60S particle blocks the assembly of the ribosome stalk (Lo et al., 2009), a structure essential for the recruitment of GTPases such as eEF2 to the GTPase association center during translation (Gao et al., 2009). Intriguingly, the GTPase Efl1 is homologous to eEF2 (Senger et al., 2001). This led us to consider the possibility that the assembly of the stalk might also be required to trigger the activity of Efl1 to promote the downstream release of Tif6 and Nmd3. We monitored Tif6 and Nmd3(AAA) localization when P0 was depleted and found that both proteins were strongly mislocalized to the cytoplasm (Fig 2C and 2D).

Although the stalk is a pentameric complex of P0 and two heterodimers of P1 and P2, only P0, which forms the base of the stalk, is essential. To further dissect the function of the different stalk proteins, we examined the localization of Yvh1, Mrt4, Tif6 and Nmd3(AAA) in P0ΔC1 and P0ΔC2 mutants, each deleted of a binding site for one of the P1/P2 dimers and in a P0ΔC1C2 mutant, which cannot bind either dimer (Krokowski et al., 2006). Contrary to the phenotype of P0 depletion, none of the P0 mutants showed defects in recycling Yvh1, Mrt4, Tif6 or Nmd3 (data not shown). Thus, P0, but not the P1-P2 dimers, is required for the release of Yvh1, Tif6 and Nmd3.

To support our conclusion that assembly of the stalk is required for the release of Yvh1 and the subsequent release of Tif6, we immunoprecipitated pre-60S particles arrested at different stages of cytoplasmic maturation. As a bait protein, we used Nmd3, which appears to be the last factor released from the pre-60S subunit in the cytoplasm. Nmd3 complexes were immunoprecipitated from *drg1-1*^{ts} mutant cells and from P_{GAL}-*P0* and P_{GAL}-*EFL1* cells, in each case from permissive versus nonpermissive conditions. Arresting the pathway early with a *drg1-1*^{ts} mutant yielded particles loaded with Tif6 and Yvh1 (Fig 2E). Depletion of P0, which prevents the release of Yvh1 and Tif6, led to an accumulation of Yvh1 whereas depletion of Efl1 led to accumulation of Tif6 but reduced levels of Yvh1 in the Nmd3 particle (Fig 2E). The accumulation of Tif6 reflects the failure of Efl1 function, whereas the dramatic decrease in Yvh1 after Efl1 depletion is explained if Efl1 acts downstream of P0. In the absence of Efl1, P0 has already acted to release Yvh1 but Tif6 and Nmd3 remain trapped on subunits. These results corroborate the fluorescence microscopy and genetic results indicating that assembly of the stalk precedes the function of Efl1.

Stalk assembly is conserved in human cells

To ascertain if stalk assembly plays a similar role in the recycling of DUSP12 (human Yvh1) and eIF6 in mammalian cells, we knocked down P0 and DUSP12 using siRNA in HeLa cells. Knockdown of P0 resulted in a dramatic relocalization of MRTO4 (human

Mrt4) to the cytoplasm (Fig 3A) and a partial mislocalization of eIF6 to the cytoplasm. The mislocalization of MRTO4 is most likely the indirect consequence of not releasing DUSP12, although we cannot rule out that P0 can act directly on MRTO4 in HeLa cells. Knockdown of P0 also caused a dramatic mislocalization of DUSP12 (Fig 3B). We observed DUSP12 distributed throughout the cell, but largely excluded from the nucleus after P0 knockdown. The distribution to both the nucleus and cytoplasm is consistent with the observation that in yeast Yvh1 can function in either compartment (Kemmler et al., 2009;Lo et al., 2009). We have previously shown that knockdown of DUSP12 in HeLa cells results in relocalization of MRTO4 (Lo et al., 2009). Knockdown of DUSP12 also caused partial mislocalization of eIF6 to the cytoplasm (Fig 3A). Thus, in human cells as in yeast, assembly of the stalk is necessary for the recycling of DUSP12 as well as the subsequent release of eIF6.

The release of Tif6 by Efl1 and Sdo1 precedes the release of Nmd3—Sdo1 and the GTPase Efl1 are both required for the release of Tif6 (Becam et al., 2001; Menne et al., 2007; Senger et al., 2001), with Sdo1 being thought to recruit Efl1 to the subunit (Menne et al., 2007). To determine if the release of Tif6 is coupled with other release events, we tested *sdo1* and *efl1* mutants for their effects on the recycling of other trans-acting factors. We monitored the localization of 60S shuttling factors Nmd3, Tif6, Arx1 and Rlp24 in an *sdo1* temperature sensitive mutant (Warren, unpublished) at nonpermissive temperature. As previously reported (Becam et al., 2001; Menne et al., 2007; Senger et al., 2001), we observed mislocalization of Tif6 to the cytoplasm (Fig 4A). We also observed mislocalization of Nmd3(AAA) (Fig 4A), suggesting that the release of Tif6 and Nmd3 are somehow coupled. The mislocalization of Nmd3(AAA) was qualitatively similar to what we have reported previously for *lsg1* mutants (Hedges et al., 2005). We did not observe appreciable changes in localization of Rlp24 or Arx1 (Fig 4A and data not shown). We then tested if release of Nmd3 would also be affected by depletion of Efl1. The effect of depleting Efl1 replicated the phenotypes of an $sdo1^{ts}$ mutant; both Tif6 and Nmd3(AAA) showed clear mislocalization to the cytoplasm in P_{GAL}*::EFL1* cells grown on glucose (Fig 4B), whereas Arx1 localization was not affected (Fig 4B). Thus mutations in both Tif6 releasing factors also affect Nmd3 release.

We next turned to the question of the order of events of Tif6 and Nmd3 release. We previously reported that the cytoplasmic GTPase Lsg1 is required for the release of Nmd3 (Hedges et al., 2005). To determine if Tif6 or Nmd3 is released first, we asked if Lsg1 is required for Tif6 release. We observed mislocalization of Nmd3(AAA) but not of Tif6-GFP in an *lsg1-1* mutant and upon overexpression of the dominant negative *LSG1-K349T* mutant (Hedges et al., 2005) (data not shown). We also tested the specificity of mutant versions of Tif6 and Nmd3 for suppression of maturation defects. *TIF6-V192F* is a dominant mutant that bypasses the requirement for Sdo1 or Efl1 in vivo because of reduced affinity of the mutant protein for the pre-60S subunit (Becam et al., 2001; Menne et al., 2007; Senger et al., 2001). Strikingly, *TIF6-V192F* fully rescued the mislocalization defect of Nmd3(AAA) when Efl1 was depleted (Fig 4C compare panels 2 and 4). Thus, bypassing the need for Efl1 allows the release of Nmd3, suggesting that the presence of Tif6 blocks the release of Nmd3. On the other hand, *nmd3(I112T, I362T)*, a mutant that suppresses certain *rpl10* and *lsg1* mutants (Hedges et al., 2006), did not suppress the growth defect of either *sdo1* or *efl1* mutants and had no effect on Tif6 mislocalization when Efl1 was depleted (data not shown). These results demonstrate that the failure to recycle Nmd3 in *sdo1* or *efl1* mutants is the indirect consequence of not releasing Tif6. Thus the release of Tif6 by Efl1 and Sdo1 is upstream of and a prerequisite for the release of Nmd3 by Lsg1. Because the *lsg1-1* mutant appears to affect only Nmd3, the Lsg1 dependent release of Nmd3 does not appear to be coupled with release of other factors.

The Drg1 pathway converges with stalk assembly in the release of Tif6

A dominant mutant of Rlp24 prevents recruitment of Drg1—Rlp24 is an essential ribosome biogenesis factor that is associated with the pre-60S subunit during export from the nucleus. It is closely related to the ribosomal protein Rpl24, which replaces Rlp24 in the cytoplasm. Depletion of Rlp24 impairs rRNA processing and results in decreased 60S subunit levels (Saveanu et al., 2003). After transport of the pre-60S to the cytoplasm, Rlp24 is removed by the AAA-ATPase Drg1, allowing for the assembly of Rpl24 into the subunit (Pertschy et al., 2007). Rlp24 and Rpl24 share a conserved N-terminal domain that binds to the ribosome (Spahn et al., 2004). Rlp24 has a C-terminal extension absent from Rpl24. Given the role of Rlp24 in ribosome biogenesis, its C-terminus could have a specialized function in biogenesis or export (Fig 5A).

To address the role of the C-terminus of Rlp24, we deleted the last 53 amino acids (Rlp24ΔC). *rlp24ΔC* was unable to complement a *rlp24*Δ mutant (Supplemental Fig S1A). Western blotting and immunoprecipitation experiments showed that the truncated protein was expressed at a level similar to wild type and that it retained the ability to bind to 60S subunits (Supplemental Fig S1B and data not shown). Mutant proteins in multiprotein complexes are often dominant negative when overexpressed because they compete with the function wild-type protein. Indeed, Rlp24ΔC strongly inhibited cell growth when overexpressed (Fig 5B) and caused a 60S subunit deficiency (data not shown). These data imply that the C-terminal domain of Rlp24 is not required for ribosome binding but rather for other interactions once it is bound to the pre-60S subunit.

Rlp24ΔC localized to the cytoplasm, in contrast to the predominantly nucleolar localization of wild type Rlp24 (Supplemental Fig S2) and remained bound to subunits, assayed by sucrose gradient sedimentation (data not shown). We also blocked subunit export with leptomycin B (LMB), a specific inhibitor of the export receptor Crm1 (Kudo et al., 1999). Rlp24ΔC showed a modest accumulation in the nucleus after 30 min in the presence of LMB (Supplemental Fig S2) indicating that it does recycle to the nucleus, although inefficiently. These results suggest that Rlp24ΔC is not efficiently released from the subunit in the cytoplasm.

The AAA-ATPase Drg1 releases Rlp24 (Pertschy et al., 2007). Thus, we considered that Rlp24ΔC prevents recruitment of Drg1 to subunits. If so, 60S subunits with Rlp24ΔC should not contain Drg1. Because the release of Rlp24 is necessary for the subsequent recruitment of Rei1 (Lebreton et al., 2006), Rei1 should also not be present. To examine this, we immunoprecipitated different pre-60S particles using Rei1 and Lsg1. Figure 5C shows that Rlp24ΔC could not be detected in the Rei1 bound particles but was highly enriched on Lsg1 particles, suggesting that Rlp24ΔC blocks the loading of Rei1 but not Lsg1. To examine Drg1 binding we immunoprecipitated 60S subunits with Rlp24 or Rlp24ΔC and assayed for the presence of Drg1 by western blotting. Whereas wild type Rlp24 coimmunoprecipitated Drg1-containing 60S subunits, Drg1 was not detected in the Rlp24ΔC pull down (Fig 5D). In contrast, Nog1, which is recruited to the pre-60S particle by Rlp24 (Hung and Johnson, 2006; Lebreton et al., 2006; Nissan et al., 2002; Saveanu et al., 2003; Strasser et al., 2000), was recovered to similar extents in the Rlp24 and Rlp24ΔC samples (Fig 5D). Thus, Rlp24ΔC appears to block Drg1 binding specifically. The block in Rlp24 release and failure in recruiting Drg1 are consistent with the view that recruitment of Drg1 is necessary for the release of Rlp24 (Pertschy et al., 2007).

Overexpression of Rlp24ΔC impairs the release of Tif6 and Arx1—*drg1* mutants accumulate Rlp24, Arx1, and Tif6 in the cytoplasm (Pertschy et al., 2007). Because Rlp24ΔC prevents the recruitment of Drg1 to the nascent subunit, we asked if over-expression of Rlp24ΔC would phenocopy a *drg1* mutant. Rlp24ΔC or wild-type Rlp24 was

overexpressed in cells expressing GFP-tagged Tif6 or Arx1. Overexpression of Rlp24ΔC, but not wild-type Rlp24, caused mislocalization of Tif6-GFP and Arx1-GFP to the cytoplasm (Fig 5E), similar to the mislocalization observed in *drg1-1^{ts}* mutant cells (Fig 5F). However, the degree of mislocalization was less in Rlp24ΔC-expressing cells, probably because of incomplete penetrance of the mutant phenotype, as the mutant protein is expressed ectopically to wild-type Rlp24. We did not observe mislocalization of Mrt4 or Nmd3(AAA) when Rlp24ΔC was overexpressed (data not shown), nor did we observe mislocalization of Nmd3(AAA) in *drg1* mutant cells (data not shown). Interestingly, we did observe mislocalization of Mrt4 but not NLS_{SV40}-Yvh1 in *drg1* mutant cells (Fig 5F). This suggests that Drg1 is needed for the release of Mrt4 and is perhaps required for the loading of Yvh1. In parallel with the localization studies we carried out coimmunoprecipitations with Lsg1 and Rei1 from wild-type and *drg1-1^{ts}* mutant cells. In support of the localization data, we observed an enrichment of Tif6 and Rlp24 on the Lsg1-containing 60S subunits in the *drg1-1^{ts}* mutant (Fig 5G). The amount of Rpl8 (reflecting 60S subunits) in the Rei1 immunoprecipitation was greatly reduced in the *drg1-1^{ts}* mutant. This loss of 60S binding by Rei1 in *drg1-1^{ts}* cells explains the loss of Arx1 in the Rei1 immunoprecipitation. We conclude that deleting the C-terminus of Rlp24 phenocopies a *drg1* mutant, impairing the release of Rlp24 itself and subsequent downstream steps.

The release of Arx1 by Rei1 and Jjj1 is upstream of Tif6 release by Efl1 and Sdo1—The recycling of Arx1 from the cytoplasm to the nucleus requires Rei1, Jjj1 and the Hsp70 Ssa (Demoinet et al., 2007; Hung and Johnson, 2006; Lebreton et al., 2006; Meyer et al., 2010; Meyer et al., 2007). The observation that *rei1* mutant cells mislocalize both Arx1 and Tif6 (Lebreton et al., 2006) suggests that the recycling of these proteins is functionally connected. We previously proposed that Rei1, Jjj1 and Ssa work together to release Arx1 from the subunit (Meyer et al., 2007). Alternatively, it has been suggested that Rei1 and Jjj1 act at different steps, with Jjj1 being required for Arx1 release (Demoinet et al., 2007) and Rei1 acting at a later step to recycle Arx1 to the nucleus (Lebreton et al., 2006).

To further address the role of Rei1, Jjj1 and Arx1 in the release of Tif6, we first recapitulated the result that Tif6 is partially mislocalized to the cytoplasm in *rei1*Δ cells (Demoinet et al., 2007; Lebreton et al., 2006) (Fig 6A). However, we also found that Tif6 mislocalized in *jjj1*Δ cells (Fig 6A). The weaker mislocalization of Tif6-GFP in *jjj1*Δ compared to *rei1*Δ cells is consistent with milder growth defect of a *jjj1*Δ mutant compared to an *rei1*Δ mutant (Demoinet et al., 2007; Meyer et al., 2007). Nevertheless, the common effect on Tif6 localization implies that Rei1 and Jjj1 both act upstream of Tif6 release. (Note that in the wild-type and mutants, the fraction of cells showing a nuclear bias for Tif6 remains high, however there is a distinct increase of Tif6 in the cytoplasm of *rei1*Δ and *jjj1*Δ cells.) Taken together, these results imply that the persistence of Arx1 on the subunit inhibits the release of Tif6. Indeed, deletion of *ARX1* restores the nuclear localization of Tif6 (Fig 6B and (Lebreton et al., 2006)). As additional support for the notion that the persistence of Arx1 on the subunit blocks the release of Tif6, we asked if a mutation in *ARX1* that bypasses the requirement for Rei1 would restore Tif6 localization in *rei1*Δ cells. We identified *arx1-S347P* in a screen for suppressors of *rei1*Δ (Lo, Bresson and Johnson, unpublished). *arx1-S347P* fully complemented an *arx1*Δ mutant and suppressed the growth defect of *rei1*Δ cells (Fig 6C). In contrast, wild-type Arx1 was detrimental to *rei1*Δ cells. Unlike wild-type Arx1, which is predominantly cytoplasmic in *rei1*Δ cells, Arx1-S347P is nuclear (Fig 6D) as is Arx1 in wild-type cells (Hung and Johnson, 2006; Lebreton et al., 2006). When *arx1-S347P* was introduced into *rei1*Δ cells, it restored the normal nuclear localization of Tif6 (Fig 6B), indicating that the release of Arx1 from subunits facilitates the release of Tif6.

As described above, the *TIF6-V192F* mutant bypasses the requirement for its releasing factors Sdo1 and Efl1 (Menne et al., 2007; Senger et al., 2001). If Rei1 were to work in

concert with Sdo1 and Efl1, one might expect that *TIF6-V192F* would also suppress the growth defect of an *rei1*Δ or *jjj1*Δ mutant. However, *TIF6-V192F* did not improve the growth of either strain and did not affect the mislocalization of Arx1 in *rei1*Δ cells (data not shown). Together, these data indicate that the release of Arx1 by Rei1-Jjj1-Ssa is upstream of and a prerequisite for the release of Tif6 by Efl1 and Sdo1.

Tif6 was mislocalized in *rei1*Δ and *jjj1*Δ cells. Similarly, deletion of *YVH1* or depletion of P0 blocked the release of Tif6 and Nmd3. Thus, these two pathways impinge on the release of Tif6. However, neither Arx1 nor Rlp24 were affected by deletion of *YVH1* or depletion of P0 (data not shown), indicating that the Drg1 and Rei1 mediated steps are independent of stalk assembly. To ask if other release steps are coupled with the release of Mrt4 or Yvh1, GFP was integrated into the genomic locus of MRT4 in *drg1-1*^{ts}, *rei1*Δ, *jji1*Δ, *lsg1-1*^{ts}, *sdo1*^{ts}, and *GAL-EFL1* strains. We observed partial mislocalization of Mrt4-GFP in *drg1-1*^{ts} cells at restrictive temperature (Fig 5F). However, we did not observe significant relocalization of NLS_{SV40}-Yvh1-GFP from the nucleus (Fig 5F). The mislocalization of Mrt4 was not as complete as in *yvh1*Δ cells, nor was it as complete as the mislocalization of Rlp24 in *drg1-1*^{ts} cells (data not shown). We did not observe mislocalization of Mrt4-GFP in *rei1*Δ, *jji1*Δ, *lsg1-1*^{ts}, *sdo1*^{ts}, and P_{GAL}-*EFL1* strains (data not shown). These results support a model in which Drg1 and Rei1/Jjj1 work upstream of the release of Tif6. Similarly, Yvh1 and P0 function upstream of the release of Tif6 but on a separate pathway of stalk assembly. Although the effect of *drg1-1* on Mrt4 recycling suggests that Drg1 is required to initiate the stalk assembly pathway, stalk assembly and the release of Arx1 do not appear to be obligatorily linked, but converge on the release of Tif6. Figure 7 presents our interpretation of the order of the cytoplasmic maturation pathway for the 60S subunit.

Discussion

Newly assembled large ribosomal subunits are exported from the nucleus in a translationally inactive state, requiring the removal of a handful of biogenesis factors and the addition of several critical ribosomal proteins (Panse and Johnson). This translationally inactive state may provide “functional compartmentalization” of nascent ribosomes, preventing their premature engagement with the translation machinery to facilitate transport or allow localized activation. Additionally, it may provide further points of control of ribosome biogenesis and quality control. The final maturation of the 60S subunit in the cytoplasm involves five different events of protein removal or loading, most of which are catalyzed by ATPases or GTPases. Here, we have ordered these events in a coherent pathway in which each step is dependent on a prior event (Fig 7A). In Figure 7B we depict the events of this pathway in the context of the 3-dimensional structure of the 60S particle. Drg1 appears to initiate two parallel branches of the pathway that converge on the Efl1- and Sdo1-dependent release of Tif6. The release of Nmd3 by Lsg1 appears to be the last step before the subunit engages in translation. Because Tif6 prevents subunit association, its release would appear to regulate subunit association and it has been assumed that Tif6 is the last factor released from the large subunit prior to translation initiation. That release of Tif6 precedes the release of Nmd3 is unexpected. The establishment of this pathway provides a conceptual framework that can be integrated with the 3-dimensional structure of the ribosome to begin to understand the structural changes during ribosome maturation. In addition, the sequential nature of changes in the nascent subunit during maturation could serve as a paradigm for understanding the dynamics of other ribonucleoprotein complexes.

The interdependence of cytoplasmic releasing events

A dominant negative RLP24 mutant blocks recruitment of Drg1 and downstream steps—We found that deletion of the C-terminus of Rlp24 is strongly

dominant negative, blocking the binding of Rei1 and the release of Arx1 and Tif6. These phenotypes are very similar to those of a *drg1* mutant. Indeed, deletion of the C-terminus of Rlp24 blocks the recruitment of Drg1 to the nascent subunit. These results are consistent with Drg1 utilizing the C-terminus of Rlp24 for recruitment to the subunit, however we did not detect interaction between these two factors in a yeast 2-hybrid assay (unpublished). The interaction of Rei1 with Rpl24 in yeast-two-hybrid assay (Lebreton et al., 2006), explains the requirement for Rpl24 loading in the recruitment of Rei1 and release of Arx1. However, the binding site for Rei1 must be more complex than Rpl24 alone, as we have observed that an *rpl24a*Δ *rpl24b*Δ double deletion mutant only partially mislocalizes Arx1 and retains Rei1 binding to the ribosome (unpublished). Indeed, Rei1 has high affinity for RNA in vitro (M Parnell, personal communication), suggesting that its binding site may be composed of an RNA element in addition to Rpl24.

Release of Arx1 is required for efficient release of Tif6—Tif6 mislocalizes to the cytoplasm in *rei1*Δ (Lebreton et al., 2006) and *jjj1*Δ mutant strains, suggesting that deletion of *REI1* and *JJJ1* impinges on the Efl1 and Sdo1-dependent release of Tif6. Deletion of *ARX1* or a mutation of Arx1 that bypasses the need for Rei1 restores Tif6 recycling to the nucleus ((Lebreton et al., 2006) and this work), implying that it is the release of Arx1, rather than the loading of Rei1 or Jjj1, that is important for subsequent release of Tif6. We suggest that the release of Arx1 is a prerequisite for the efficient release of Tif6. In contrast to *rei1* and *jjj1* mutants, inactivation of Sdo1 or depletion of Efl1 did not trap Arx1 in the cytoplasm. In addition, an allele of *TIF6* that suppress *efl1* and *sdo1* mutants did not suppress the growth defect of an *rei1*Δ or *jjj1*Δ mutant. Thus, Rei1 and Jjj1 are required for efficient recycling of Tif6 but Efl1 and Sdo1 do not impact Rei1 and Jjj1 function. Our results place the Sdo1-Efl1-dependent release of Tif6 downstream of and functionally linked to Rei1-Jjj1-Ssa release of Arx1. How the release of Arx1 from the polypeptide exit tunnel impinges on the release of Tif6 on the joining face is not clear. However, cryo-electron microscopy identifies Rpl24, on the edge of the subunit joining face, as part of the binding site for Tif6 (Gartmann et al., 2010) (Fig 7B). Because Rpl24 also recruits Rei1, it may provide a means of coupling these two events.

The release of Tif6 is a prerequisite for Lsg1 release of Nmd3—We found that inactivation of Efl1 or Sdo1 prevented the release of both Tif6 and Nmd3 from 60S subunits in the cytoplasm. Although the block in Tif6 release was previously well documented (Becam et al., 2001; Menne et al., 2007; Senger et al., 2001), the block in Nmd3 recycling was unexpected. A mutant Tif6 with weakened affinity for the pre-60S subunit and that suppresses *sdo1* or *efl1* mutations also suppressed the release defect of Nmd3. In contrast, inactivation of Lsg1 specifically blocked the release of Nmd3, and a mutant Nmd3 that bypasses a mutation in *lsg1* did not suppress *efl1* or *sdo1*. These results show that Tif6 must be released prior to the release of Nmd3. Thus, the two releasing factors Efl1 and Lsg1 work in series with Efl1 acting upstream of Lsg1.

How do *efl1* or *sdo1* mutants block the release of Nmd3? Because the release of Nmd3 requires Lsg1, one possibility is that the binding of Lsg1 to the subunit is sterically blocked until Tif6 is released. However, Lsg1 can coimmunoprecipitate subunits containing Rlp24 and Tif6 (Fig 5G Lsg1 IP) indicating that it can bind considerably upstream of its point of function. That Lsg1 is present on the subunit during the time of Efl1 function suggests that it does not bind to the GTPase-associated center (GAC) of the ribosome as Efl1 likely does (Graindorge et al., 2005). In addition *lsg1* mutants do not inhibit the release of Tif6, indicating that they do not interfere with Sdo1 or Efl1 function. Another possibility is that the activity of Lsg1 is inhibited by the presence of Tif6. We do not yet know what acts as the effector for the GTPase activity of Lsg1. Lsg1 may sense a conformational change in the subunit, perhaps associated with the release of Tif6.

We have recently determined the position of Nmd3 to be relatively centered on the joining face of the 60S subunit (Sengupta et al., 2010). This is close to the position of Tif6, which binds on the edge of the joining face, interacting with Rpl23, Rpl24 and the sarcin-ricin loop (Gartmann et al., 2010). Indeed, there is evidence from a genome-wide protein complementation assay for their physical proximity (Tarassov et al., 2008). The binding sites of Tif6 and Nmd3 are consistent with functional coupling between their release events.

Functional proofreading in ribosome maturation

We previously proposed that "structural proofreading" may be employed to ensure that only properly assembled subunits are exported from the nucleus (Johnson et al., 2002). Thus, recruitment of the essential export adapter Nmd3 would depend on presenting a binding site that depends on proper subunit biogenesis. However, this does not provide a mechanism to check ribosome function. We now propose that the 60S subunit undergoes a quasi-functional assessment step during maturation in the cytoplasm. The ribosome stalk recruits and activates translation factors, including the elongation factor eEF2 (Berk and Cate, 2007). Here, we have identified a second essential function of the stalk, to promote the release of Tif6 and Nmd3 during the final maturation steps of the pre-60S subunit. The release of Tif6 requires Sdo1 and the GTPase Efl1 (Becam et al., 2001; Menne et al., 2007; Senger et al., 2001) and Efl1 is closely related to translation elongation factor 2 (Senger et al., 2001). This raises the intriguing possibility that a translation-like step is used during ribosome assembly. We suggest that assembly of the ribosome stalk is necessary for recruitment of the elongation factor-like GTPase Efl1 to the GAC. Correct assembly of the ribosome would lead to activation of Efl1 in a translocation-like event to promote final maturation of the subunit. This model utilizes the known function of the stalk in a previously unrecognized step in subunit maturation. Activation of Efl1 to release Tif6 is at the convergence of two branches of the 60S maturation pathway and may represent a critical quality control step controlling the progression of subunits into the actively translating pool.

Experimental Procedures

Strains, plasmids, and media

All *S. cerevisiae* strains used in this study are listed in Table S1. Cells were grown at 30°C, unless otherwise indicated, in rich medium (yeast extract-peptone) or synthetic dropout medium, containing 2% glucose. Plasmids used in this work are listed in Table S2. Details about strains and plasmid construction are in Supplemental Materials.

Microscopy

Overnight cultures of cells were diluted into fresh media and cultured for 3-4 hours at permissive temperature. The temperature sensitive strains and isogeneic wild type strains were shifted to 37°C for the indicate times, as described in the figure legends, before cell harvest. For LMB treatment, cells were concentrated ten-fold and LMB was added to a final concentration of 0.1μg/ml. Fluorescence was visualized on a Nikon E800 microscope fitted with an X100 objective and a Diagnostic Instruments SPOT II camera controlled by NIS-Elements AR2.10 software. Images were prepared using Adobe Photoshop 7.0.

Immunoprecipitation

For immunoprecipitations cultures were grown to an OD_{600} of ~0.5 in selective medium. The *rei1* and *jjj1* mutants were grown continuously at room temperature. Temperature-sensitive mutants were shifted to 37°**C** before cell harvest: the *Drg1ts* mutant was shifted for 1 hour, *sdo1ts* for 30 minutes and *lsg1-1* for 3 hours.

Cells were resuspended in IP buffer (20mM Tris pH 7.5, 100mM NaCl, 6mM $MgCl_2$, 10% glycerol, 0.1% NP40, 1mM PMSF and 1μ M leupeptin and 1μ M pepstatin A), lysed by vortexing with glass beads and clarified by centrifugation. Immunoprecipitation was done with α -c-myc or α -HA antibody and protein A agarose beads. Proteins were eluted in Laemmli sample buffer and detected by Western blotting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Basu U, Si K, Warner JR, Maitra U. The Saccharomyces cerevisiae TIF6 gene encoding translation initiation factor 6 is required for 60S ribosomal subunit biogenesis. Molecular and cellular biology. 2001; 21:1453–1462. [PubMed: 11238882]

Becam AM, Nasr F, Racki WJ, Zagulski M, Herbert CJ. Ria1p (Ynl163c), a protein similar to elongation factors 2, is involved in the biogenesis of the 60S subunit of the ribosome in Saccharomyces cerevisiae. Mol Genet Genomics. 2001; 266:454–462. [PubMed: 11713675]

Berk V, Cate JH. Insights into protein biosynthesis from structures of bacterial ribosomes. Current opinion in structural biology. 2007; 17:302–309. [PubMed: 17574829]

Bradatsch B, Katahira J, Kowalinski E, Bange G, Yao W, Sekimoto T, Baumgartel V, Boese G, Bassler J, Wild K, et al. Arx1 functions as an unorthodox nuclear export receptor for the 60S preribosomal subunit. Molecular cell. 2007; 27:767–779. [PubMed: 17803941]

Demoinet E, Jacquier A, Lutfalla G, Fromont-Racine M. The Hsp40 chaperone Jjj1 is required for the nucleo-cytoplasmic recycling of preribosomal factors in Saccharomyces cerevisiae. Rna. 2007; 13:1570–1581. [PubMed: 17652132]

Fromont-Racine M, Senger B, Saveanu C, Fasiolo F. Ribosome assembly in eukaryotes. Gene. 2003; 313:17–42. [PubMed: 12957375]

Gadal O, Strauss D, Kessl J, Trumpower B, Tollervey D, Hurt E. Nuclear export of 60s ribosomal subunits depends on Xpo1p and requires a nuclear export sequence-containing factor, Nmd3p, that associates with the large subunit protein Rpl10p. Molecular and cellular biology. 2001; 21:3405–3415. [PubMed: 11313466]

Gao YG, Selmer M, Dunham CM, Weixlbaumer A, Kelley AC, Ramakrishnan V. The structure of the ribosome with elongation factor G trapped in the posttranslocational state. Science. 2009; 326:694–699. [PubMed: 19833919]

Gartmann M, Blau M, Armache JP, Mielke T, Topf M, Beckmann R. Mechanism of eIF6-mediated inhibition of ribosomal subunit joining. J Biol Chem. May 14; 2010 285(20):14848–51. Epub 2010 Mar 31. [PubMed: 20356839]

Gonzalo P, Reboud JP. The puzzling lateral flexible stalk of the ribosome. Biology of the cell / under the auspices of the European Cell Biology Organization. 2003; 95:179–193. [PubMed: 12867082]

Graindorge JS, Rousselle JC, Senger B, Lenormand P, Namane A, Lacroute F, Fasiolo F. Deletion of EFL1 results in heterogeneity of the 60 S GTPase-associated rRNA conformation. J Mol Biol. 2005; 352:355–369. [PubMed: 16095611]

Hedges J, Chen YI, West M, Bussiere C, Johnson AW. Mapping the functional domains of yeast NMD3, the nuclear export adapter for the 60 S ribosomal subunit. The Journal of biological chemistry. 2006; 281:36579–36587. [PubMed: 17015443]

Hedges J, West M, Johnson AW. Release of the export adapter, Nmd3p, from the 60S ribosomal subunit requires Rpl10p and the cytoplasmic GTPase Lsg1p. The EMBO journal. 2005; 24:567–579. [PubMed: 15660131]

Ho JH, Kallstrom G, Johnson AW. Nmd3p is a Crm1p-dependent adapter protein for nuclear export of the large ribosomal subunit. The Journal of cell biology. 2000; 151:1057–1066. [PubMed: 11086007]

Hung NJ, Johnson AW. Nuclear recycling of the pre-60S ribosomal subunit-associated factor Arx1 depends on Rei1 in Saccharomyces cerevisiae. Molecular and cellular biology. 2006; 26:3718–3727. [PubMed: 16648468]

Hung NJ, Lo KY, Patel SS, Helmke K, Johnson AW. Arx1 Is a Nuclear Export Receptor for the 60S Ribosomal Subunit in Yeast. Mol Biol Cell. 2008; 19:735–744. [PubMed: 18077551]

Johnson, AW. Nuclear Export of Ribosomes. In: Kehlenbach, R., editor. Nuclear Transport. Landes Bioscience; Austin, TX: 2009.

Johnson AW, Lund E, Dahlberg J. Nuclear export of ribosomal subunits. Trends Biochem Sci. 2002; 27:580–585. [PubMed: 12417134]

Kallstrom G, Hedges J, Johnson A. The putative GTPases Nog1p and Lsg1p are required for 60S ribosomal subunit biogenesis and are localized to the nucleus and cytoplasm, respectively. Molecular and cellular biology. 2003; 23:4344–4355. [PubMed: 12773575]

Kemmler S, Occhipinti L, Veisu M, Panse VG. Yvh1 is required for a late maturation step in the 60S biogenesis pathway. The Journal of cell biology. 2009; 186:863–880. [PubMed: 19797079]

Kressler D, Hurt E, Bassler J. Driving Ribosome Assembly. Biochim Biophys Acta. 2009 in press.

Krokowski D, Boguszewska A, Abramczyk D, Liljas A, Tchorzewski M, Grankowski N. Yeast ribosomal P0 protein has two separate binding sites for P1/P2 proteins. Molecular microbiology. 2006; 60:386–400. [PubMed: 16573688]

Kudo N, Matsumori N, Taoka H, Fujiwara D, Schreiner EP, Wolff B, Yoshida M, Horinouchi S. Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. Proceedings of the National Academy of Sciences of the United States of America. 1999; 96:9112–9117. [PubMed: 10430904]

Lebreton A, Saveanu C, Decourty L, Rain JC, Jacquier A, Fromont-Racine M. A functional network involved in the recycling of nucleocytoplasmic pre-60S factors. The Journal of cell biology. 2006; 173:349–360. [PubMed: 16651379]

Lo KY, Li Z, Wang F, Marcotte EM, Johnson AW. Ribosome stalk assembly requires the dual-specificity phosphatase Yvh1 for the exchange of Mrt4 with P0. The Journal of cell biology. 2009; 186:849–862. [PubMed: 19797078]

Menne TF, Goyenechea B, Sanchez-Puig N, Wong CC, Tonkin LM, Ancliff PJ, Brost RL, Costanzo M, Boone C, Warren AJ. The Shwachman-Bodian-Diamond syndrome protein mediates translational activation of ribosomes in yeast. Nature genetics. 2007; 39:486–495. [PubMed: 17353896]

Meyer AE, Hoover LA, Craig EA. The cytosolic J-protein, Jjj1, and Rei1 function in the removal of the pre-60 S subunit factor Arx1. J Biol Chem. Jan 8; 2010 285(2):961–8. Epub 2009 Nov 9. [PubMed: 19901025]

Meyer AE, Hung NJ, Yang P, Johnson AW, Craig EA. The specialized cytosolic J-protein, Jjj1, functions in 60S ribosomal subunit biogenesis. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:1558–1563. [PubMed: 17242366]

Nissan TA, Bassler J, Petfalski E, Tollervey D, Hurt E. 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. The EMBO journal. 2002; 21:5539–5547. [PubMed: 12374754]

Panse VG, Johnson AW. Maturation of eukaryotic ribosomes: acquisition of functionality. Trends Biochem Sci.

Pertschy B, Saveanu C, Zisser G, Lebreton A, Tengg M, Jacquier A, Liebminger E, Nobis B, Kappel L, van der Klei I, et al. Cytoplasmic recycling of 60S pre-ribosomal factors depends on the AAA-protein Drg1. Molecular and cellular biology. 2007; 27:6581–6592. [PubMed: 17646390]

Rodriguez-Mateos M, Garcia-Gomez JJ, Francisco-Velilla R, Remacha M, de la Cruz J, Ballesta JP. Role and dynamics of the ribosomal protein P0 and its related trans-acting factor Mrt4 during

ribosome assembly in Saccharomyces cerevisiae. Nucleic Acids Res. 2009; 37:7519–7532. [PubMed: 19789271]

Saveanu C, Namane A, Gleizes PE, Lebreton A, Rousselle JC, Noaillac-Depeyre J, Gas N, Jacquier A, Fromont-Racine M. Sequential protein association with nascent 60S ribosomal particles. Molecular and cellular biology. 2003; 23:4449–4460. [PubMed: 12808088]

Senger B, Lafontaine DL, Graindorge JS, Gadal O, Camasses A, Sanni A, Garnier JM, Breitenbach M, Hurt E, Fasiolo F. The nucle(ol)ar Tif6p and Efl1p are required for a late cytoplasmic step of ribosome synthesis. Molecular cell. 2001; 8:1363–1373. [PubMed: 11779510]

Sengupta J, Bussiere C, Pallesen J, West M, Johnson AW, Frank J. Characterization of the nuclear export adaptor protein Nmd3 in association with the 60S ribosomal subunit. J Cell Biol. Jun 28; 2010 189(7):1079–86. [PubMed: 20584915]

Si K, Maitra U. The Saccharomyces cerevisiae homologue of mammalian translation initiation factor 6 does not function as a translation initiation factor. Molecular and cellular biology. 1999; 19:1416–1426. [PubMed: 9891075]

Spahn CM, Gomez-Lorenzo MG, Grassucci RA, Jorgensen R, Andersen GR, Beckmann R, Penczek PA, Ballesta JP, Frank J. Domain movements of elongation factor eEF2 and the eukaryotic 80S ribosome facilitate tRNA translocation. The EMBO journal. 2004; 23:1008–1019. [PubMed: 14976550]

Strasser K, Bassler J, Hurt E. Binding of the Mex67p/Mtr2p heterodimer to FXFG, GLFG, and FG repeat nucleoporins is essential for nuclear mRNA export. The Journal of cell biology. 2000; 150:695–706. [PubMed: 10952996]

Strunk BS, Karbstein K. Powering through ribosome assembly. RNA. 2009

Tarassov K, Messier V, Landry CR, Radinovic S, Serna Molina MM, Shames I, Malitskaya Y, Vogel J, Bussey H, Michnick SW. An in vivo map of the yeast protein interactome. Science. 2008; 320:1465–1470. [PubMed: 18467557]

Tschochner H, Hurt E. Pre-ribosomes on the road from the nucleolus to the cytoplasm. Trends in cell biology. 2003; 13:255–263. [PubMed: 12742169]

Vale RD. AAA proteins. Lords of the ring. The Journal of cell biology. 2000; 150:F13–19. [PubMed: 10893253]

Valenzuela DM, Chaudhuri A, Maitra U. Eukaryotic ribosomal subunit anti-association activity of calf liver is contained in a single polypeptide chain protein of Mr = 25,500 (eukaryotic initiation factor 6). The Journal of biological chemistry. 1982; 257:7712–7719. [PubMed: 7085645]

Venema J, Tollervey D. Ribosome synthesis in Saccharomyces cerevisiae. Annual review of genetics. 1999; 33:261–311.

West M, Hedges JB, Chen A, Johnson AW. Defining the order in which Nmd3p and Rpl10p load onto nascent 60S ribosomal subunits. Molecular and cellular biology. 2005; 25:3802–3813. [PubMed: 15831484]

Zakalskiy A, Hogenauer G, Ishikawa T, Wehrschutz-Sigl E, Wendler F, Teis D, Zisser G, Steven AC, Bergler H. Structural and enzymatic properties of the AAA protein Drg1p from Saccharomyces cerevisiae. Decoupling of intracellular function from ATPase activity and hexamerization. The Journal of biological chemistry. 2002; 277:26788–26795. [PubMed: 12006565]

Zemp I, Kutay U. Nuclear export and cytoplasmic maturation of ribosomal subunits. FEBS letters. 2007; 581:2783–2793. [PubMed: 17509569]

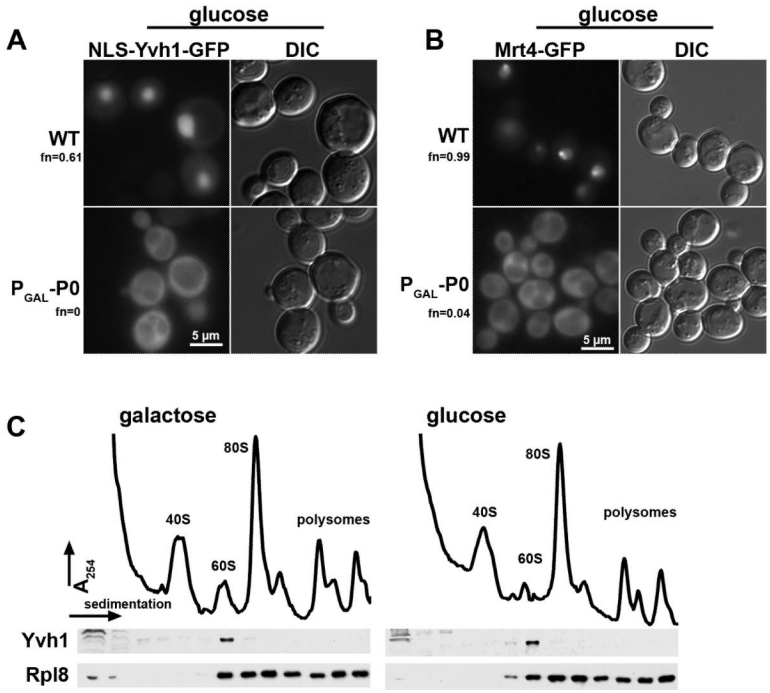

Figure 1. P0 is required for Yvh1 release

(A) The localization of NLS_{SV40}-Yvh1-GFP (pAJ2481) was visualized in wild-type (W303) and *P_{GAL1}::P0* strain (AJY3057). Cells were diluted from galactose-containing medium into glucose-containing medium and cultured for 6 hrs. (B) Mrt4-GFP localization in wild-type (AJY3100) and *P_{GAL1}::P0* (AJY3102) strains cultured as described in A. (C) NLS_{SV40}-Yvh1-GFP persists on 60S subunits when P0 is depleted. Strain AJY3110 (*P_{GAL}::P0 yvh1*Δ) containing pAJ2481 (*NLS_{SV40}-YVH1-GFP*) was cultured as described in A. Extracts were prepared and fractionated by sedimentation through 7-47% sucrose density gradients as described in (Lo et al., 2009). Western blotting was done with anti-GFP to detect NLS_{SV40}-Yvh1-GFP and anti-Rpl8 as a marker for 60S subunits. The fraction of cells showing stronger nuclear fluorescence than cytoplasmic (fraction nuclear, fn) is given for each panel.

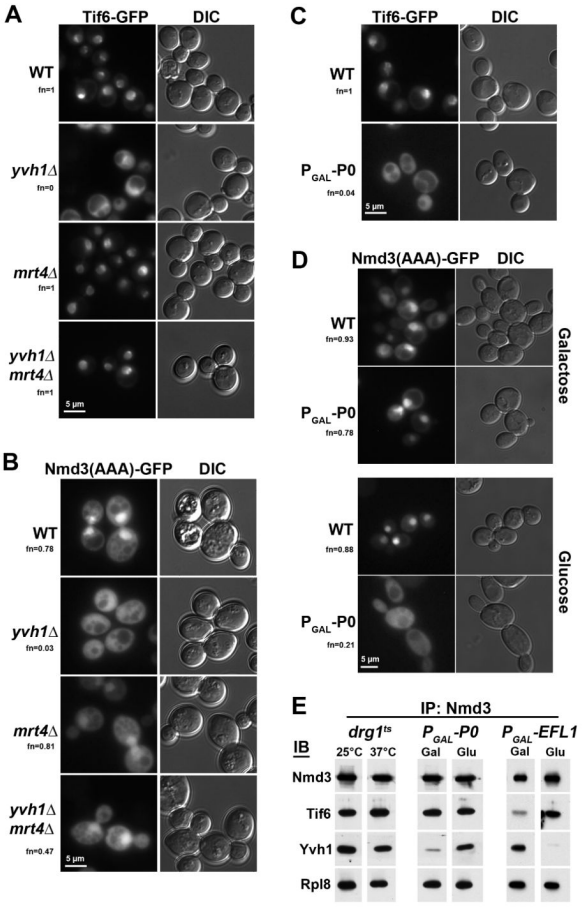

Figure 2. The assembly of the stalk is required for the function of the GTPases Efl1 and Lsg1

(A) Tif6-GFP was visualized in AJY2909 (wild-type), AJY3073 (*yvh1*Δ *TIF6-GFP*), AJY3075 (*mrt4*Δ *TIF6-GFP*), and AJY3098 (*yvh1*Δ *mrt4*Δ *TIF6-GFP*). (B) Nmd3(AAA)-GFP (pAJ754) was visualized in BY4741 (wild-type), AJY2976 (*yvh1*Δ), AJY2548 (*mrt4*Δ), and AJY2553 (*yvh1*Δ *mrt4*Δ). (C) Tif6-GFP was visualized in AJY3078 (wild-type) and in AJY3080 (P_{GAL1}*::P0 TIF6-GFP*). Strains containing P_{GAL}*::EFL1* or P_{GAL}*::P0* were cultured in galactose or shifted to glucose medium for 24 hrs (*EFL1*) or 3 hrs (*P0*). (D) Nmd3(AAA)-GFP (pAJ754) was expressed in wild-type (W303) and P_{GAL1}*::P0* (AJY3057). (E) Pre-60S particles were immunoprecipitated from conditional Drg1, P0 and EFL1 mutants. Nmd3-myc (pAJ538) was expressed in AJY3079 (*drg1-1*^{ts} *TIF6-GFP*), AJY3083 (P_{GAL1}*::EFL1 TIF6-GFP*) and AJY3080 (P_{GAL1}*::P0 TIF6-GFP*). Extracts were prepared from mid log phase cultures and immunoprecipitated as described in Materials and Methods. Proteins were separated by SDS-PAGE and western blotting was done using antibodies against c-myc (Nmd3), GFP (Tif6), Yvh1 and Rpl8.

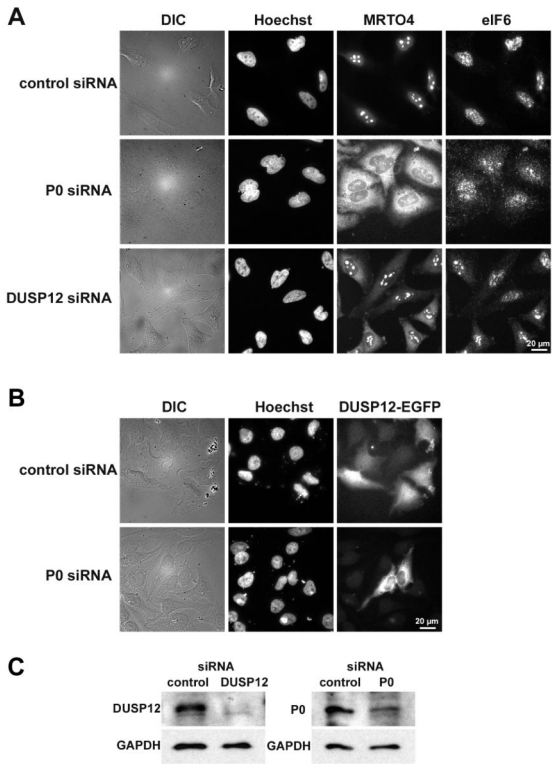

Figure 3. Depletion of P0 from HeLa cells affects the shuttling of DUSP12, MRTO4 and eIF6

(**A**) HeLa cells were transfected with either control siRNA or siRNA against P0 or DUSP12. The localization of MRTO4 and eIF6 was detected by indirect immunofluorescence with anti-MRTO4 (Santa Cruz Biotechnology, Inc) and anti-eIF6 antibody (Cell Signaling) 48 hours after transfection. Nuclei were visualized by staining with Hoechst 33342. (**B**) HeLa cells were transfected with control siRNA or siRNA against P0. After 48hr cells were transfected with DUSP12-eGFP (pcDNA3-DUSP12-EGFP). Twelve hours later cells were fixed and nuclei were stained with Hoechst 33342. (**C**) The efficiency of knockdown was monitored by western blotting whole cell extracts for DUSP12 and P0.

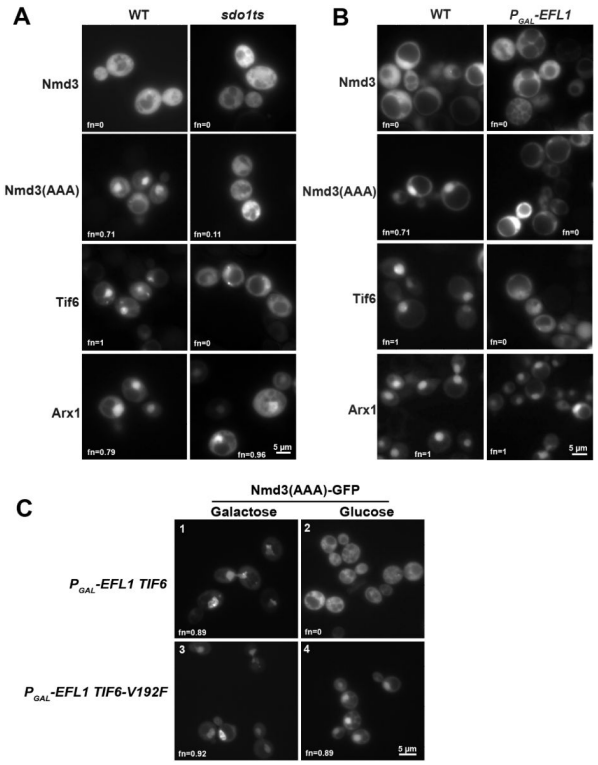

Figure 4. Mutation of *sdo1* or depletion of Efl1 blocks cytoplasmic release of Nmd3 which can be suppressed by TIF6(V192F)

(A) The localization of Nmd3, Tif6 and Arx1 was examined in wild-type versus *sdo1*^{ts} mutant cells. Nmd3-GFP (pAJ582), Nmd3(AAA)-GFP (pAJ754) or Tif6-GFP (pAJ1003) were expressed in wild-type (Y5563) or *sdo1*^{ts} mutant (BSY28) cells. Arx1-GFP was expressed from its genomic locus in wild-type (AJY3090) and *sdo1*^{ts} (AJY3086) cells. The cells were cultured at 30°C and then shifted to 37°C for 30 minutes before visualization by microscopy. (B) The localization of GFP-tagged proteins in Efl1-depleted cells. Nmd3-GFP (pAJ582) or Nmd3(AAA)-GFP (pAJ754) were expressed in wild-type (W303′) or P_{GAL1}-*EFL1* (AJY2981) cells. Tif6-GFP and Arx1-GFP were expressed from their genomic loci in wild-type (W303′) and P_{GAL1}-*EFL1* (AJY2981) cells. The cells were cultured in galactose-containing medium and then shifted to glucose medium for 26 hours before microscopy. (C) Nmd3(AAA) (pAJ754) localization was detected in P_{GAL1}*::EFL1 tif6*Δ cells expressing *TIF6* (AJY3013) or *TIF6(V192F)* (AJY3014) grown in galactose or glucose to repress *EFL1* expression.

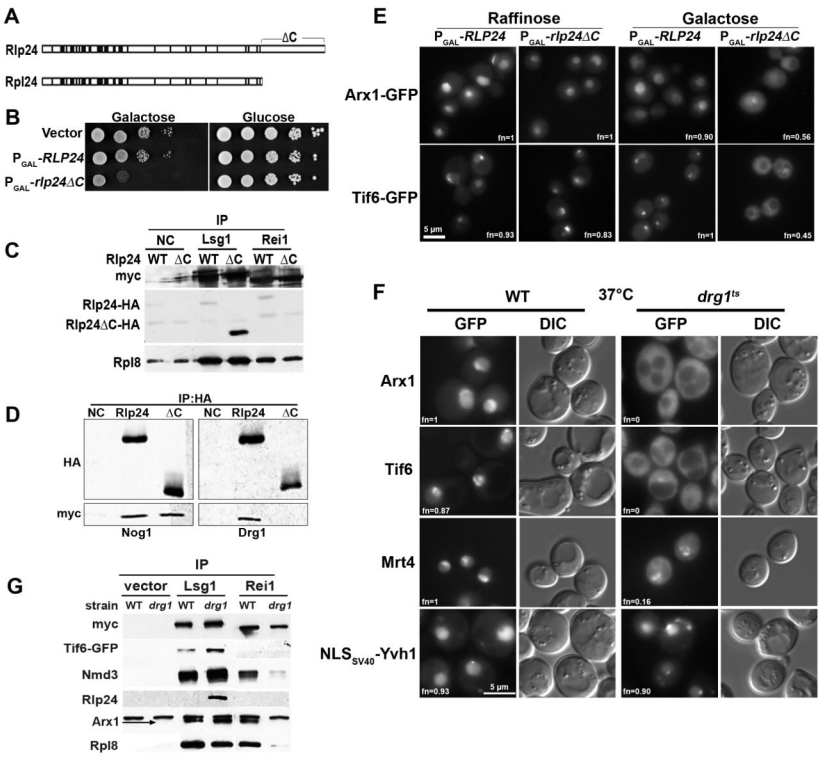

Figure 5. A dominant *rlp24* mutant phenocopies a *drg1* mutant

(A) Comparison of Rpl24 and Rlp24 generated using MACAW. Vertical bars indicate amino acid identities. The approximate truncation of the C-terminus unique to Rlp24 is indicated. (B) Serial dilutions of cultures of BY4741 containing vector, P_{GAL}*::RLP24* (pAJ2064) or P_{GAL}*::rlp24*Δ*C* (pAJ2065) were spotted onto glucose- or galactose-containing media. (C) Extracts were prepared from wild-type (BY4741) expressing Lsg1-myc (pAJ903) or Rei1-myc (pAJ1028) in combination with Rlp24-HA (pAJ1139) or Rlp24ΔC-HA (pAJ1895) and immunoprecipitated with anti-c-myc antibody. Western blotting of SDS-PAGE separated proteins was carried out against the myc epitope, Rlp24 and Rpl8. (D) Extracts from wild-type (BY4741) expressing Rlp24-HA (pAJ1139) or Rlp24ΔC-HA (pAJ1895) in combination with either Nog1-myc (pAJ2074) or Drg1-myc (pAJ2075) were immunoprecipitated with anti-HA antibody. Western blotting was done with anti-HA or anti-myc antibodies. (E) P_{GAL}*::RLP24* (pAJ2064) and P_{GAL}*::rlp24*Δ*C* (pAJ2065) were transformed in Arx1-GFP (AJY1948) and Tif6-GFP (AJY2909) expressing strains. Cells were grown in drop-out medium with raffinose or induced with galactose for 5 hours. (F) The localization of Arx1-GFP, Tif6-GFP, Mrt4-GFP and NLS_{SV40}-Yvh1-GFP was visualized in W303 and *drg1-1*^{ts} cells. Cells were cultured at 30°C and then shifted to 37°C for 1 hour before microscopy. (G) W303 or *drg1-1*^{ts} cells expressing Lsg1-myc (pAJ903), or Rei1-myc (pAJ1028) were cultured at 30°C until OD_{600} ~0.5 and then shifted to 37°C for 1 hour. Immunoprecipitation was carried out with anti-c-myc antibody and protein-A beads. Precipitated proteins were eluted in Laemmli buffer and separated by SDS-PAGE. Western blotting was performed against myc, GFP (Tif6), Nmd3, Rlp24, Arx1, and Rpl8. The position of Arx1 is indicated by an arrow. The band above Arx1 that is present in all fractions is a non-specific cross-reacting protein.

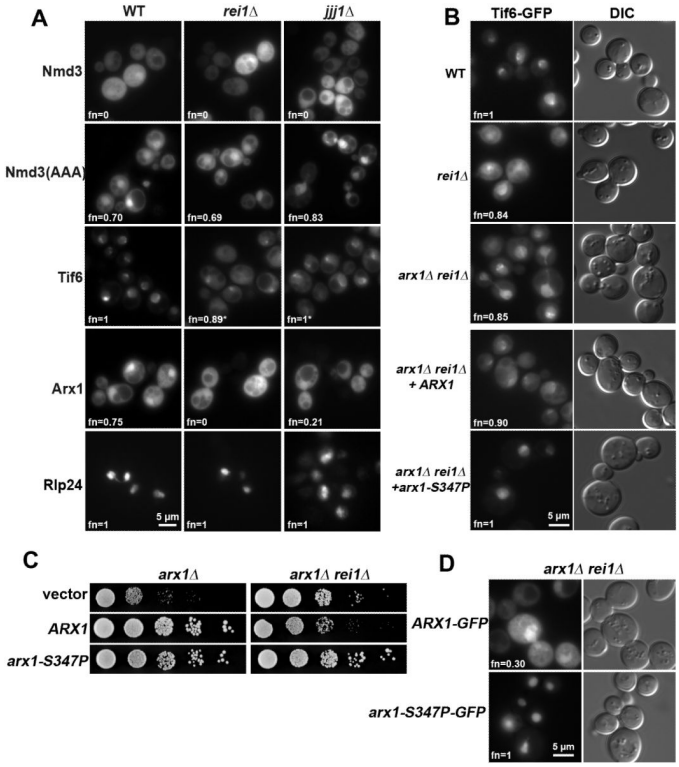

Figure 6. Tif6 is mislocalized in both *rei1*Δ and *jjj1*Δ mutant cells and the mislocalization can be suppressed by a functional *arx1* mutant

(A) The localization of Nmd3-GFP (pAJ582), Nmd3(AAA)-GFP (pAJ754) and Rlp24HA (pAJ1139) was visualized in wild-type (BY4741), *rei1*Δ (AJY1917) and *jjj1*Δ (AJY2474) cells. Tif6-GFP and Arx1-GFP were expressed genomically in the appropriate strains (see strains, Table S1). The cells were cultured at 25°C to mid-log phase. *Note that although the fraction of cells showing predominantly nuclear Tif6 is high for *rei1*Δ and *jjj1*Δ cells, in both cases, there is a uniform and significant mislocalization of Tif6 to the cytoplasm. (B) Tif6-GFP localization was visualized in wild-type (AJY2909), *rei1*Δ (AJY3074) and *arx1*Δ*rei1*Δ (AJY3093), and in *arx1*Δ *rei1*Δ cells expressing *ARX1* or *arx1-S347P*. (C) *arx1*Δ (AJY1901) or *arx1*Δ *rei1*Δ (AJY1903) cells were transformed with vector, *ARX1* (pAJ2425) or *arx1-S347P* (pAJ1682). Ten-fold serial dilutions were plated onto selective media and incubated 3 or 4 days, for *arx1*Δ or *arx1*Δ *rei1*Δ, respectively. (D) The localization of Arx1-GFP (pAJ1015) or Arx1-S347P-GFP (pAJ2423) was visualized in *arx1*Δ *rei1*Δ (AJY1903) cells.

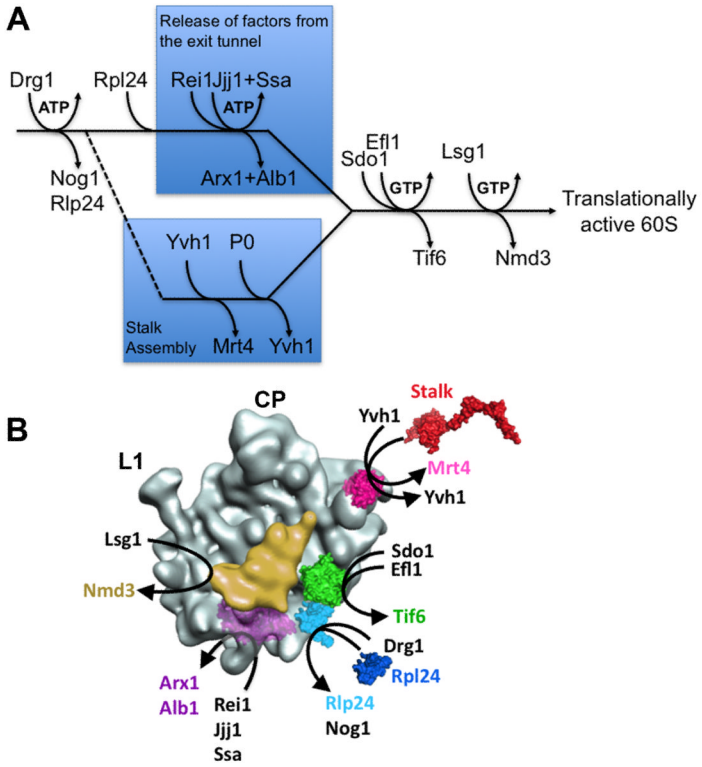

Figure 7. Proposed pathway of 60S maturation in the cytoplasm

(A) Drg1 facilitates the replacement of Rlp24 by Rpl24, which then recruits Rei1. The latter, together with Jjj1 and Ssa1/Ssa2, enables the release of the export receptor Arx1, located near the polypeptide exit tunnel. In parallel, Yvh1 enables replacement of Mrt4 with P0 to construct the ribosome stalk. In turn, the stalk recruits the GTPase Efl1 to the GTPase-associated center to release Tif6 from the subunit joining face of the particle. The release of Tif6 leads to activation of Lsg1 to release export adapter Nmd3, also from the joining face. It is important to note that the events indicated represent the order of action of these factors but not necessarily their order of association with the pre-60S particle. (B) Cartoon showing the events depicted in (A) in the context of the 60S particle in “crown” view, looking at the joining surface. Where possible, proteins have been positioned in their approximate locations on the particle. Mrt4 and Rlp24 are assumed to occupy the sites of P0 and Rpl24, respectively, in the mature subunit. The positions of Tif6 and Nmd3 are based on cryo-EM reconstructions of complexes in vitro (Gartmann et al., 2010, Sengupta et al., 2010). Arx1 is shaded to suggest that it binds on the back side of the particle, in the vicinity of the exit tunnel. CP: central protuberance; L1: L1 stalk.