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Assembly of *Saccharomyces cerevisiae* 60S ribosomal subunits: role of factors required for 27S pre-rRNA processing

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The precise functions of most of the ~200 assembly factors and 79 ribosomal proteins required to construct yeast ribosomes *in vivo* remain largely unexplored. To better understand the roles of these proteins and the mechanisms driving ribosome biogenesis, we examined in detail one step in 60S ribosomal subunit assembly—processing of $27SA_3$ pre-rRNA. Six of seven assembly factors required for this step (A_3 factors) are mutually interdependent for association with preribosomes. These A_3 factors are required to recruit Rrp17, one of three exonucleases required for this processing step. In the absence of A_3 factors, four ribosomal proteins adjacent to each other, rpL17, rpL26, rpL35, and rpL37, fail to assemble, and preribosomes are turned over by Rat1. We conclude that formation of a neighbourhood in preribosomes containing the A_3 factors establishes and maintains stability of functional preribosomes containing 27S pre-rRNAs. In the absence of these assembly factors, at least one exonuclease can switch from processing to turnover of pre-rRNA.

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Introduction

Formation of mature ribosomal subunits involves interplay between folding, modification, and processing of pre-rRNA, and binding of ribosomal proteins (r-proteins) to pre-rRNA (reviewed in Sykes and Williamson, 2009). Biogenesis of ribosomal subunits in *Saccharomyces cerevisiae* also requires the activity of ~200 transiently associating assembly factors (reviewed in Henras *et al*, 2008; Kressler *et al*, 2009). These factors bind preribosomes at specific steps of the assembly pathway, perform their functions, and eventually dissociate before the formation of mature ribosomal subunits. The precise mechanisms by which these assembly factors and r-proteins exert their roles during ribosome assembly are only now beginning to be understood.

Initially, studies of ribosome assembly in yeast focused on identification of intermediates in pre-rRNA processing, discovery of assembly factors, and determining which factors were required for which steps in pre-rRNA processing or nuclear export of pre-rRNPs. Subsequently, physical and genetic interactions between factors were identified, assembly intermediates were purified and their constituents determined, and assembly subcomplexes were discovered (reviewed in Henras *et al*, 2008; Kressler *et al*, 2009). It appears that most assembly factors have been found and initially assigned to one or more steps in subunit biogenesis. Thus, it is now possible to more comprehensively examine how all factors known to participate in one step work together to drive each assembly step and the biogenesis pathway. Such focused approaches have provided more detailed insights into mechanisms of late steps in maturation of pre-40S and pre-60S particles (reviewed in Panse and Johnson, 2010).

To better understand the mechanism of biogenesis of 60S subunits in yeast, we have focused on the pre-rRNA processing step involving the exonucleolytic removal of ITS1 sequences of $27SA_3$ pre-rRNA to form $27SB_{1S}$ pre-rRNA ('A_3 processing step') (Figure 1A and C). Proper processing of $27SA_3$ pre-rRNA is important because it generates the 5′-end of the major form of mature 5.8S rRNA, $5.8S_S$ rRNA. Furthermore, this processing event may initiate a conformational switch necessary to form functional ribosomes. Secondary structure models predict that ITS1 sequences in $27SA_3$ pre-rRNA, removed by the A_3 processing step, basepair with sequences in what will become the 5′-end of $5.8S_S$ rRNA (Yeh *et al*, 1990; van Nues *et al*, 1994). In mature ribosomes, the same sequences of $5.8S_S$ rRNA basepair with 25S rRNA and provide a binding site for the r-protein rpL17 (Taylor *et al*, 2009; Ben-Shem *et al*, 2010; Supplementary Figure S1). Exonucleolytic trimming of $27SA_3$ pre-rRNA to $27SB_{1S}$ pre-rRNA therefore provides an excellent framework to understand how the interplay between RNA folding, RNA processing, and protein binding enables maturation of preribosomes.

Approximately 80 different assembly factors have been found in one or more 66S preribosomes that are precursors to mature 60S ribosomal subunits. Effects on 60S subunit biogenesis have been examined for 70 of these 80 factors. A defect in $27SA_3$ pre-rRNA processing is evidenced by the accumulation of the $27SA_3$ pre-rRNA precursor and reduction of the immediate downstream product, $27SB_{1S}$ pre-rRNA (Dunbar *et al*, 2000; Pestov *et al*, 2001; Adams *et al*, 2002;

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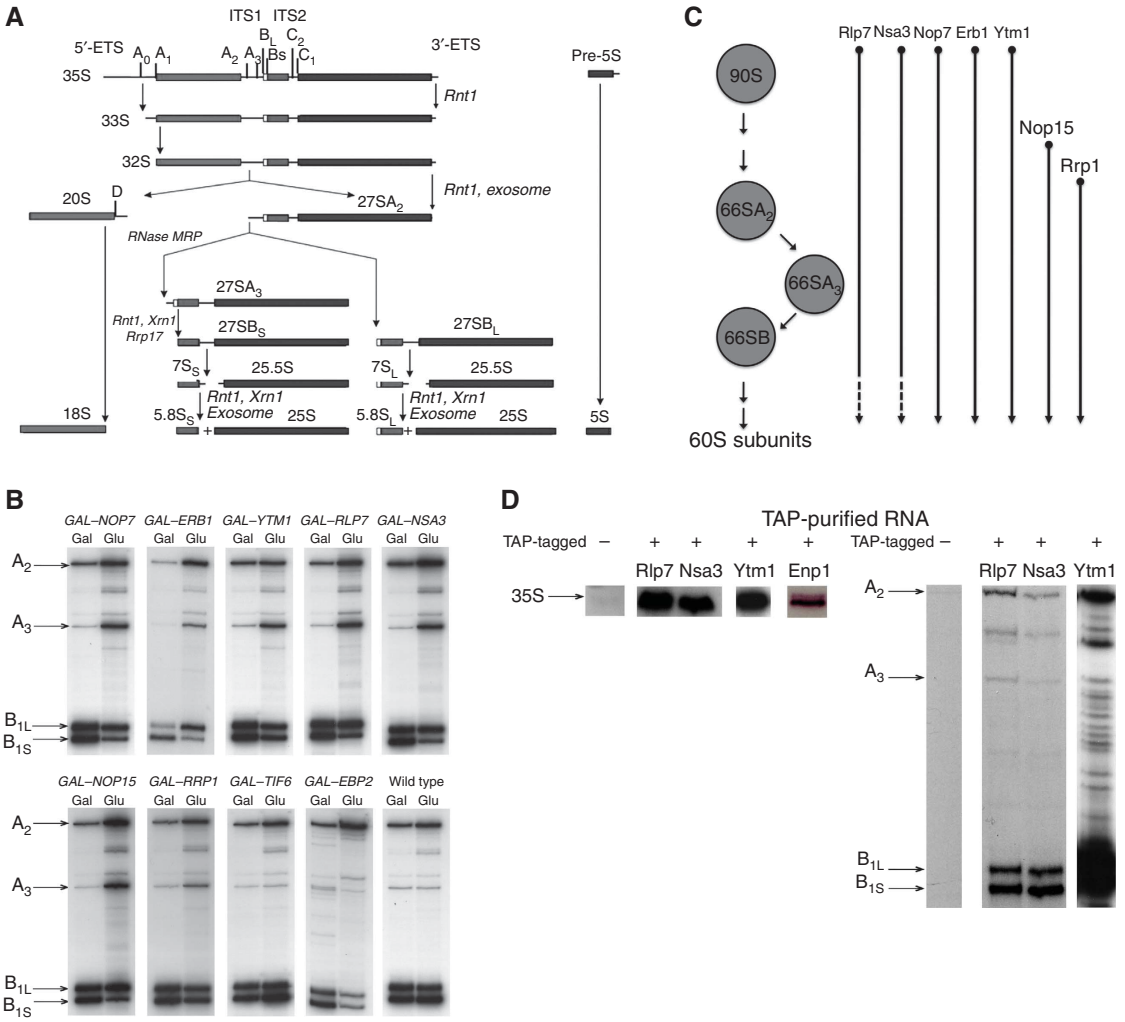

Figure 1 Pre-rRNA processing pathway in *S. cerevisiae* and assembly factors involved in processing of $27SA_3$ pre-rRNA. (**A**) Pre-rRNA processing pathway in *S. cerevisiae*. The initial 35S pre-rRNA transcript, synthesized by RNA Polymerase I, contains sequences for mature 18S, 5.8S, and 25S rRNAs along with internal and external transcribed spacer sequences (ITS/ETS). Processing intermediates that form during ribosome assembly are indicated. Exo- and endonucleases, where known, are mentioned alongside the step in which they function. (**B**) Defects in pre-rRNA processing in A_3 factor mutants. RNA was extracted from lysates prepared from cells grown in galactose (left lane in each pair) or shifted to glucose to deplete respective proteins (right lanes), and subjected to primer extension to assay the A_2, A_3, B_{1S}, and B_{1L} ends of pre-rRNAs. The B_{1S} and B_{1L} ends include both 27S and 7S pre-rRNA species. Depletion of Tif6 and Ebp2 are shown as controls for assembly mutants that do not specifically block processing of $27SA_3$ pre-rRNA. A wild-type control is included to indicate effects of the carbon source shift. Ratios of each processing intermediate in unshifted and shifted samples were quantified and are shown in Supplementary Table S3. All samples were run on the same gel, except *GAL–ERB1*, which was run on a separate gel with *GAL–RLP7* as a control. (**C**) Timing of association of A_3 factors with nascent ribosomes. The assembly pathway of 60S ribosomal subunits is shown. Preribosomes (grey circles) are aligned with processing intermediates in (**A**). Each line represents the duration of time for which an individual protein associates with preribosomes. The broken line indicates that the association of the protein with those preribosomal intermediates has not been determined. Arrowheads represent direction of the assembly pathway, and where determined, the point of exit of assembly factors. (**D**) Entry point of association of Rlp7, Nsa3/Cic1, and Ytm1 with nascent ribosomes. TAP-tagged strains as indicated were used to purify preribosomes containing Rlp7, Nsa3, or Ytm1. Pre-rRNAs present in these preribosomes were assayed by primer extension. Untagged parent strain is used as the negative control. *ENP1–TAP* strain is shown as positive control for co-IP of 35S pre-rRNA. Primer extension products for Rlp7 and Nsa3 samples were run on one gel, for Ytm1 on a second, and for Enp1 on a third, each with an untagged negative control. Figure source data can be found with the Supplementary Information.

Gadal *et al*, 2002; Oeffinger *et al*, 2002; Oeffinger and Tollervey, 2003; Horsey *et al*, 2004; Miles *et al*, 2005; Figure 1B; Supplementary Table S3). Thus far, mutation or depletion of only seven of these 70 assembly factors tested unambiguously results in a pre-rRNA processing phenotype diagnostic of a role in $27SA_3$ pre-rRNA processing. These proteins, which we refer to as the 'A_3 factors', include potential scaffolding proteins (Nop7, Erb1, and Ytm1), RNA-binding proteins (Rlp7 and Nop15), and proteins with no predicted function (Nsa3/Cic1, Rrp1).

The 5′–3′ exonucleases Rat1, Xrn1, and Rrp17 together with Rai1 (Rat1-interacting protein) also are required for processing $27SA_3$ pre-rRNA, and normally halt precisely at the B_{1S} site (Henry *et al*, 1994; Xue *et al*, 2000; Oeffinger *et al*, 2009). When 60S subunit assembly is aborted upon depletion of A_3 factors, 27S pre-rRNAs undergo turnover (Dunbar *et al*, 2000; Pestov *et al*, 2001; Adams *et al*, 2002; Gadal *et al*, 2002; Oeffinger *et al*, 2002; Oeffinger and Tollervey, 2003; Horsey *et al*, 2004; Miles *et al*, 2005). Interestingly, Rat1 has been shown to function in the turnover of poly(A) + 27S

pre-rRNAs in strains defective for the exosome (Fang *et al*, 2005). This result led us to ask whether Rat1 might have a role in turnover of defective 27S pre-rRNAs generated in A_3 factor mutants.

In order to understand the events underlying the A_3 processing step, and to understand the principles involved in eukaryotic ribosome biogenesis, we have addressed the following issues: (1) the timing of association of A_3 factors with preribosomes, (2) the interdependence among A_3 factors for association with preribosomes, (3) the role of A_3 factors, if any, in recruitment of exonucleases to preribosomes, and in stopping exonucleases precisely at the B_{1S} site, (4) whether A_3 factors enable association of other assembly factors and r-proteins with preribosomes, which might be required for subsequent remodelling events within preribosomes, and (5) the fate of preribosomes in A_3 factor mutants, specifically whether they are turned over by the Rat1 exonuclease.

Results

A_3 factors associate with preribosomes in a concerted manner, well before processing of $27SA_3$ pre-rRNA

To begin to understand how $27SA_3$ pre-rRNA processing occurs, we asked when and how all assembly factors required for this processing step are recruited into preribosomes. Nop7 and Erb1 first associate with 90S preribosomes containing 35S pre-rRNA, whereas Rrp1 and Nop15 associate subsequently with 66S preribosomes containing $27SA_2$ pre-rRNA (Oeffinger and Tollervey, 2003; Horsey *et al*, 2004; Miles *et al*, 2005; Figure 1C). In order to determine the timing of entry of the remaining A_3 factors—Rlp7, Nsa3/Cic1, and Ytm1, we assayed with which pre-rRNA species each protein copurifies. The 90S factor Enp1 was used as a positive control for coimmunoprecipitation (co-IP) of 35S pre-rRNA. Co-IP of 35S pre-rRNA revealed that, like the other A_3 factors, Rlp7, Nsa3/Cic1, and Ytm1 assemble early into preribosomes, well before their requirement for processing of $27SA_3$ pre-rRNA (Figure 1C and D).

Since A_3 factors are required for the same pre-rRNA processing step and some of them physically interact with each other (Miles *et al*, 2005; Tang *et al*, 2008), we examined whether they are interdependent for their association with preribosomes. We purified preribosomes from strains in which each A_3 factor was depleted and asked what happened to the other A_3 factors. Because all seven assembly factors are essential, we engineered conditional *GAL* promoter strains to regulate expression of each gene (Longtine *et al*, 1998). We used TAP-tagged assembly factor Rpf2 to purify preribosomes, since Rpf2 is present in all precursors to 60S subunits and does not have a known role in processing of $27SA_3$ pre-rRNA (Zhang *et al*, 2007). Preribosomes were purified from strains grown in galactose and after shifting from galactose to glucose-containing medium for 16 h to deplete each assembly factor.

As shown before, Nop7, Erb1, and Ytm1 are interdependent for assembly into preribosomes (Figure 2A; Tang *et al*, 2008). Silver staining and mass spectrometry also revealed that Rlp7, Nop15, and Nsa3/Cic1 were significantly decreased in preribosomes lacking Nop7, Erb1, or Ytm1 (Figure 2A; unpublished observations). Likewise, upon depletion of Rlp7, Nop15, or Nsa3/Cic1, the five other A_3 factors (except Rrp1) were greatly diminished in preribosomes (Figure 2A and B; Supplementary Figure S2A). The SDS–PAGE profiles of preribosomes upon depletion of each of these six interdependent proteins were remarkably similar (Figure 2A), and appeared to be unique to factors required for $27SA_3$ pre-rRNA processing. Very different changes in the composition of Rpf2-containing preribosomes were seen in strains mutant for any of 20 other assembly factors that function in different steps of pre-rRNA processing, for example upon depletion of Rea1 (Figure 2A; unpublished observations). Therefore, the observed gel profiles of preribosomal proteins in A_3 factor mutants are specific and not simply a secondary effect of prolonged growth in glucose-containing medium, to deplete the A_3 factors.

Western blotting of preribosomes purified from the *GAL–RLP7* strain is shown here as an example of interdependent association of A_3 factors (Figure 2B). In the absence of Rlp7, levels of the A_3 factors Nop7, Ytm1, Nsa3/Cic1, and Nop15 are diminished in preribosomes (Erb1 was not tested). Although not in preribosomes under these conditions, the three proteins Nop7, Erb1, and Ytm1 are present as a heterotrimeric subcomplex (Miles *et al*, 2005) (Figure 2C). In contrast, factors required for other steps in pre-rRNA processing and assembly were unchanged when Rlp7 was depleted (Figures 2A and B and 3A and B; Supplementary Figure S2B). When Rrp1 was depleted from cells, few changes in preribosome composition could be detected by inspection of stained proteins resolved by SDS–PAGE (Figure 2D).

Thus, six A_3 factors—Nop7, Erb1, Ytm1, Rlp7, Nop15, and Nsa3/Cic1 are interdependent for their association with preribosomes, whereas Rrp1 appears to associate independently. Owing to the interdependence of A_3 factors (Figure 2), all experiments described henceforth were performed only in the *GAL–RLP7* strain.

To more thoroughly assay global changes in the composition of preribosomes upon depletion of A_3 proteins, we took a proteomic approach, using iTRAQ (Ross, 2004). We compared preribosomes purified from the *GAL–RLP7 RPF2–TAP* strain grown in galactose or shifted to glucose, to identify proteins that either increased or decreased in the absence of Rlp7 (Figure 3A and B; Supplementary Figure S2B; Supplementary Table S1). We identified 177 proteins from 1859 spectra ($\geqslant$95% confidence), including 51 assembly factors (Figure 3A), 63 r-proteins (Figure 3B), and 62 other proteins (Supplementary Figure S2C).

Levels of $\sim$15 assembly factors associated with preribosomes were increased upon depletion of Rlp7 (Figure 3A; see also Figure 2A). These assembly factors function in early steps of ribosome biogenesis, before processing of $27SA_3$ pre-rRNA (Dragon *et al*, 2002; reviewed in Henras *et al*, 2008; Kressler *et al*, 2009). Their accumulation in preribosomes when $27SA_3$ pre-rRNA processing is blocked is in agreement with the observed enrichment of early pre-rRNAs in these mutants. Consistent with the SDS–PAGE and immunoblot analysis (Figure 2A and B), levels of Nop7, Nop15, and Nsa3/Cic1 in preribosomes were reduced in the absence of Rlp7. Erb1 and Ytm1 were not identified by iTRAQ, as is often the case in our experience. Assembly factors Nog2, Nsa2, and Alb1 also were reduced, consistent with their joining preribosomes after completion of $27SA_3$ pre-rRNA processing (Saveanu *et al*, 2001, 2003; Lebreton *et al*, 2006). Cgr1, Spb1, Ybl028c, Nop16, and Dbp10 proteins exhibited the greatest reduction in preribosomes when A_3 factors were

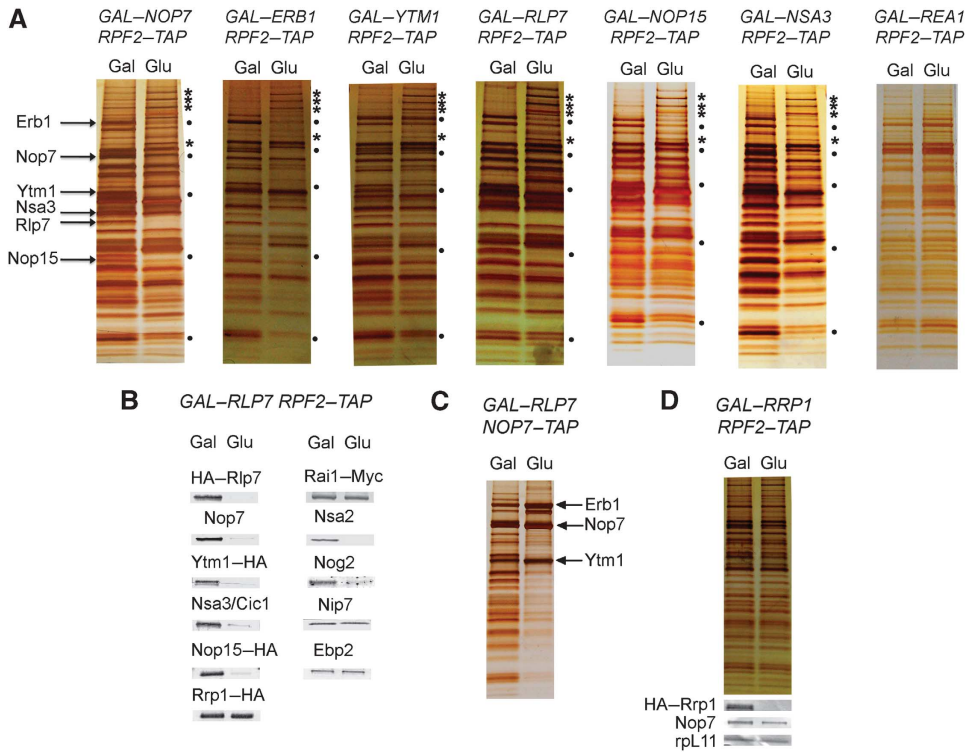

Figure 2 A_3 assembly factors are mutually interdependent for association with preribosomes. (**A**) Depletion of each of the six A_3 factors results in mutant preribosomes with similar changes in protein constituents. Precursors to 60S subunits were isolated using TAP-tagged assembly factor Rpf2, from strains in which A_3 factors were depleted using the conditional *GAL* promoter. Proteins present in the purified preribosomes were resolved by SDS–PAGE, and stained with silver. * Indicates proteins whose levels increase upon depletion of each protein, ● indicates proteins whose levels decrease upon depletion of each protein. A_3 factors are labelled. The *GAL–REA1 RPF2–TAP* strain is shown as a control for specificity of the phenotype. (**B**) Five other A_3 factors are specifically missing from preribosomes in the absence of Rlp7. Western blotting was used to specifically assay the presence of selected proteins, including A_3 factors, in mutant and wild-type preribosomes. (**C**) The Nop7 subcomplex remains intact, but is not associated with preribosomes in the absence of Rlp7. TAP-tagged Nop7 was used for affinity purification from wild-type (left lane) and Rlp7-depleted (right) extracts. Silver-stained protein bands corresponding to Nop7 subcomplex proteins Erb1, Nop7 and Ytm1 are indicated. (**D**) In the absence of Rrp1, the association of A_3 factors with preribosomes is not measurably reduced. Proteins present in Rpf2–TAP-containing preribosomes purified from the *GAL–RRP1* strain were assayed by SDS–PAGE, silver staining, and western blotting. rpL11 is the loading control.

absent. However, it is not known when these assembly factors are present in preribosomes. While Dbp10 and Spb1 function after the A_3 processing step (Kressler *et al*, 1999; Burger *et al*, 2000; Moy *et al*, 2002; Fleischer *et al*, 2006), mutants for Cgr1, Nop16, and Ybl028c have not been thoroughly assayed. Therefore, it remains possible that one or more of these assembly factors might be as yet unidentified A_3 factors. Levels of most other assembly factors did not change, consistent with SDS–PAGE profiles and western blotting (Figures 2A and B and 3A; Supplementary Figure S2B).

The 5′–3′ exonuclease Rrp17, but not Rat1 or Xrn1, requires A_3 factors for association with preribosomes

Since the exonucleases Rat1, Xrn1, and Rrp17 are required for processing $27SA_3$ pre-rRNA, we hypothesized that A_3 factors might recruit these enzymes to preribosomes. Therefore, in the absence of A_3 factors, these exonucleases would no longer be present in preribosomes, resulting in accumulation of unprocessed $27SA_3$ pre-rRNA. iTRAQ and western blotting revealed that this was the case for Rrp17 (Figures 3A and 4A). In contrast, western blotting revealed that the remaining two exonucleases, Rat1 and Xrn1, still were able to associate with preribosomes in the absence of Rlp7 (Figure 4A). Therefore, we conclude that the absence of Rrp17 is responsible, at least in part, for the accumulation of $27SA_3$ pre-rRNA observed in A_3 factor mutants.

Rat1 is not directed to preribosomes by its cofactor Rai1 and enters preribosomes before creation of its substrate, the 5′-end of $27SA_3$ pre-rRNA

Since Rat1 appears to be a major exonuclease involved in $27SA_3$ pre-rRNA processing (Henry *et al*, 1994), but is not recruited to preribosomes by A_3 factors, we wanted to explore other mechanisms by which Rat1 might be recruited to preribosomes. Rai1 physically interacts with Rat1 (Xue *et al*, 2000), and stimulates its exonuclease activity (Xiang *et al*, 2009). Therefore, we investigated whether Rai1 directs Rat1 to preribosomes, by assaying levels of Rat1 in preribosomes purified from the *rai1*Δ strain. Rat1 was present in these preribosomes, indicating that Rai1 is not required for assembly of Rat1 into preribosomes (Figure 4B).

To test whether Rat1 is recruited to preribosomes by its RNA substrate, the 5′-end of $27SA_3$ pre-rRNA, we assayed preribosomes when cleavage at the A_3 site is blocked. To do so, we depleted Pop3 (Dichtl and Tollervey, 1997), a protein component of RNase MRP that cleaves $27SA_2$ pre-rRNA at the A_3 site (Lygerou *et al*, 1996). Rat1–HA was still present in

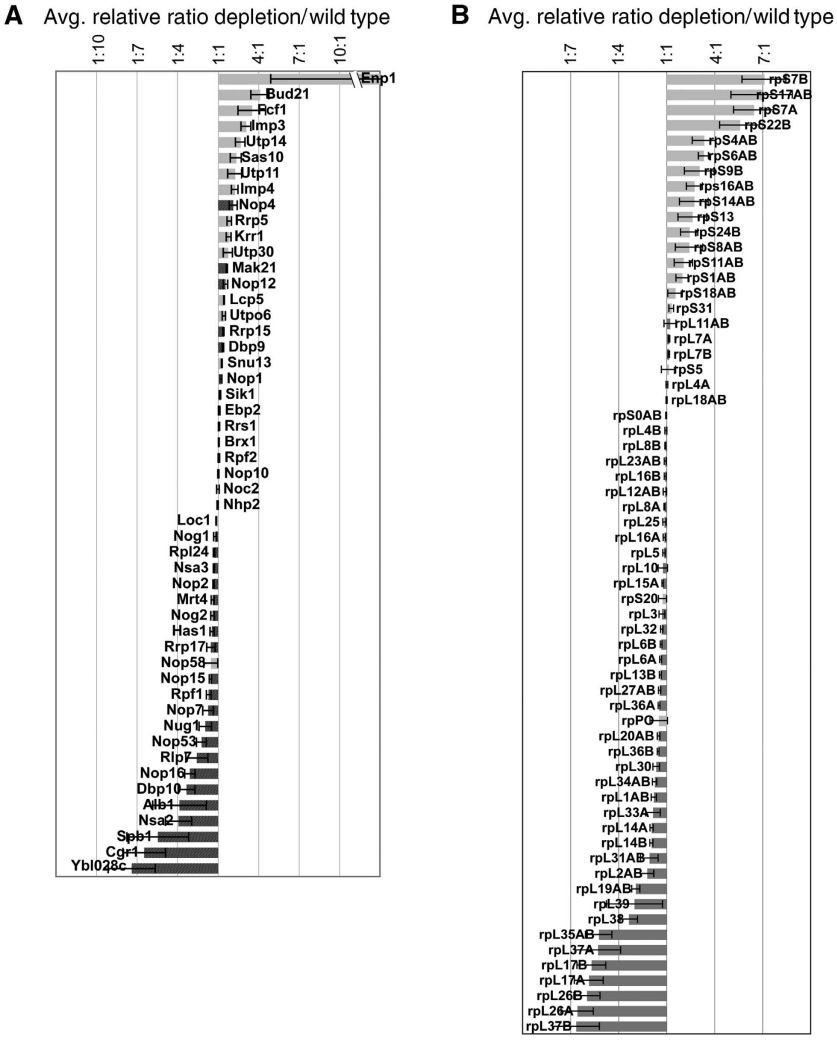

Figure 3 iTRAQ analysis of changes in composition of preribosomes upon depletion of Rlp7. (**A**) Effects of depleting Rlp7 on ribosome assembly factors. The relative abundance of ribosome assembly factors is shown as the ratio from Rlp7-depleted cells to Rlp7-expressed cells (average relative ratio depletion/wild type). Light bars: proteins involved in 40S subunit assembly. Dark bars: proteins involved in 60S subunit biogenesis. s.e.m. are given. Results here and in (**B**) are from two independent biological replicates (two mutant, two wild type). The data were collected as a four-plex after a single LCLC MALDITOFTOF run. (**B**) Effects of depleting Rlp7 on ribosomal proteins. The relative abundance of r-proteins is shown as the ratio from Rlp7-depleted cells to Rlp7-expressed cells (average relative ratio depletion/wild type). Light bars: 40S ribosomal proteins. Dark bars: 60S ribosomal proteins. s.e.m. are given.

preribosomes in the absence of Pop3, suggesting that the 5′-end of $27SA_3$ pre-rRNA does not have to be generated for Rat1 to be recruited to preribosomes (Figure 4C).

The results above suggest that Rat1 may be present in preribosomes before the A_3 end is generated. Therefore, we assayed with which pre-rRNAs Rat1 is associated. Primer extension revealed that Rat1 co-IPed with large amounts of $27SA_2$ pre-rRNA (Figure 4D), indicating that it joins preribosomes before generation of $27SA_3$ pre-rRNA. Therefore, Rat1 might be recruited to preribosomes by other early associating assembly factors.

rpL17, rpL26, rpL35, and rpL37 cannot stably associate with preribosomes lacking A_3 factors

Of the 63 r-proteins identified in preribosomes by iTRAQ, 18 were from the 40S ribosomal subunit (rpS proteins). The levels of most rpS proteins were significantly increased in Rpf2-containing preribosomes in the absence of Rlp7. This is consistent with the presence of 40S subunit r-proteins in 90S preribosomes, and the accumulation of early ribosomal intermediates including 90S preribosomes when $27SA_3$ pre-rRNA processing is blocked. In contrast, r-proteins of the 60S subunit were affected to varying extents. Some were unaffected, whereas others were modestly reduced (Figure 3B). Notably, four r-proteins were significantly diminished in preribosomes when Rlp7 was depleted—rpL17, rpL26, rpL35, and rpL37 (Figure 3B). Immunoblotting confirmed this result from iTRAQ experiments (Figure 5A). These four r-proteins were also diminished when two other A_3 factors, Nop7 and Nop15, were depleted (Supplementary Figure S4).

Based on the crystal structure of the yeast 60S ribosomal subunit (Ben-Shem *et al*, 2010), rpL17, rpL26, rpL35, and rpL37, lie close to each other, adjacent to $5.8S_S$ rRNA, whose 5′-end is generated by $27SA_3$ pre-rRNA processing (Figure 5B and C; Supplementary Figure S1). rpL17 is particularly interesting because it contacts helix 2, formed between the 5′-end

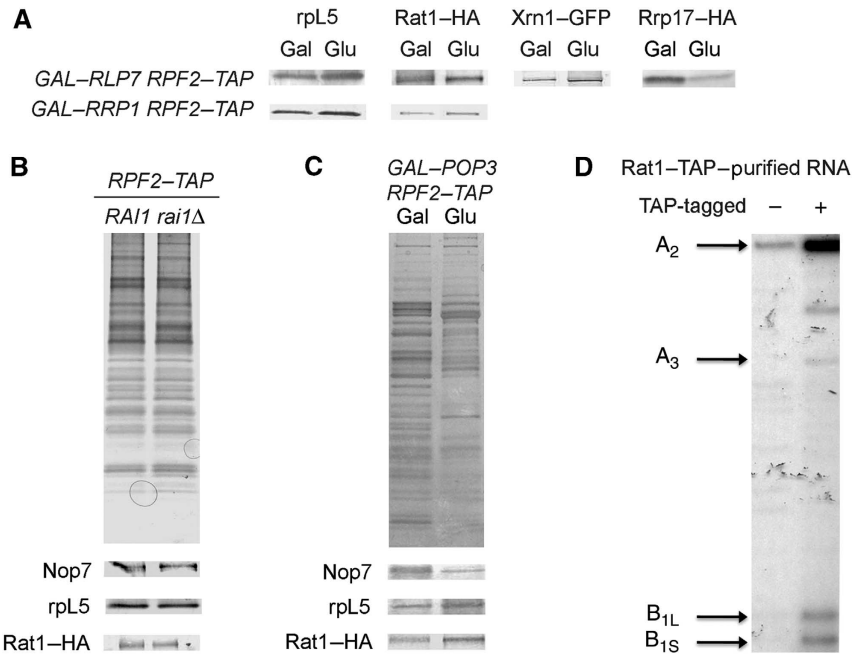

Figure 4 Recruitment of exonucleases Rat1, Xrn1, and Rrp17 to preribosomes. (**A**) Rrp17, but not Rat1 and Xrn1, is dependent on A_3 factors to associate with preribosomes. The presence of Rat1, Xrn1, and Rrp17 in preribosomes purified from A_3 factor mutants was assayed by SDS–PAGE and western blotting. rpL5 is the loading control. Binding of Rat1 to beads was not detected when extracts from untagged strains were used for purification (unpublished observations). (**B**) In the absence of Rai1, association of Rat1 with preribosomes is not affected. Preribosomes were purified from *RAI1* (left) and *rai1*Δ (right) strains. Proteins were assayed by SDS–PAGE, silver staining, and western blotting. rpL5 is the loading control. (**C**) Generation of the 5′-end of $27SA_3$ pre-rRNA is not required for association of Rat1 with preribosomes. 66S preribosomes were isolated using TAP-tagged assembly factor Rpf2, from a strain in which RNase MRP was inactivated by depleting Pop3 using the conditional *GAL* promoter. Preribosomes were assayed by SDS–PAGE, silver staining, and western blotting against indicated proteins. (**D**) Rat1 is recruited to preribosomes early in the pathway of 60S subunit biogenesis. TAP-tagged Rat1 was used to purify Rat1-containing preribosomes. Pre-rRNAs present in these preribosomes were assayed by primer extension. In all, 50% of TAP-purified RNA was used to assay pre-rRNAs containing A_2, A_3, B_{1S}, and B_{1L} ends. The remaining RNA was used to detect 35S pre-rRNA (not shown).

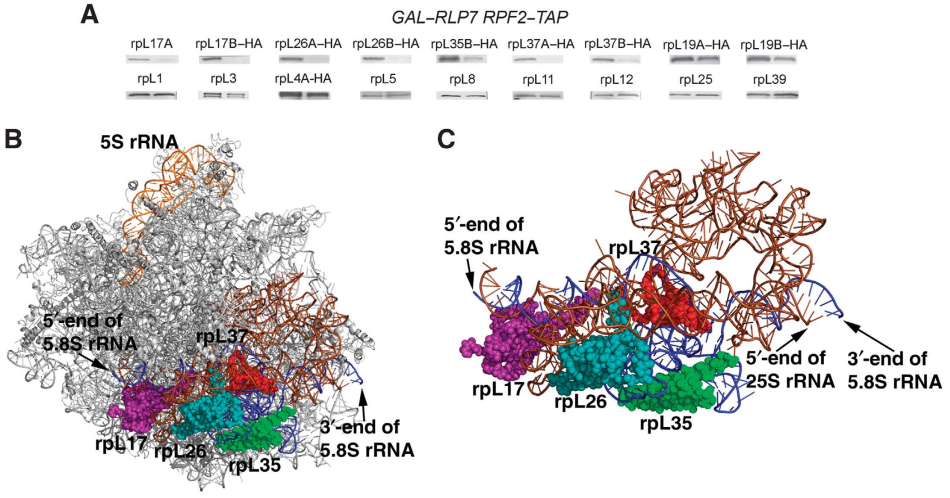

Figure 5 Ribosomal proteins adjacent to 5.8S rRNA in mature ribosomes are missing from preribosomes lacking A_3 factors. (**A**) Specific ribosomal proteins are reduced in preribosomes in the absence of Rlp7. Western blotting of ribosomal proteins in preribosomes purified from *GAL–HA–RLP7 RPF2–TAP* strain are shown. Indicated ribosomal proteins are 3xHA-tagged. R-proteins in the bottom panel were unaffected in the absence of Rlp7. (**B**) R-proteins dependent on A_3 factors cluster around 5.8S rRNA. In both **B** and **C**, a 180° rotation of the crown view of the mature 60S subunit is shown. Domain I of rRNA is highlighted, with the region corresponding to 5.8S rRNA in blue and 25S rRNA in brown. Colour coding of ribosomal proteins: rpL17—purple, rpL26—teal, rpL35—green, rpL37—red. 5S rRNA is highlighted in orange. Pymol images here and in (**C**) were generated using PDB file 3O58 (Ben-Shem *et al*, 2010). Affected r-proteins are close to helices formed by basepairing between 5.8S and 25S rRNA. Only domain I of 5.8S/25S rRNAs is shown. rRNA and r-proteins are colour coded as in (**B**).

of $5.8S_S$ rRNA and 25S rRNA. We imagined that binding of rpL17 to helix 2 might form an RNP structure that stops Rat1 precisely at the B_{1S} site. Therefore, we wanted to understand when and how rpL17 associates with preribosomes.

rpL17 becomes more stably associated with preribosomes after processing of $27SA_3$ pre-rRNA

The absence of rpL17 from preribosomes in A_3 factor mutants might indicate that it associates with pre-rRNPs only after

processing of $27SA_3$ pre-rRNA is completed. Therefore, in mutants where $27SA_3$ pre-rRNA processing is blocked, rpL17 might not yet have assembled into nascent ribosomes. Alternatively, rpL17 might associate with preribosomes before the $27SA_3$ pre-rRNA processing step, but its binding may be strengthened by rRNP remodelling. In A_3 factor mutants, where such rearrangements might not occur, weakly bound rpL17 might readily dissociate from preribosomes. To begin to distinguish between these possibilities, we determined the timing of association of rpL17 with nascent ribosomes, by assaying with which pre-rRNA processing intermediates it co-IPs (Figure 6A). rpL17 co-IPed $27SA_2$ and 27SB pre-rRNAs,

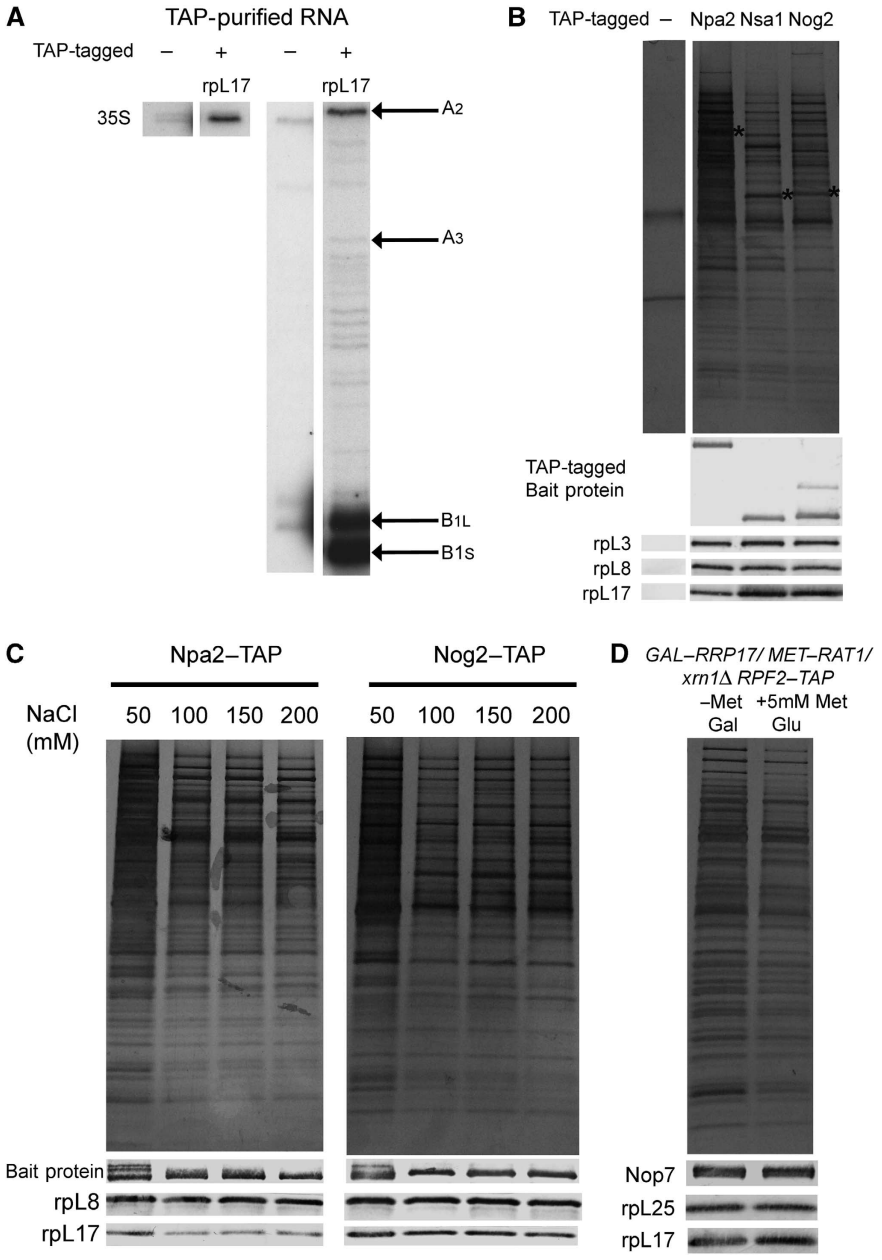

Figure 6 rpL17 becomes more stably associated with 66S preribosomes after $27SA_3$ pre-rRNA processing. (**A**) rpL17 is present in $27SA_2$ and 27SB containing preribosomes. rpL17–TAP strain was used to purify preribosomes containing rpL17. The untagged parent strain is used as a negative control. Pre-rRNAs that copurified were assayed by primer extension. The two samples on the left are from one gel and the two on the right are from a second gel. (**B**) rpL17 copurifies in larger amounts with 'later' 66S preribosomes than with 'early' 66S intermediates. Preribosomes were purified using indicated TAP-tagged early (Npa2) or middle assembly factors (Nsa1, Nog2), and assayed by SDS–PAGE, silver staining, and western blotting. Purification with the untagged parent strain is shown as a negative control. Purified bait proteins are indicated with asterisks. The higher molecular weight band in the untagged control strain corresponds to IgG heavy chain, and the lower molecular weight band is TEV protease. All protein samples were assayed on the same gel and blot. (**C**) Association of rpL17 with Npa2-containing preribosomes, but not Nog2-containing preribosomes, is salt sensitive. Preribosomes were purified using indicated TAP-tagged strains. After binding of cell lysates, beads were washed with buffer containing increasing concentrations of NaCl, as indicated. SDS–PAGE, silver staining, and western blotting were used to assay preribosomes. (**D**) Binding of rpL17 to preribosomes does not depend on processing of $27SA_3$ pre-rRNA. The three exonucleases required for $27SA_3$ pre-rRNA processing were depleted using the *GAL–RRP17 MET–RAT1 xrn1*Δ strain. The strain was grown in indicated media and preribosomes were purified using TAP-tagged Rpf2. Preribosomes were assayed by SDS–PAGE, silver staining, and western blotting. Figure source data can be found with the Supplementary Information.

and a small amount of 35S pre-rRNA (Figure 6A). No $27SA_3$ pre-rRNA was detected copurifying with rpL17, most likely due to the very low abundance of this pre-rRNA species in cells under wild-type conditions.

To further investigate the timing and degree of association of rpL17 with preribosomes, we measured its copurification with 'early' (Npa2) or 'middle' (Nsa1, Nog2) assembly intermediates. Consistent with experiments above, we found that rpL17 is present in early preribosomes purified using the TAP-tagged early assembly factor Npa2 (Figure 6B). Npa2 is thought to leave the assembly pathway during or immediately after processing of $27SA_2$ pre-rRNA (Rosado *et al*, 2007). However, reproducibly more rpL17 copurified with later assembly factors such as Nsa1 or Nog2 (Figure 6B and C) (Saveanu *et al*, 2003; Kressler *et al*, 2008). No such difference in copurification could be observed for the other r-protein tested, rpL8 (Figure 6B and C). Taken together, the above results suggest that rpL17 normally associates with preribosomes before processing of $27SA_3$ pre-rRNA. Thus, there are two possible explanations for the reduced association of rpL17 in A_3 factor mutants. On the one hand, rpL17 might completely fail to associate with preribosomes in the absence of A_3 factors. Alternatively, rpL17 might be weakly bound to preribosomes early in the assembly pathway, and may dissociate from pre-rRNPs if there is a block in $27SA_3$ pre-rRNA processing.

The consistently elevated amounts of rpL17 observed in Nsa1 and Nog2-containing preribosomes, compared with Npa2-containing pre-rRNPs, suggests that rpL17 is in fact more tightly associated with later preribosomes, than with early pre-rRNPs. We therefore tested salt sensitivity of association of rpL17 with preribosomes. Association of rpL17 with Npa2–TAP, but not Nog2–TAP, was salt sensitive (Figure 6C), indicating that association of rpL17 with later preribosomes is stronger than with early preribosomes. No such difference in association was observed for the other r-protein tested, rpL8 (Figure 6C). The difference in recovery of rpL17 in preribosomes at 50 mM NaCl compared with higher concentrations of salt is not merely an artefact of contamination with mature ribosomes, since levels of rpL8 that copurify with preribosomes remain constant at different salt concentrations (Figure 6C). Taken together, these results suggest that rpL17 assembles into early preribosomes before the $27SA_3$ pre-rRNA processing step, but its association is strengthened after subsequent remodelling events, coincident with production of $27SB_{1S}$ pre-rRNA.

We hypothesized that exonucleolytic processing of $27SA_3$ pre-rRNA might be the event that triggers more stable association of rpL17 with preribosomes. To test this idea, we used the *GAL–RRP17 MET–RAT1 xrn1*Δ strain, in which all three exonucleases required for $27SA_3$ pre-rRNA processing can be conditionally repressed (Oeffinger *et al*, 2009). Because A_3 factors remained in preribosomes when Rat1, Xrn1, and Rrp17 were depleted, using this strain allowed us to prevent removal of ITS1 sequences in $27SA_3$ pre-rRNA, in a manner independent of the A_3 factors (Figure 6D). However, equal amounts of rpL17 could still associate with preribosomes (Figure 6D), indicating that $27SA_3$ pre-rRNA processing is not required for strengthening association of rpL17. It is possible that rpL17 is stably bound to helix 2, which might have already formed before $27SA_3$ pre-rRNA processing occurs. The above result also suggests that the stable association of rpL17 with preribosomes depends on the presence of A_3 factors or some other remodelling event induced by their presence.

rpL17 is required for processing within ITS2

The presence of rpL17 near the 5′-end of helix 2 in mature ribosomes suggests that it could serve as a roadblock to Rat1, causing it to stop precisely at the B_{1S} site during 5′–3′ processing of ITS1. This idea is further supported by the results above that rpL17 is associated with preribosomes before processing of $27SA_3$ pre-rRNA. To test whether rpL17 functions to halt Rat1, we determined the effect of depleting rpL17 on pre-rRNA processing. In the absence of rpL17, we observed accumulation of pre-rRNA species whose 5′-ends are ~10 nts downstream of the B_{1S} site, very close to the end of helix 2 (Figure 7, left). This 5′-truncated pre-rRNA has not been observed in the absence of any other assembly factor or r-protein tested, including rpL35 (Zhang *et al*, 2007; Pöll *et al*, 2009; Babiano and de la Cruz, 2010; Supplementary Figure S3). This shorter pre-rRNA may form due to the inability of Rat1 to stop at the B_{1S} site in the absence of rpL17. To test whether Rat1 is required for the formation of this shorter pre-rRNA species, we constructed a temperature-sensitive *rat1-1* strain where rpL17 was depleted using the *GAL* promoter. When rpL17 was depleted and Rat1-1 was inactivated by shifting to 37 °C, the 5′-truncated product observed in the absence of rpL17 alone no longer accumulated (Figure 7, right), revealing a direct role for Rat1 in the formation of these pre-rRNAs. Therefore, it appears that in a small fraction of preribosomes lacking rpL17, Rat1 starts processing at the A_3 site, but cannot stop at the B_{1S} site. Instead, it is stopped ~10 nts downstream of this site.

The significance of this 5′-truncated pre-rRNA species, however, is unclear, as it appears that the major fraction of

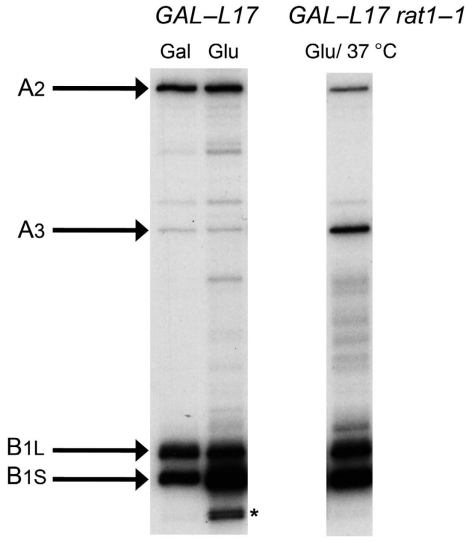

Figure 7 rpL17 is required for processing within ITS2, and to a smaller extent within ITS1. RNA was extracted from indicated strains grown in galactose or grown in galactose and shifted to glucose. The *GAL–RPL17 rat1-1* strain was grown in glucose for 12 h at 25 °C to deplete rpL17 and then shifted to 37 °C for 5 h to inactivate Rat1-1. Primer extension was used to assay the A_2, A_3, B_{1S}, and B_{1L} ends of pre-rRNA. * Indicates 5′-truncated pre-rRNAs formed in the absence of rpL17. All samples were assayed on the same gel. Figure source data can be found with the Supplementary Information.

$27SA_3$ pre-rRNA is properly processed by the exonucleases to form $27SB_{1S}$ pre-rRNA. We observed an increase in $27SB_{1S}$ pre-rRNA (Figure 7, left), indicating that rpL17 is required for the next step in pre-rRNA processing—conversion of the $27SB_{1S}$ pre-rRNA to 25.5S and 7S pre-rRNAs. Consistent with these data, it has been shown that there is a reduction in the amount of 7S pre-rRNA in the absence of rpL17 (Pöll *et al*, 2009).

Rat1 initiates turnover of misassembled preribosomes when A_3 factors are absent

Pulse-chase and steady-state assays of pre-rRNA processing in A_3 factor mutants suggest that 27S pre-rRNA is turned over when 60S subunit assembly is aborted (Dunbar *et al*, 2000; Pestov *et al*, 2001; Adams *et al*, 2002; Gadal *et al*, 2002; Oeffinger *et al*, 2002; Oeffinger and Tollervey, 2003; Horsey *et al*, 2004; Miles *et al*, 2005). Because Rat1 is present in preribosomes when A_3 factors are depleted (Figure 4A), we hypothesized that Rat1 might degrade the $27SA_3$ pre-rRNA in abortive preribosomes, instead of properly processing it. Thus, turnover intermediates degraded from their 5′-ends might be detectable. Primer extension assays revealed the presence of such turnover intermediates. We detected low levels of pre-rRNA species with 5′-ends 67–69 nts downstream of the B_{1S} site, when Rat1 was present in preribosomes lacking A_3 factors (Figure 8A, lanes 1–12).

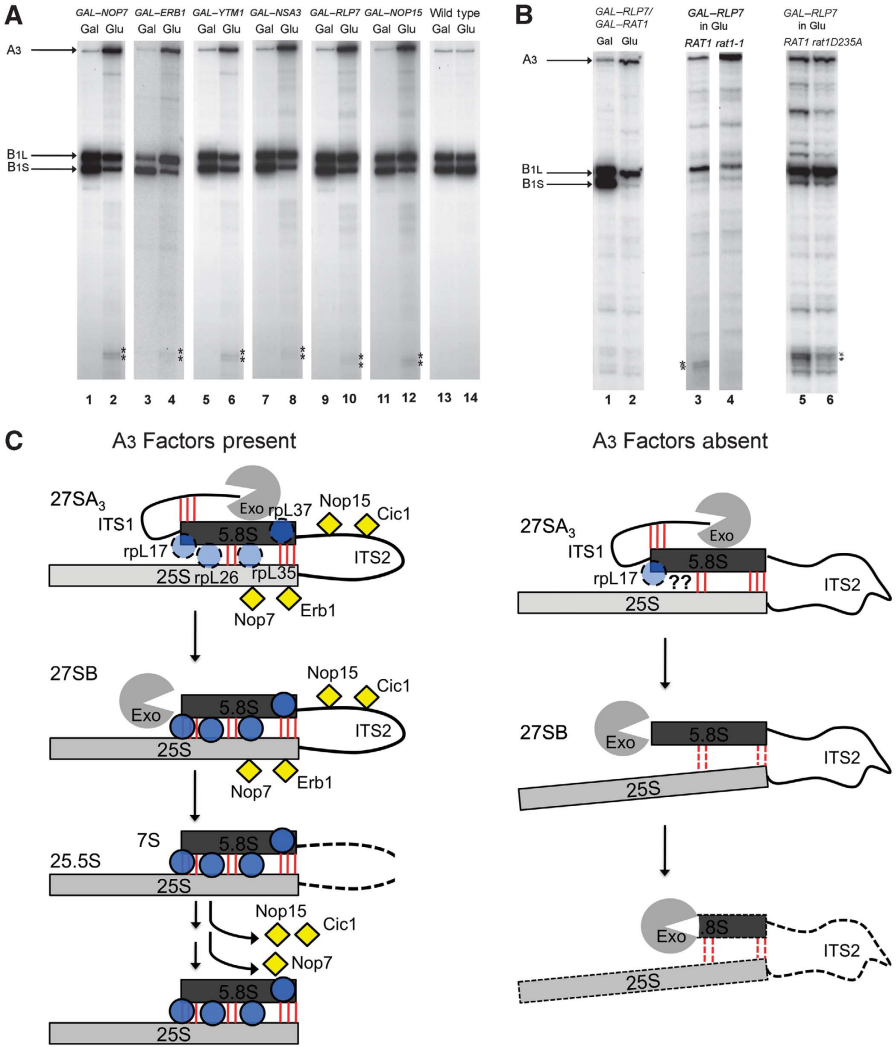

Figure 8 In the absence of A_3 factors, Rat1 initiates turnover of misassembled preribosomes. (**A**) 5′-Truncated 27SB pre-rRNAs are formed in the absence of A_3 factors. RNA was extracted from whole-cell lysates prepared from the indicated strains, and assayed by primer extension to detect $A_{2,}$ A_3, B_{1S}, and B_{1L} 5′-ends. For each panel, asterisks indicate primer extension products that are 67–69 nts shorter than $27SB_{1S}$ pre-rRNA at the 5′-end. All samples were run on the same gel, except *GAL–ERB1,* which was run won a separate gel with *GAL–RLP7* as a control. (**B**) Rat1 activity is required for formation of the shorter pre-rRNAs. RNA was extracted and assayed as described in (**A**) above. The *GAL–RPL17 rat1-1* strain was grown in glucose for 12 h at 25 °C to deplete rpL17 and then shifted to 37 °C for 5 h to inactivate Rat1-1. Samples from lanes 3 and 4 were run on the same gel. (**C**) Model for the role of A_3 factors during ribosome assembly. Basepairing between 5.8S and 25S RNAs is indicated by short red lines. Dashed red lines indicate that the basepairing is affected. Dashed black lines indicate that the pre-rRNAs are being turned over. Dashed circles show that the r-proteins might not be tightly associated with preribosomes. (Left panel) Binding of A_3 factors (yellow diamonds) determined by CRAC (Granneman *et al*, 2011) allows basepairing between 5.8S and 25S rRNAs. It also enables stable association of rpL17, rpL26, rpL35, and rpL37 (blue circles), and all proteins together help Rat1 to stop at the B_{1S} site. rpL17 is also required for processing within ITS2. (Right panel) Preribosomes undergo turnover in the absence of A_3 factors, and the four r-proteins. Figure source data can be found with the Supplementary Information.

To confirm that Rat1 is required for formation of these shorter RNAs when A_3 factors are absent, we constructed a strain in which both Rlp7 and Rat1 were depleted. In this strain, no 5′-truncated pre-rRNAs accumulated (Figure 8B, lanes 1 and 2). Similar results were obtained upon depletion of Rlp7 in a temperature-sensitive *rat1-1* strain or *rat1D235A* catalytic mutant strain (Figure 8B, lanes 1–4). Taken together, these results indicate a direct role for Rat1 exonuclease activity in turnover of 27S pre-rRNA in A_3 factor mutants.

Discussion

The work described here has enabled us to bring into sharper focus the assembly factors known to be involved in the processing of $27SA_3$ pre-rRNA. At the same time, we have also been able to make connections between these assembly factors and r-proteins in a manner that is consistent with the structure of *S. cerevisiae* 60S ribosomes.

We have shown that all known A_3 factors associate with preribosomes well before their requirement for processing of $27SA_3$ pre-rRNA. Six of seven A_3 factors assemble into preribosomes in a concerted manner. A_3 factors enable stable association of one of the 5′–3′ exonucleases, Rrp17, but not Rat1 or Xrn1, with preribosomes. Four ribosomal proteins—rpL17, rpL26, rpL35, and rpL37 specifically cannot associate with preribosomes when A_3 factors are depleted. These four r-proteins bind adjacent to each other on 5.8S rRNA in mature 60S ribosomes in *S. cerevisiae* (Ben-Shem *et al*, 2010). This result indicates that the presence of A_3 factors, which are required for proper formation of the 5′-end of $5.8S_S$ rRNA, stabilizes this neighbourhood of r-proteins within assembling ribosomes. We also show here that in the absence of A_3 factors and rpL17, rpL26, rpL35, and rpL37, Rat1 cannot stop at the B_{1S} site and proceeds beyond this site to turn over 27S pre-rRNA (Figure 8C).

Recruiting functions of A_3 factors during ribosome assembly

The seven assembly factors required for processing of $27SA_3$ pre-rRNA enable stable association of three classes of proteins with preribosomes—other A_3 assembly factors, an exonuclease, and r-proteins. First, A_3 factors ensure productive assembly of other A_3 factors into preribosomes. Such a pattern of interdependence could reflect both direct and indirect interactions—directly by protein–protein contacts, and indirectly via a network of RNA–protein and RNA–RNA contacts. Indeed, recent proteome-wide protein complementation analysis has revealed that these proteins, except Rrp1, are in the vicinity of each other (Tarassov *et al*, 2008). Consistent with these data, Nop7, Erb1, and Ytm1 form a stable heterotrimer that can be isolated from preribosomes (Miles *et al*, 2005; Figure 2C). It has been proposed that this Nop7 subcomplex might serve as a scaffold upon which further assembly can occur (Lapik *et al*, 2004). CRAC analysis (Granneman *et al*, 2009) revealed that Nop7 and Erb1 bind RNA sequences that lie close to each other in 25S rRNA near the ITS2 junction, whereas Nop15 and Cic1 bind sequences within ITS2 (Granneman *et al*, 2011). As Ytm1 has been shown to interact directly with Erb1 (Miles *et al*, 2005), it follows that its binding site on preribosomes is very close to those of Erb1 and Nop7. Since these five A_3 factors are potentially present in such close proximity to each other in preribosomes, one can well imagine how their association with preribosomes would be interdependent.

Second, A_3 factors are required for the association of Rrp17, one of the three 5′–3′ exonucleases that processes $27SA_3$ pre-rRNA to $27SB_{1S}$ pre-rRNA (Oeffinger *et al*, 2009). Thus, the absence of Rrp17 could account for the observed accumulation of some unprocessed $27SA_3$ pre-rRNA in A_3 factor mutants. Two-hybrid assays revealed no physical interactions between any of the A_3 factors and Rrp17 (unpublished observations), suggesting that A_3 factors might affect association of Rrp17 with preribosomes indirectly through their effect on the structure of nascent ribosomes. Interestingly, it appears that the association of the remaining exonucleases, Rat1 and Xrn1, is not affected in A_3 mutants by such changes in preribosomal architecture. They might be anchored to preribosomes or properly positioned within pre-rRNPs by other assembly factors and/or r-proteins that remain stably associated with preribosomes in the absence of A_3 factors.

A third important function of A_3 factors is to ensure stable association of r-proteins rpL17, rpL26, rpL35, and rpL37 with preribosomes. Interestingly, the binding sites in mature ribosomes of rpL17, rpL26, rpL35, and rpL37 are near each other in domains I and III of 5.8S/25S rRNAs (Ben-Shem *et al*, 2010). This appears to be close to the binding sites of Erb1 in domain I, Nop7 in domain III, and potentially Nop15 and Nsa3/Cic1 in ITS2 within preribosomes (Granneman *et al*, 2011). We have not detected physical interactions between A_3 factors and these r-proteins (unpublished observations), suggesting that binding of these r-proteins to preribosomes also depends on remodelling or stabilization of preribosome structure mediated by A_3 factors. It is conceivable that the binding of Nop7 and Erb1 to domains I and III of 5.8S/25S rRNAs, respectively, might help bring these two rRNA domains in close proximity, thereby providing binding sites for the four r-proteins and stabilizing their association with preribosomes.

Rat1 might function as a processing or degradative enzyme depending on the presence or absence of roadblocks

It has been observed that in mutants defective in ribosome biogenesis, pre-rRNA processing intermediates do not accumulate to high levels, suggesting that when assembly fails, pre-rRNAs are turned over. Mistakes made in the course of normal ribosome assembly in wild-type cells may also trigger turnover. In fact, in wild-type cells, polyadenylation targets pre-rRNAs for 3′–5′ degradation by the exosome (LaCava *et al*, 2005). In strains defective for the exosome, depletion of Rat1 results in increased accumulation of poly(A)+ pre-rRNAs (Fang *et al*, 2005), suggesting that the 5′–3′ exonuclease activity of Rat1 plays a role in turnover of aberrant pre-rRNAs during ribosome assembly. Our work provides evidence for the role of Rat1 in 5′–3′ degradation of specific aberrant pre-rRNA intermediates when ribosome assembly is aborted in A_3 factor mutants. This is evident by the accumulation of shorter Rat1-dependent RNAs in the absence of A_3 factors and rpL17 (Figures 7 and 8A). Consistent with this idea, there is greater accumulation of $27SA_3$ pre-rRNA in the *GAL–RLP7 rat1-1* double mutant compared with the *GAL–RLP7* single mutant (Figure 8B). However, this Rat1-mediated turnover might not be a very efficient process, since in the

absence of A3 factors, significant amounts of $27SA_3$ intermediate still can be detected relative to the short degradation products. It is also likely that turnover by Rat1 is not the only pathway for degradation of aberrant pre-rRNA intermediates.

An important question in ribosome biogenesis is why cells employ both endonucleases and exonucleases for pre-rRNA processing. The exonucleases Rat1 and Xrn1 are implicated in generating precise 5′-ends of two mature rRNAs—$5.8S_S$ and 25S rRNAs (Geerlings *et al*, 2000). While at first glance the use of an exonuclease to create precise 5′-ends of rRNA might appear counterintuitive, it is in fact an ingenious display of cellular economy. Exonucleases, such as Rat1, could process pre-rRNA to create appropriate 5′-ends when stopped by roadblocks such as RNA secondary structure or RNA–protein contacts present in preribosomes. However, under conditions of aberrant ribosome assembly as signalled by the absence of the roadblock, the same exonuclease might not stop at a specific site to create a precise 5′-end. Instead, its processing function would now be converted to a turnover function, without necessitating recruitment of other factors explicitly for the purpose of turnover. Such a dual role has recently been proposed for the mammalian homologue of Rat1, Xrn2, during 60S ribosome biogenesis (Wang and Pestov, 2011).

rpL17 binds to the 5′-end of 5.8S rRNA (Ben-Shem *et al*, 2010), immediately downstream of the B_{1S} site where exonucleolytic processing precisely halts in wild-type cells. rpL17 is present in preribosomes before processing of $27SA_3$ pre-rRNA is completed. We therefore predicted that rpL17 might serve as a roadblock to the exonucleases Rat1, Xrn1, and Rrp17 during processing of $27SA_3$ pre-rRNA, causing them to stop at the B_{1S} site. Our results indicate that in part rpL17 is a roadblock to Rat1 (Figure 7), as seen by the small fraction of Rat1-dependent 5′-truncated pre-rRNAs observed in the absence of rpL17. However, rpL17 most likely does not act alone to stop Rat1. Instead, it is likely that an RNP structure created by the stable association of A_3 factors and all four r-proteins with preribosomes might serve as the roadblock to Rat1.

A_3 factors may be necessary to help fold ITS2 RNA, and bring together 5.8S and 25S rRNA sequences to stabilize 27S pre-rRNAs during ribosome biogenesis

Because all seven A_3 factors are present in preribosomes after $27SA_3$ pre-rRNA processing is completed (Figure 1C), they might also function in later steps of ribosome biogenesis, for example processing of 27SB pre-rRNA into 25.5S plus 7S pre-rRNAs. This is thought to be the last step that occurs before nucleolar release of pre-60S particles (Kressler *et al*, 2009). Consistent with this idea, preribosomes are found to remain in the nucleolus in *ytm1-1*, *cic1-2*, and *rlp7-1* mutants (Gadal *et al*, 2002; Fatica *et al*, 2003; Miles *et al*, 2005). Furthermore, the AAA^+-ATPase Rea1 releases the Nop7–Erb1–Ytm1 subcomplex from preribosomes just prior to the exit of pre-60S particles from the nucleolus (Baßler *et al*, 2010). Therefore, A_3 factors might also play a structural role to enable proper processing of 27SB pre-rRNA, before they are released from 66S preribosomes.

Indeed, three observations suggest a role for A_3 factors in facilitating proper folding and processing of ITS2, and thus contributing to $27SB_{1S}$ pre-rRNA processing. (1) Nop15 and Nsa3/Cic1 bind ITS2 sequences, whereas Nop7 and Erb1 bind 25S rRNA sequences close to the ITS2-25S junction (Granneman *et al*, 2011). (2) Cleavage at the C_2 site within ITS2 occurs prematurely in the *cic1-2* mutant (Fatica *et al*, 2003). (3) Mutations in Pes1 and Bop1, the mammalian homologues of Nop7 and Erb1, respectively, slow processing of ITS2 (Strezoska *et al*, 2000; Lapik *et al*, 2001).

Three of the four r-proteins most affected in the A_3 mutants are not required for $27SA_3$ pre-rRNA processing, but are required for 27SB pre-rRNA processing. In the absence of rpL17, rpL35 or rpL37, 27SB pre-rRNA, but not $27SA_3$ pre-rRNA accumulates (Babiano and de la Cruz, 2010; M Gamalinda, personal communication). Consistent with these observations, A_3 factors are present in preribosomes even in the absence of rpL17, rpL35, or rpL37 (M Gamalinda, personal communication). The block in processing of 27SB pre-rRNA indicates that these three r-proteins are required to establish or maintain structures required for processing of 27SB pre-rRNA, but not $27SA_3$ pre-rRNA. Recently, we also found that rpL17 is required for the stable association of several assembly factors necessary for proper processing of 27SB to 7S + 25.5S pre-rRNAs (M Gamalinda, personal communication).

Binding of A_3 factors to domains I and III of rRNA sequences in preribosomes, coupled with the assembly of the r-protein neighbourhood adjacent to 5.8S rRNA, might therefore be required for maintaining the preribosomal architecture necessary for proper processing of $27SA_3$ pre-rRNA and stabilization of $27SB_{1S}$ pre-rRNA, before cleavage and removal of ITS2 (Figure 8C). We propose that the A_3 factors and r-proteins facilitate folding of $5.8S_S$ and 25S rRNA sequences in pre-rRNAs, including formation of helices 2, 4, and 10 between them (Supplementary Figure S1), and influence proper folding of ITS2 within 27S pre-rRNAs. Proper maintenance of these helices is likely required not just for precise processing of $27SA_3$ pre-rRNA, but also for the correct processing of later intermediates such as $27SB_{1S}$, 7S, and 25.5S pre-rRNAs. Once A_3 factors dissociate from preribosomes, it is imperative that this basepairing between 5.8S and 25S rRNAs be maintained. Stable association of rpL17, rpL26, rpL35, and rpL37 may play a role in maintaining basepairing between these two rRNAs after release of A_3 factors, and in mature functioning ribosomes (Supplementary Figure S1).

Materials and methods

3xHA-, GFP-, 13xMYC-, TAP-tagged genes, *GAL1* promoter fusions, and gene disruptions were generated as described in Longtine *et al* (1998). Yeast strains used in this study are listed in Supplementary Table S1.

Single-step purifications of preribosomes from whole-cell extracts using magnetic Dynabeads were performed as described in Oeffinger *et al* (2007), with the following modifications. Cells were grown in either galactose, to express *GAL* promoter fusion genes, or shifted to glucose for 16 h to repress these genes, and harvested at mid-log phase (3×10^7 cells/ml). Cultures were centrifuged using a GS-3 rotor at a speed of 5000 r.p.m., for 5 min. Cell pellets were resuspended in RNP buffer (20 mM HEPES (pH 7.4), 110 mM KOAc, 0.5% Triton, 0.1% Tween 20, 40 mM NaCl), and subjected to glass bead lysis. After binding of lysates to IgG-coated Dynabeads at 4 °C for 30 min, beads were washed three times with RNP buffer to eliminate non-specifically bound proteins. Bead-bound preribosomes were eluted by cleaving the TEV protease site within the TAP-tag, using 10 U of TEV Protease (Invitrogen). Proteins were recovered from the eluate by precipitation in 10% TCA, and were subsequently suspended in SDS sample buffer and separated by SDS–PAGE on 4–20% polyacrylamide NOVEX gels (Invitrogen).

For purifications done under high salt conditions, the following modifications were made. Cell lysis was performed using RNP buffer containing 40 mM NaCl. After binding of lysates to Dynabeads, three washes were done with RNP buffer containing the indicated concentration of NaCl. These were followed by three washes with RNP buffer containing 40 mM NaCl to get rid of excess salt that might affect the efficiency of TEV Protease.

Silver staining was done according to standard procedure. Proteins present in whole-cell extracts or purified preribosomes were assayed by western blot analysis (Ausubel *et al*, 1994), with the following modification. After electroblotting, the nitrocellulose membrane was cut into smaller sections based on known mobility of different proteins to enable probing of multiple proteins from one blot. TAP-tagged proteins were detected using alkaline phosphatase conjugated to IgG (Pierce). 3HA-tagged proteins were identified with mouse monoclonal antibody 12CA5, GFP-tagged proteins with rabbit polyclonal anti-GFP antibody, and myc-tagged proteins with anti-mouse 9e10 antibody. Otherwise, antibodies specific for ribosomal proteins or assembly factors were used.

Proteins copurifying with TAP-tagged Rpf2 were identified by mass spectrometry as described in Horsey *et al* (2004). For semi-quantitative iTRAQ analysis, preribosomes were purified as described above. Following TCA precipitation, pellets were resuspended in 20 µl of 20 mM HEPES pH 7.4. iTRAQ labelling and quantification were done as described in Jiang *et al* (2007). The hash that represents these data is iCHfI8YFKDk4nxNV79z9Z + AcPF 8B6tPwYtsNRV8FK + Dq7qQPZpgLpS0bbrEfsgaxlmjUBK6pnxlLoDZ jbjGQkK9Kfv8AAAAAACipjQ = =, located at the network Proteome Commons.org Tranche, under the title 'Assembly of Saccharomyces cerevisiae 60S Ribosomal Subunits: Role of Factors Required for 27S Pre-rRNA Processing'.

RNA from whole-cell lysates was extracted as described in Horsey *et al* (2004). RNA enriched from purified preribosomes was extracted as follows. After binding of cell-free lysate to magnetic beads, the bead-bound preribosomes were treated with 5 µl of Proteinase K (Roche) for 30 min at 37 °C to degrade proteins. RNA was extracted from the eluate using phenol chloroform isoamyl alcohol. Primer extension was carried out using ^{32}P- radiolabelled oligonucleotide sequences complementary to 27S pre-rRNA (Miles *et al*, 2005).

PyMOL images of ribosome structure were generated using PDB File 3O58 (Ben-Shem *et al*, 2010).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Adams CC, Jakovljevic J, Roman J, Harnpicharnchai P, Woolford Jr JL (2002) *Saccharomyces cerevisiae* nucleolar protein Nop7p is necessary for biogenesis of 60S ribosomal subunits. *RNA* **8:** 150–165

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1994) *Current Protocols in Molecular Biology*. John Wiley & Sons Inc.: NY, USA

Babiano R, de la Cruz J (2010) Ribosomal protein L35 is required for 27SB pre-rRNA processing in Saccharomyces cerevisiae. *Nucl Acids Res* **38:** 5177–5192

Baßler J, Kallas M, Pertschy B, Ulbrich C, Thoms M, Hurt E (2010) The AAA-ATPase Rea1 drives removal of biogenesis factors during multiple stages of 60S ribosome assembly. *Mol Cell* **38:** 712–721

Ben-Shem A, Jenner L, Yusupova G, Yusupov M (2010) Crystal structure of the eukaryotic ribosome. *Science* **330:** 1203–1209

Burger F, Daugeron MC, Linder P (2000) Dbp10p, a putative RNA helicase from Saccharomyces cerevisiae, is required for ribosome biogenesis. *Nucl Acids Res* **28:** 2315–2323

Dichtl B, Tollervey D (1997) Pop3p is essential for the activity of the RNase MRP and RNase P ribonucleoproteins *in vivo*. *EMBO J* **16:** 417–429

Dragon F, Gallagher JE, Compagnone-Post PA, Mitchell BM, Porwancher KA, Wehner KA, Wormsley S, Settlage RE, Shabanowitz J, Osheim Y, Beyer AL, Hunt DF, Baserga SJ (2002) A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. *Nature* **417:** 967–970

Dunbar DA, Dragon F, Lee SJ, Baserga SJ (2000) A nucleolar protein related to ribosomal protein L7 is required for an early step in large ribosomal subunit biogenesis. *Proc Natl Acad Sci USA* **97:** 13027–13032

Fang F, Phillips S, Butler JS (2005) Rat1p and Rai1p function with the nuclear exosome in the processing and degradation of rRNA precursors. *RNA* **11:** 1571–1578

Fatica A, Oeffinger M, Tollervey D, Bozzoni I (2003) Cic1p/Nsa3p is required for synthesis and nuclear export of 60S ribosomal subunits. *RNA* **9:** 1431–1436

Fleischer TC, Weaver CM, McAfee KJ (2006) Systematic identification and functional screens of uncharacterized proteins associated with eukaryotic ribosomal complexes. *Genes Dev* **20:** 1294–1307

Gadal O, Strauss D, Petfalski E, Gleizes PE, Gas N, Tollervey D, Hurt E (2002) Rlp7p is associated with 60S preribosomes, restricted to the granular component of the nucleolus, and required for pre-rRNA processing. *J Cell Biol* **157:** 941–951

Geerlings TH, Vos JC, Raué HA (2000) The final step in the formation of 25S rRNA in Saccharomyces cerevisiae is performed by 5′→3′ exonucleases. *RNA* **12:** 1698–1703

Granneman S, Kudla G, Petfalski E, Tollervey D (2009) Identification of protein binding sites on U3 snoRNA and pre-rRNA by UV cross-linking and high-throughput analysis of cDNAs. *Proc Natl Acad Sci USA* **106:** 9613–9618

Granneman S, Petfalski E, Tollervey D (2011) A cluster of ribosome synthesis factors regulate pre-rRNA folding and 5.8S rRNA maturation by the Rat1 exonuclease. *EMBO J* (advance online publication 2 August 2011; doi:10.1038/emboj.2011.256)

Henras AK, Soudet J, Gérus M, Lebaron S, Caizergues-Ferrer M, Mougin A, Henry Y (2008) The post-transcriptional steps of eukaryotic ribosome biogenesis. *Cell Mol Life Sci* **14:** 1–26

Henry Y, Wood H, Morrissey JP, Petfalski E, Kearsey S, Tollervey D (1994) The 5′ end of yeast 5.8S rRNA is generated by exonucleases from an upstream cleavage site. *EMBO J* **13:** 2452–2463

Horsey EW, Jakovljevic J, Miles TD, Harnpicharnchai P, Woolford Jr JL (2004) Role of the yeast Rrp1 protein in the dynamics of pre-ribosome maturation. *RNA* **5:** 813–827

Jiang M, Sullivan SM, Walker AK, Strahler JR, Andrews PC, Maddock JR (2007) Identification of novel *Escherichia coli* ribosome-associated proteins using isobaric tags and multidimen-

sional protein identification techniques. *J Bacteriol* **189:** 3434–3444

Kressler D, Hurt E, Baßler J (2009) Driving ribosome assembly. *Biochim Biophys Acta* **1803:** 673–683

Kressler D, Rojo M, Linder P, de la Cruz J (1999) Spb1p is a putative methyltransferase required for 60S ribosomal subunit biogenesis in *Saccharomyces cerevisiae*. *Nucl Acids Res* **27:** 4598–4608

Kressler D, Roser D, Pertschy B, Hurt E (2008) The AAA ATPase Rix7 powers progression of ribosome biogenesis by stripping Nsa1 from pre-60S particles. *J Cell Biol* **181:** 935–944

LaCava J, Houseley J, Saveanu C, Petfalski E, Thompson E, Jacquier A, Tollervey D (2005) RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. *Cell* **121:** 713–724

Lapik YR, Fernandes CJ, Lau LF, Pestov DG (2004) Physical and functional interaction between Pes1 and Bop1 in mammalian ribosome biogenesis. *Mol Cell* **15:** 17–29

Lebreton A, Saveanu C, Decourty L, Jacquier A, Fromont-Racine M (2006) Nsa2 is an unstable, conserved factor required for the maturation of 27SB pre-rRNAs. *J Biol Chem* **281:** 27099–27108

Longtine MS, McKenzie III A, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* **14:** 953–961

Lygerou Z, Mitchell P, Petfalski E, Séraphin B, Tollervey D (1996) The POP1 gene encodes a protein component common to the RNase MRP and RNase P ribonucleoproteins. *Genes Dev* **8:** 1423–1433

Miles TD, Jakovljevic J, Horsey EW, Harnpicharnchai P, Tang L, Woolford Jr JL (2005) Ytm1, Nop7, and Erb1 form a complex necessary for maturation of yeast 66S preribosomes. *Mol Cell Biol* **25:** 10419–10432

Moy TI, Boettner D, Rhodes JC, Silver PA, Askew DS (2002) Identification of a role for Saccharomyces cerevisiae Cgr1p in pre-rRNA processing and 60S ribosome subunit synthesis. *Microbiol* **148:** 1081–1090

Oeffinger M, Leung A, Lamond A, Tollervey D (2002) Yeast Pescadillo is required for multiple activities during 60S ribosomal subunit synthesis. *RNA* **5:** 626–636

Oeffinger M, Tollervey D (2003) Yeast Nop15p is an RNA-binding protein required for pre-rRNA processing and cytokinesis. *EMBO J* **22:** 6573–6583

Oeffinger M, Wei KE, DeGrasse JA, Rogers JD, Chait BT, Rout MP (2007) Comprehensive analysis of diverse ribonucleoprotein complexes. *Nat Methods* **4:** 951–956

Oeffinger M, Zenklusen D, Ferguson A, Wei KE, El Hage A, Tollervey D, Chait BT, Singer RH, Rout MP (2009) Rrp17 is a eukaryotic exonuclease required for 5′ end processing of pre-60S ribosomal RNA. *Mol Cell* **36:** 768–781

Panse VG, Johnson AW (2010) Maturation of eukaryotic ribosomes: acquisition of functionality. *Trends Biochem Sci* **35:** 260–266

Pestov DG, Stockelman MG, Strezoska Z, Lau LF (2001) ERB1, the yeast homolog of mammalian Bop1, is an essential gene required for maturation of the 25S and 5.8S ribosomal RNAs. *Nucl Acids Res* **29:** 3621–3630

Pöll G, Braun T, Jakovljevic J, Neueder A, Jakob S, Woolford Jr JL, Tschochner H, Milkereit P (2009) rRNA maturation in yeast cells depleted of large ribosomal subunit proteins. *PLoS One* **4:** 1–13

Ross PL (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* **3:** 1154–1169

Rosado IV, Dez C, Lebaron S, Caizergues-Ferrer M, Henry Y, de la Cruz J (2007) Characterization of *Saccharomyces cerevisiae* Npa2p (Urb2p) reveals a low-molecular-mass complex containing Dbp6p, Npa1p (Urb1p), Nop8p, and Rsa3p involved in early steps of 60S ribosomal subunit biogenesis. *Mol Cell Biol* **27:** 1207–1221

Saveanu C, Bienvenu D, Namane A, Gleizes PE, Gas N, Jacquier A, Fromont-Racine M (2001) Nog2p, a putative GTPase associated with pre-60S subunits and required for late 60S maturation steps. *EMBO J* **20:** 6475–6484

Saveanu C, Namane A, Gleizes PE, Lebreton A, Rousselle JC, Noaillac-Depeyre J, Gas N, Jacquier A, Fromont-Racine M (2003) Sequential protein association with nascent 60S ribosomal particles. *Mol Cell Biol* **23:** 4449–4460

Strezoska Z, Pestov DG, Lau LF (2000) Bop1 is a mouse WD40 repeat nucleolar protein involved in 28S and 5.8S rRNA processing and 60S ribosome biogenesis. *Mol Cell Biol* **20:** 5516–5528

Sykes MT, Williamson JR (2009) A complex assembly landscape for the 30S ribosomal subunit. *Annu Rev Biophys* **38:** 197–215

Tang L, Sahasranaman A, Jakovljevic J, Schleifman E, Woolford Jr JL (2008) Interactions among Ytm1, Erb1, and Nop7 required for assembly of the Nop7- subcomplex in yeast preribosomes. *Mol Biol Cell* **19:** 2844–2856

Tarassov K, Messier V, Landry CR, Radinovic S, Serna Molina MM, Shames I, Malitskaya Y, Vogel J, Bussey H, Michnik SW (2008) An *in vivo* map of the yeast protein interactome. *Science* **320:** 1465–1470

Taylor DJ, Devkota B, Huang AD, Topf M, Narayanan E, Sali A, Harvey SC, Frank J (2009) Comprehensive molecular structure of the eukaryotic ribosome. *Structure* **17:** 1591–1604

van Nues RW, Rientjes JM, van der Sande CA, Zerp SF, Sluiter C, Venema J, Planta RJ, Raué HA (1994) Separate structural elements within internal transcribed spacer 1 of *Saccharomyces cerevisiae* precursor ribosomal RNA direct the formation of 17S and 26S rRNA. *Nucl Acids Res* **22:** 912–919

Wang M, Pestov DG (2011) 5′-end surveillance by Xrn2 acts as a shared mechanism for mammalian pre-rRNA maturation and decay. *Nucl Acids Res* **39:** 1811–1822

Xiang S, Cooper-Morgan A, Jiao X, Kiledjian M, Manley JL, Tong L (2009) Structure and function of the 5′→3′ exoribonuclease Rat1 and its activating partner Rai1. *Nature* **458:** 784–788

Xue Y, Bai X, Lee I, Kallstrom G, Ho J, Brown J, Stevens A, Johnson AW (2000) *Saccharomyces cerevisiae* RAI1 (YGL246c) is homologous to human DOM3Z and encodes a protein that binds the nuclear exoribonuclease Rat1p. *Mol Cell Biol* **20:** 4006–4015

Yeh LC, Thweatt R, Lee JC (1990) Internal transcribed spacer 1 of the yeast precursor ribosomal RNA. Higher order structure and common structural motifs. *Biochemistry* **29:** 5911–5918

Zhang J, Harnpicharnchai P, Jakovljevic J, Tang L, Guo Y, Oeffinger M, Rout MP, Hiley SL, Hughes T, Woolford Jr JL (2007) Assembly factors Rpf2 and Rrs1 recruit 5S rRNA and ribosomal proteins rpL5 and rpL11 into nascent ribosomes. *Genes Dev* **21:** 2580–2592

