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# Yeast polypeptide exit tunnel ribosomal proteins L17, L35 and L37 are necessary to recruit late-assembling factors required for 27SB pre-rRNA processing

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## ABSTRACT

**Ribosome synthesis involves the coordinated folding and processing of pre-rRNAs with assembly of ribosomal proteins. In eukaryotes, these events are facilitated by *trans*-acting factors that propel ribosome maturation from the nucleolus to the cytoplasm. However, there is a gap in understanding how ribosomal proteins configure pre-ribosomes *in vivo* to enable processing to occur. Here, we have examined the role of adjacent yeast r-proteins L17, L35 and L37 in folding and processing of pre-rRNAs, and binding of other proteins within assembling ribosomes. These three essential ribosomal proteins, which surround the polypeptide exit tunnel, are required for 60S subunit formation as a consequence of their role in removal of the ITS2 spacer from 27SB pre-rRNA. L17-, L35- and L37-depleted cells exhibit turnover of aberrant pre-60S assembly intermediates. Although the structure of ITS2 does not appear to be grossly affected in their absence, these three ribosomal proteins are necessary for efficient recruitment of factors required for 27SB pre-rRNA processing, namely, Nsa2 and Nog2, which associate with pre-60S ribosomal particles containing 27SB pre-rRNAs. Altogether, these data support that L17, L35 and L37 are specifically required for a recruiting step immediately preceding removal of ITS2.**

## INTRODUCTION

Ribosomes are complex macromolecular machines that catalyze the fundamental process of translation. Assembly of ribosomes is a complicated multi-step process that involves transcription, folding, modification and processing of pre-rRNAs, as well as the concomitant assembly of ribosomal proteins (r-proteins) (1–4). In eukaryotes, assembly of both 40S and 60S ribosomal subunits begins in the nucleolus with the transcription of pre-35S and pre-5S rRNA precursors by RNA polymerase I (Pol I) and RNA polymerase III (Pol III), respectively (Figure 1A). Assembly proceeds through a dynamic flux of protein–protein, protein–rRNA and rRNA–rRNA interactions toward final maturation in the cytoplasm (1,6–9). More than 200 *trans*-acting assembly factors facilitate these remodeling events that trigger the successive nucleolytic removal of transcribed spacer sequences and the hierarchical addition of r-proteins into nascent subunits (6,10) (Figure 1A and B).

Pioneering *in vitro* reconstitution studies of bacterial 30S subunits established the hierarchical succession of small subunit r-protein assembly with 16S rRNA (11–13). rRNA structural probing showed that binding of an initial subset of r-proteins causes structural rearrangements in 16S rRNA. These rearrangements form binding sites that allow subsequent association of another set of r-proteins and stabilize rRNA configurations (14–19). What emerged from these studies is the principle that ribosome synthesis is highly cooperative and occurs in parallel intermediate pathways (20–22). However, ribosome assembly *in vivo* is much more complex, involving (i) coordination of transcription with assembly, (ii) removal of spacer sequences, (iii) export of pre-ribosomes from the nucleus to the cytoplasm and (iv) controlled action of assembly factors.

The discovery of more than 200 ribosome biogenesis factors has led to extensive investigations on how they facilitate processing of pre-rRNAs and structural rearrangements to steer maturation of assembling pre-ribosomal complexes. Although the field has learned a great deal from studying them, *trans*-acting factors are

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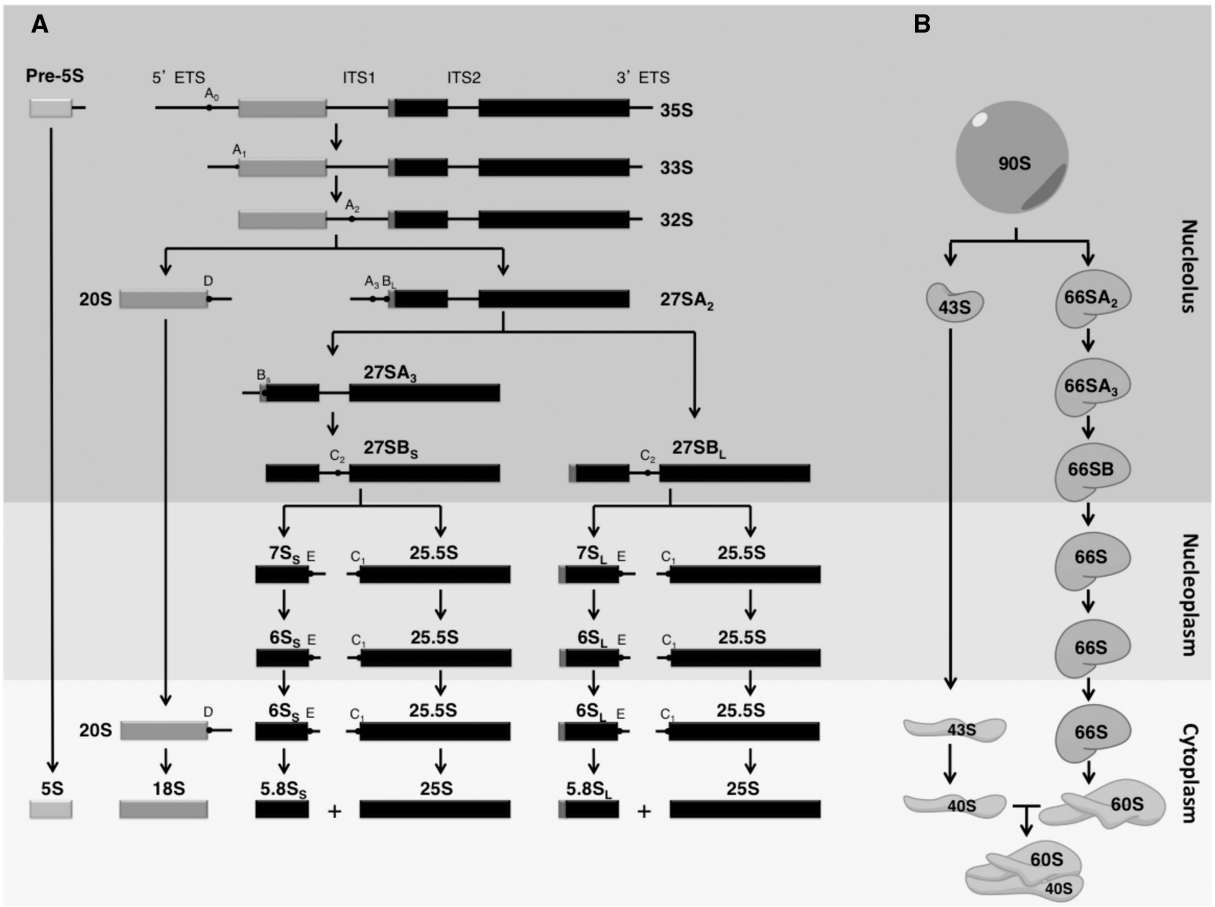


**Figure 1.** Ribosome assembly pathway in *Saccharomyces cerevisiae*. (**A**) Pre-rRNA processing pathway indicating exonucleolytic trimming and endonucleolytic reactions. Note that cotranscriptional processing at the $A_3$ site also occurs (5) (not shown). (**B**) Maturation of pre-ribosomal particles from the nucleus to the cytoplasm.

only one part of the grand design that is ribosome biogenesis. Ribosomes are multimolecular complexes wherein r-proteins embellish a core of rRNA; hence, r-proteins likely serve as molecular scaffolds necessary not only for ribosome function but also assembly. Comprehensive studies of how r-proteins facilitate ribosomal subunit biogenesis are greatly underrepresented. Recent work has identified for which steps in pre-rRNA processing different r-proteins are required, but it is not clear exactly how they participate in these steps (23–35). It is presumed that they do not function directly in pre-rRNA processing; r-proteins are thought to have structural rather than enzymatic functions. The roles of r-proteins in establishing pre-ribosomal particles and recruitment of assembly factors and other r-proteins to enable subsequent maturation are even less well studied. Thus far, examination of how r-proteins affect the composition of pre-ribosomes to propel pre-rRNA maturation has only been reported for a handful of r-proteins from both r-subunits (36–39). Hence, more studies of this kind are needed to fully comprehend the assembly process.

Initial systematic investigation of 60S subunit assembly categorized r-proteins based on their requirement for early, intermediate, and late pre-rRNA maturation steps [(30), unpublished data]. Following up on Pöll *et al.* (30), we recently characterized in more detail a subset of r-proteins that function early in ribosome assembly, during the exonucleolytic trimming of ITS1 sequences in $27SA_3$ pre-rRNAs (39). In this work, we have focused on r-proteins L17, L35 and L37, which had been implicated in removal of ITS2, and are adjacent to each other in the structure of mature 60S subunits (40–42) (Figure 2). L17 and L35 are eukaryotic homologs of bacterial L22 and L29, respectively, whereas L37 does not have a bacterial homolog. Together, these three r-proteins interact with all six secondary structure domains of 5.8S/25S rRNA, having multiple contact sites with each domain (40–42). They may therefore be important in promoting and stabilizing long-range inter-domain interactions that ultimately influence the architecture of assembling ribosomes. Thus, these r-proteins may help create local or global structural prerequisites for pre-rRNA processing and association of proteins. They are also of interest because, together with the nonessential r-protein L26 (43), L17, L35 and L37 are missing from pre-ribosomes in mutants unable to process early pre-rRNA intermediates (39,44). This is attributed to misfolding of rRNA domains to which they bind, as well as reflecting the coupling of pre-rRNA processing with r-protein assembly. In addition,

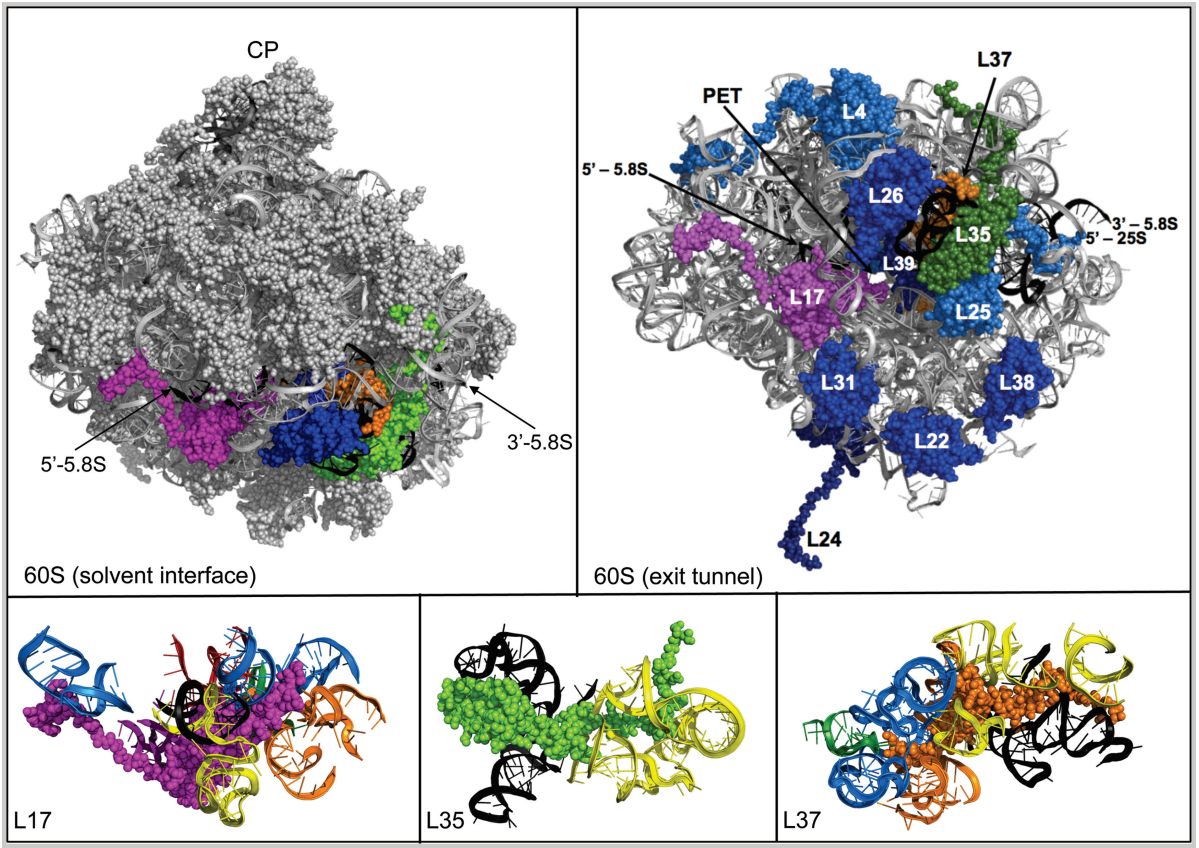


**Figure 2.** Localization of L17, L35, and L37 around the polypeptide exit tunnel (PET). (Top left) Location of L17, L35, and L37 in the structure of mature 60S ribosomal subunits (PDB: 3U5D and 3U5E) (41). CP denotes the central protuberance. (Top right) The 60S ribosomal subunit structure is viewed from the rim of the polypeptide exit tunnel looking straight through the subunit. 5.8S rRNA is in black, 25S rRNA is in grey, r-proteins of interest are shown as colored spheres. Six r-proteins (L22, L24, L26, L31, L38 and L39) adjacent to L17, L35 and L37 are nonessential. (Bottom, left to right) rRNA domains with which r-proteins L17, L35 and L37 interact. 5.8S rRNA is shown in black, while domains I, II, III, IV, V and VI of 25S rRNA are shown in yellow, blue, orange, green, red, and purple, respectively.

since these r-proteins lie around the rim of the nascent polypeptide exit tunnel, one might be able to relate the construction of this rRNP neighborhood to intermediate pre-rRNA maturation steps.

We wanted to take a comprehensive look at the function of these three r-proteins and how they affect ribosome assembly. We tested their role in recruiting assembly factors to begin to understand how their absence leads to a distinct pre-rRNA processing defect. We have compared the phenotypes upon depleting each of these r-proteins and found that they exhibit very similar defects in biogenesis of 60S subunits even though they are not interdependent for association with pre-ribosomal particles (pre-rRNPs). Our data indicate that these r-proteins affect recruitment of a specific set of assembly factors necessary for 27SB pre-rRNA processing, and in their absence pre-rRNPs are gradually turned over.

## MATERIALS AND METHODS

### Generation of yeast strains and *in vivo* depletion of r-proteins

Yeast strains used in this study are derivatives of either JWY6147 (*MATa ura3-52 trp1-1 lys2-801 his3-Δ200 leu2-Δ1*) or BY4741 (*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*) as listed in Supplementary Table S1. Gene disruptions, *GAL1* promoter fusions, and 3xHA-, 13xMYC- and TAP-tagged genes were constructed using polymerase chain reaction (PCR)-based chromosomal modifications (45,46). Oligonucleotide primers for PCR and plasmids used in this study are available upon request.

Like most yeast r-proteins, L17, L35 and L37 are encoded by duplicate (paralogous) genes (47). These r-protein pairs have highly similar, or identical, amino acid sequences (Supplementary Figure S1). Conditional null mutant strains of *RPL17* (*GAL-HA-RPL17*; *MATa ura3-52 trp1-1 lys2-801 his3-Δ200 leu2-Δ1 rpl17b::KANMX6 rpl17a::GAL-HA-RPL17A TRP1*) and *RPL35* (*GAL-HA-RPL35*; *MATa ura3-52 trp1-1 lys2-801 his3-Δ200 leu2-Δ1 rpl35b::KANMX6 rpl35a::GAL-HA-RPL35A TRP1*) were constructed by first disrupting one r-protein gene copy. The promoter of the more highly expressed copy of each duplicate r-protein gene (48) that confers a stronger phenotype upon deletion (data not shown) was the *GAL1* promoter. Thus, we replaced the complete open reading frame (ORF) of *RPL17B*/YJL177W or *RPL35B*/YDL136W with the *KANMX6* cassette that confers resistance to the drug geneticin (G418). PCR-generated *KANMX6* amplicons from the

pFA6a-kanMX6 plasmid were transformed into JWY6147, transformants were screened for G418 resistance, and replacements were verified by genomic PCR. From these single gene disruption backgrounds, the endogenous promoter of the other r-protein gene copy in its genomic locus (*RPL17A*/YKL180W or *RPL35A*/YDL191W) was replaced with a *GAL1* promoter cassette together with the *TRP1* selectable marker. This cassette also contains a 3xHA-epitope to place an HA-tag at the N-terminus of the respective r-proteins, to enable their detection via western blotting. PCR modules containing *GAL1* promoter sequences were transformed into the single r-protein gene deletion strains; transformants were selected for growth in medium containing galactose but lacking tryptophan. Trp+ transformants were screened for correct integration of the *GAL1* promoter and the N-terminal HA-epitope fusion by western blotting with anti-HA antisera.

After numerous attempts, creating a chromosomal fusion of the *GAL1* promoter to either gene copy of *RPL37* proved to be unsuccessful. For this reason, an *RPL37* conditional mutant strain (*pGAL-RPL37*; *MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 rpl37a::HIS3MX6 rpl37b::KANMX4 pGAL-RPL37A LEU2*) derived from BY4741 was used for characterization. The *rpl37* mutant strain used has both chromosomal copies of *RPL37* (*RPL37A*/YLR185W and *RPL37B*/YDR500C) deleted and contains a plasmid bearing a conditional allele of *RPL37A*, without an in-frame N-terminal HA epitope fusion [procedure described in (26) and (30)].

C-terminal 3xHA-, 13xMYC- and TAP-fusion cassettes for tagging genes by transformation were PCR-amplified from plasmids bearing the sequences of the tags and a selectable marker (45,46). His+ or Ura+ transformants were screened by immunoblotting with anti-HA, -MYC or anti-TAP antisera to validate correct cassette integration.

Conditional null mutant strains were grown at 30°C in either rich or synthetic minimal medium containing 2% galactose to express *GAL1* fusion genes, or shifted to medium containing 2% glucose to repress expression of the r-protein gene.

### Cloning of yeast r-protein genes into appropriate vectors

Unique restriction sites were incorporated 800–1000 nucleotides upstream and 50–100 nucleotides downstream of the start and stop codons of r-protein genes, respectively, via a two-step PCR reaction. Plasmids from the Yeast Genomic Tiling Collection (Open Biosystems) that bear intact *RPL17A*, *RPL35A* and *RPL37A* ORFs as the template and oligonucleotides used were designed to contain palindromic sequences recognized by restriction enzymes that produce sticky ends (oligonucleotide sequences available upon request). Upstream and downstream PCR oligonucleotides to amplify each r-protein gene contained restriction sites as follows: BamHI and HinDIII for *RPL17A*; BamHI and SalI for *RPL35A*; and, BamHI and EcoRI for *RPL37A*. Resulting PCR products were digested with the corresponding restriction enzymes and then ligated into vectors digested with the same restriction enzymes. *RPL17A*- and *RPL35A*-containing PCR amplicons were cloned into pRS315 (*LEU2*) whereas the cassette bearing the *RPL37A* ORF was cloned into pRS316 (*URA3*). Correct plasmid constructs were verified by sequencing.

### Sucrose gradient centrifugation

Pre-ribosomes, 40S and 60S r-subunits, 80S ribosomes, and polyribosomes were fractionated using a modified protocol derived from Deshmukh *et al.* (49). Briefly, 100 ml of logarithmically growing yeast cultures were treated with 5 mg cycloheximide 10 min prior to harvesting. Forty $A_{254}$ units of whole-cell lysates were loaded into 7–47% (w/v) sucrose gradients and subjected to centrifugation at 27 000 rpm using a Sorvall AH-629 swinging bucket rotor. Fractions were collected and $A_{254}$ was monitored using a Teledyne ISCO Foxy R1 density gradient fraction collector.

### Steady-state analyses of whole-cell mRNAs, pre-rRNA intermediates and mature rRNAs

Steady-state levels of pre-rRNAs were assayed by primer extension and northern hybridization protocols adapted from Horsey *et al.* (50). An oligonucleotide (51) complementary to a region immediately upstream of the $C_2$ cleavage site in ITS2 was used to assay for 5′-ends of 27S pre-rRNA intermediates by primer extension. The same oligonucleotide was used to assay for 7S pre-rRNA by northern blotting. Transcription of *GAL1*-driven *RPL17A*, *RPL35A* and *RPL37A* genes in expressive and repressive conditions was also monitored by northern hybridization using oligonucleotides complementary to respective mRNAs (oligonucleotide sequences available upon request). U2 snRNA was used as loading control in all northern hybridization assays. To detect amounts of mature 25S and 18S rRNAs, total RNA was extracted from cells, separated on a 1% agarose gel, and visualized by ethidium bromide staining. Signal intensities of mature rRNAs were quantified using Image Gauge software (Fujifilm).

### Kinetic pulse-chase labeling analysis

Metabolic pulse-chase experiments to assay the kinetics of pre-rRNA processing *in vivo* were carried out as described in Horsey *et al.* (50) with the following modifications: yeast cultures at $OD_{610} = 0.4–0.5$ were pulse-labeled with [$^3$H-methyl]-methionine for 5 min then chased with an excess of non-radioactive methionine. RNA was extracted from samples collected at 0, 2, 5, 10, 20, 40 and 60 min after chase. Equal counts were loaded onto agarose-formaldehyde gels for each RNA sample from *GAL-HA-RPL17* and *pGAL-RPL37* strains grown in galactose-containing medium or shifted for 16–17 h to glucose-containing medium, respectively.

### Affinity purification of assembly intermediates

TAP-tagged assembly factors were used to affinity-purify pre-ribosomes from whole-cell extracts using the

single-step affinity purification protocol described by Sahasranaman *et al.* (44).

### Silver staining and western blotting

Proteins from whole-cell extracts or purified pre-ribosomes were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 4% to 20% Tris–Glycine precast gels (Invitrogen) after which a standard silver-staining protocol was followed. Proteins on SDS-PAGE profiles were labeled based on comigration with silver-stained proteins previously identified by mass spectrometry, decrease in protein band intensity when expression of the corresponding gene is turned off, or shift in mobility of epitope-tagged versions of the corresponding protein. Detection of proteins by western blotting was carried out using the protocol adapted from Sahasranaman *et al.* (44). Nog2, which comigrates with IgG on 4% to 20% precast Novex gels, was assayed using 4% to 12% Bis–Tris precast gels (Invitrogen).

### Co-immunoprecipitation of pre-rRNAs with GFP-tagged r-proteins

Pre-rRNAs copurifying with GFP-tagged L17 and L37 were assayed using the one-step GFP-Trap®_A protocol (Chromotek) as previously described (43). Briefly, RNA enriched from purified pre-ribosomes containing the GFP-tagged protein was extracted, and pre-rRNAs were assayed by northern hybridization following procedures described by Venema *et al.* (52). Autoradiographs were analysed using an FLA-5100 imaging system (Fujifilm) at the Biology Service (CITIUS) from the University of Seville.

### Fluorescence microscopy

Export of pre-ribosomal particles from the nucleolus through the nucleoplasm to the cytoplasm was assayed by tracking the localization of L25-eGFP or S3-eGFP as previously described (36,53–55). For this purpose, each conditional mutant strain was transformed with a plasmid carrying either L25- or S3-eGFP, and Nop1-mRFP (55,56). Cells were imaged using a Carl Zeiss LSM-510_META_UV_DuoScan inverted spectral confocal microscope and analysed using ImageJ (57).

Timing of r-protein assembly was addressed by transforming YCplac111-*RPL17B-eGFP* and YCplac111-*RPL37B-eGFP* into *rpl17* and *rpl37* null strains, respectively. Each strain was then cotransformed with either pRS316-*GAL-NMD3Δ100* or the pRS316-*GAL-NMD3FL* plasmid, expressing a *GAL* promoter fusion of a dominant negative truncation or a wild-type allele of the *NMD3* gene, respectively (58). Images were acquired using a Leica DMR microscope equipped with a DC camera and processed using Adobe Photoshop 7.0.

### *In vivo* ITS2 structure probing

ITS2 secondary structure was assayed by *in vivo* dimethyl sulfate (DMS) probing using a protocol adapted from Dutca *et al.* (59). Conditional null strains were grown in expressive and repressive media as indicated above, to $OD_{610}$ = 0.4–0.5. Ten milliliter aliquots of yeast cultures were treated with 200 µl of a fresh dilution of DMS (Sigma Aldrich) in 95% ethanol (1:4 v/v) to a final concentration of 50 mM. Treated cells were incubated with shaking at 30°C for 2 min. Reactions were quenched by placing the tubes on ice and adding 5 ml of 0.6 M 2-mercaptoethanol and 5 ml of water-saturated isoamyl alcohol. As a control for the effectiveness of the stop reaction, a control sample was included where DMS treatment was done after the addition of 2-mercaptoethanol and isoamyl alcohol. Cells were pelleted by centrifugation at $5000 \times g$ for 5 min, and the liquid phase was carefully removed. Cell pellets were suspended in 5 ml 0.6 M 2-mercaptoethanol, and centrifuged again. Total RNA was immediately extracted from the cells. Nucleotide modifications were assayed by primer extension and gel electrophoresis as described earlier, using oligonucleotides complementary to ITS2 (available upon request).

## RESULTS

Previous evidence indicates that both L17 and L35 are required for processing of the ITS2 spacer separating mature 5.8S and 25S rRNA sequences (30,31,44). The role of L37 in ribosome assembly has not been previously reported; however, our initial characterization led us to believe that L37 is also involved in the same assembly step. In cells where transcription of *RPL35* is repressed, hence depleting the protein, mature 60S subunits are not produced as consequence of a block in 27SB pre-rRNA processing (31) (Supplementary Figures S2 and S3). Nucleocytoplasmic export is impaired when L35 is depleted (Supplementary Figure S3), and pre-60S ribosomal particles are gradually turned over (31). Furthermore, L35 assembles into the pre-60S ribosomal particles containing $27SA_2$ pre-rRNA ($66SA_2$ assembly intermediates; Figure 1B). However, despite its role in 27SB pre-rRNA processing, no information is available on how L35 affects the structure of ITS2 or recruitment of specific assembly factors or r-proteins into pre-rRNPs. Here, we start by filling the gap in our knowledge of the roles of L17 and L37 in production of mature r-subunits, pre-rRNA processing, and when they are stably loaded into preribosomes. We then proceed to show the effects of depleting L17, L35 or L37 on the structure of ITS2, and the association of *trans*-acting factors and other r-proteins with pre-rRNPs.

### Depletion of r-proteins L17 and L37 is detrimental to cellular growth and synthesis of 60S subunits

To assess the function of the essential L17 and L37 r-proteins (48,60) in ribosome biogenesis, we utilized conditional null mutant systems (*GAL-HA-RPL17* and *pGAL-RPL37*) where the corresponding r-protein gene is expressed under the control of the *GAL1* promoter (see 'Materials and Methods' section). These strains failed to grow on glucose-containing solid medium (Figure 3A) where transcription of *GAL1*-driven r-protein genes is repressed (Supplementary Figure S2), and stopped dividing

6–9 h after shifting from galactose to glucose-containing liquid medium (Figure 3B). Consistently, significant reduction of the total cellular amounts of HA-L17 within this timeframe is evident (Supplementary Figure S2). Plasmids bearing wild-type *RPL17A* and *RPL37A* alleles complemented the growth defect of *GAL-HA-RPL17* and *pGAL-RPL37* strains on glucose-containing solid medium, respectively (Figure 3A).

To test the effect of depleting each protein on production of ribosomes, we assayed levels of mature rRNAs and ribosomal subunits. Upon depletion of either L17 or L37, steady-state levels of 25S rRNA relative to 18S rRNA decreased. In each case, the effect was rapid; the ratio dropped within 1.5 h after shifting from galactose to glucose (Figures 4A and Supplementary Figure S4). Consistent with these observations, amounts of free 60S r-subunits were dramatically reduced relative to free 40S subunits in each of the two strains where L17 or L37 were depleted (Figure 4B). In addition, halfmer polyribosomes were present upon depletion of r-proteins, indicative of a deficit in 60S r-subunits. These observations indicate that L17 and L37 are specifically required for the proper formation of mature 60S r-subunits.

### L17 and L37 are required for processing of 27SB pre-rRNA and formation of stable 66S assembly intermediates

To test how these r-proteins affect formation of mature rRNAs, we assayed for the synthesis and turnover of pre-rRNAs by pulse-chase experiments using [$^3$H-methyl]-methionine in *GAL-HA-RPL17* and *pGAL-RPL37* strains. Under permissive conditions where L17 and L37 are expressed, 35S pre-rRNA was rapidly processed into 27S and 20S precursors, which

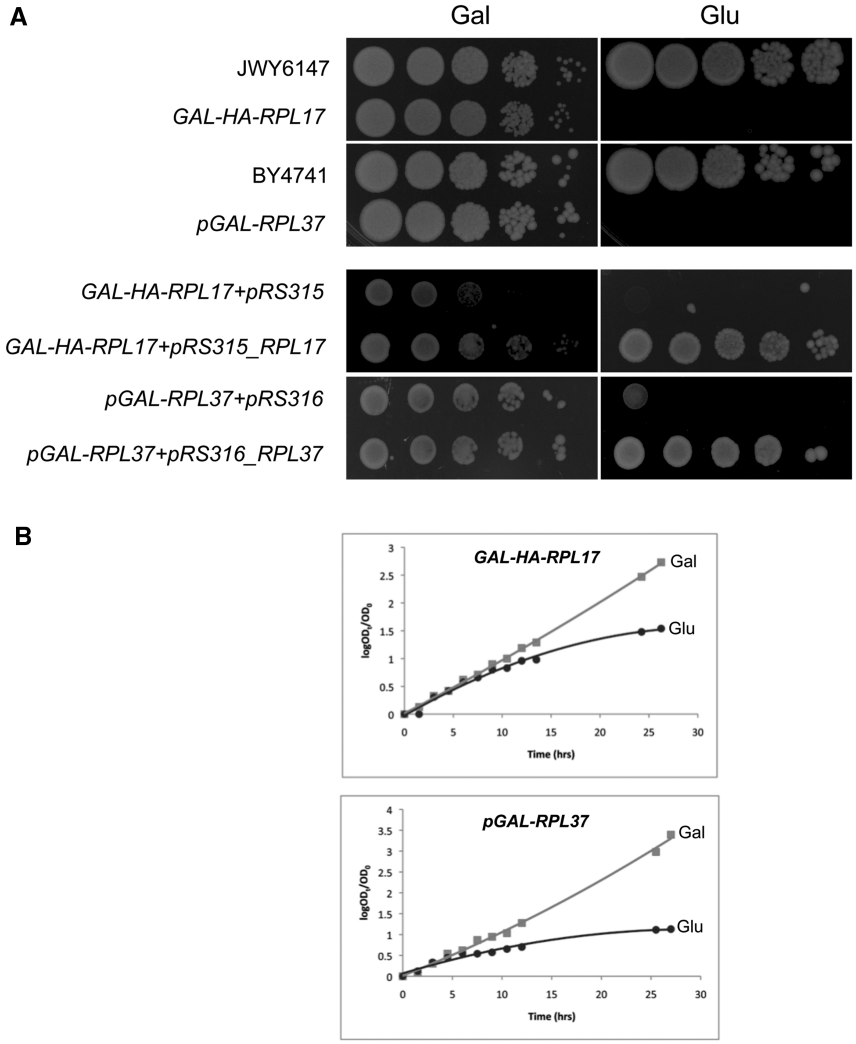


**Figure 3.** Depletion of either L17 or L37 leads to cellular growth arrest. (**A**) Growth on solid medium of each conditional null mutant strain relative to the wild-type control strains JWY6147 or BY4741. *GAL-HA-RPL17*, *pGAL-RPL37*, wild-type strains and conditional strains episomally expressing wild-type alleles of r-protein genes were grown in galactose-containing (Gal) liquid medium and diluted to an $OD_{610}$ of 0.5. Ten microliters of 10- to 100 000-fold serial dilutions were spotted on galactose-containing (Gal) and glucose-containing (Glu) solid medium, and then incubated at 30°C. (**B**) Logarithmically growing *GAL-HA-RPL17* and *pGAL-RPL37* cells were retained in galactose-containing media or transferred to glucose-containing liquid media at 30°C. Strains were diluted as necessary to maintain logarithmic growth, and cell densities ($OD_{610}$) were continuously measured.

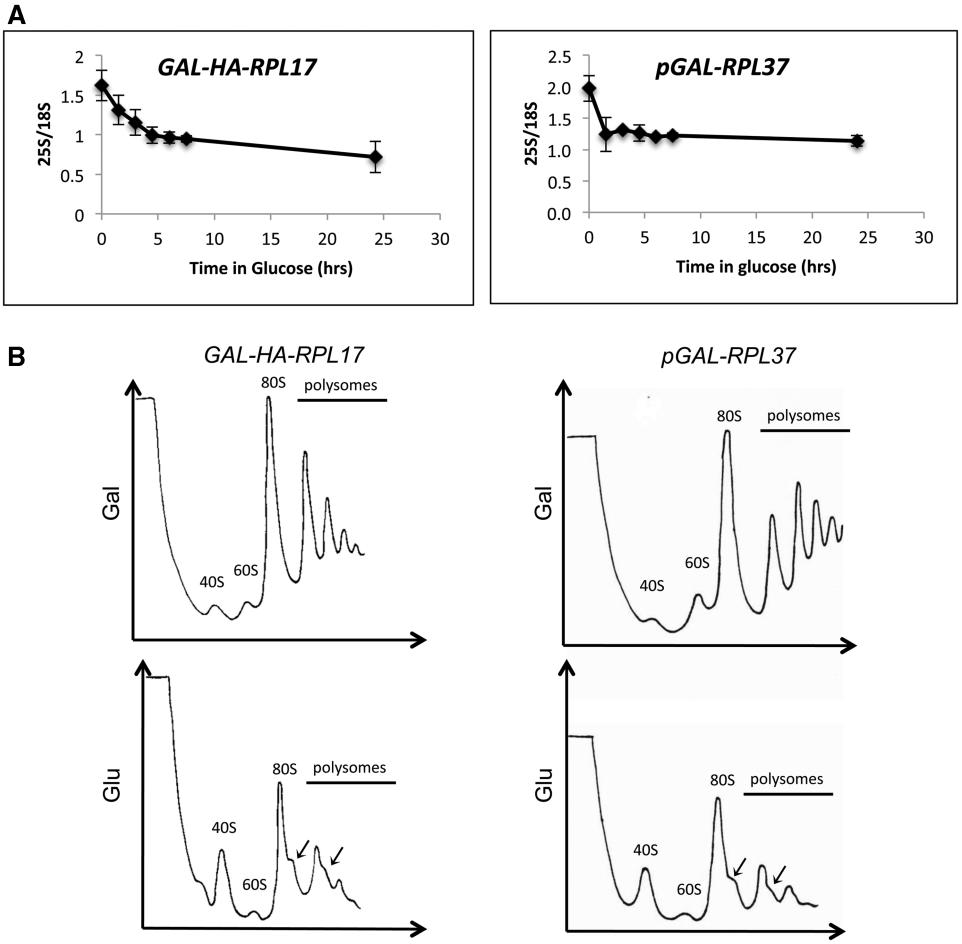


**Figure 4.** Cells depleted of L17 or L37 exhibit a deficit of 60S ribosomal subunits. (**A**) Total RNA was extracted from *GAL-HA-RPL17* and *pGAL-RPL37* strains shifted to glucose-containing media at specified time points. Samples were separated on agarose gels, and stained with ethidium bromide. Ratios of 25S relative to 18S mature rRNA levels for at least three biological replicates are shown in the graph. (**B**) Polysomes from *GAL-HA-RPL17* and *pGAL-RPL37* strains grown in galactose-containing media and shifted to glucose-containing media for 17 h were sedimented through 7 to 47% sucrose gradients. 40S and 60S r-subunits, 80S ribosomes and polyribosomes were monitored at $A_{254}$ using a Teledyne ISCO Foxy R1 density gradient fraction collector. Arrows indicate halfmers.

were subsequently converted to mature 25S and 18S rRNAs, respectively (Figure 5A). However, in repressive conditions where L17 and L37 are depleted, synthesis of 25S rRNA was inhibited relative to 18S rRNA. Upon depletion of either r-protein, 27SB pre-rRNA persisted even after 10 min of chase, indicating a delay in processing of this precursor species. Interestingly, we observed a gradual decrease in 27SB pre-rRNA to undetectable levels 20 min after the chase began, suggesting efficient turnover of this precursor (Figure 5A). 23S pre-rRNA was also detected in each mutant, albeit less so in the absence of L37, suggesting a delay in processing at sites $A_0$, $A_1$ and $A_2$. The abundance of this intermediate, previously found in many 60S r-subunit assembly mutants (28,53,61), is most likely a secondary consequence of inefficient recycling of assembly factors required for the early cleavage steps, which fail to be released from aberrant 66S pre-ribosomes [discussed in (62)].

We also assayed steady-state levels of pre-rRNA intermediates in r-protein depleted strains by primer extension and northern hybridization. Upon depletion of either L17 or L37, we found no differences in amounts of the primer extension stops at the $A_2$ and $A_3$ sites. However, we observed a significant accumulation of the primer extension stop at the $B_{1S}$ site and a more modest accumulation at the $B_{1L}$ site (Figure 5B). This might be a manifestation of the faster relative rate of turnover of abortive 66S intermediates containing $27SB_{1L}$ pre-rRNA in mutants lacking L17 or L37 (28). Northern blot analysis showed that levels of 7S pre-rRNAs decreased in each mutant (Figure 5C). Therefore, this indicates that the observed increase in $B_{1L}$ and $B_{1S}$ ends represent 27SB pre-rRNAs. Finally, consistent with previous data, L17-depleted cells also accumulated 5′ truncated intermediates caused by Rat1 exonucleolytic digestion past the $B_{1S}$ site during $27SA_3$ pre-rRNA processing (44) (Figure 5B).

We conclude that L17 and L37 are required for 27SB pre-rRNA processing at the $C_2$ site in ITS2; in their absence, abortive intermediates are formed but subsequently targeted for turnover. Interestingly, these observations are very similar to the pre-rRNA processing

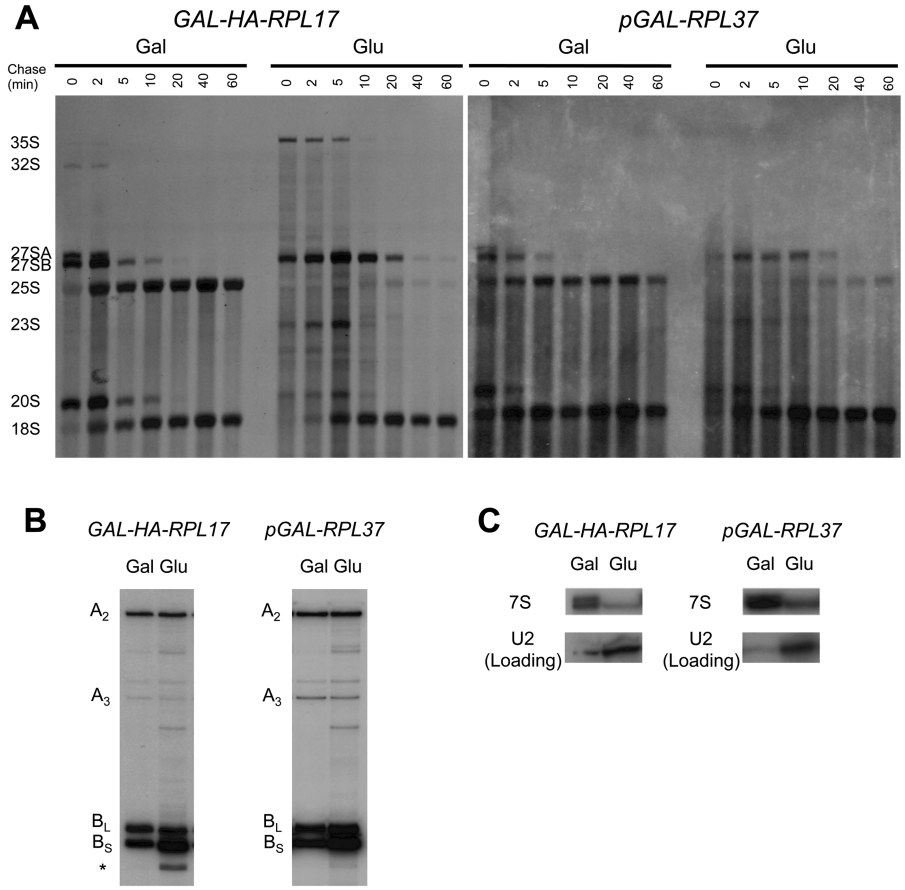


**Figure 5.** Depletion of L17 or L37 leads to a defect in processing of 27SB pre-rRNA and its eventual turnover. (**A**) Synthesis and turnover of pre-rRNAs were assayed by pulse-chase experiments. *GAL-HA-RPL17* and *pGAL-RPL37* cells were grown in galactose- or glucose-containing medium lacking methionine for 16–17 h. Cells were pulse-labeled with [$^3$H-methyl]-methionine for 5 min and chased with an excess of nonradioactive methionine. Total RNA was extracted, and equal numbers of radioactive counts were loaded onto denaturing agarose gels, transferred to nitrocellulose membranes, and exposed to X-ray films. (**B**) Total RNA was extracted from *GAL-HA-RPL17* and *pGAL-RPL37* strains grown in galactose or shifted to glucose for 16–17 h at 30°C. Samples were subjected to primer extension analysis of 5′-ends of 27S and 7S pre-rRNAs. The asterisk indicates 5′-truncated pre-rRNAs formed specifically in the absence of L17. (**C**) Total RNA was extracted as described earlier, and 7S pre-rRNAs were assayed by northern hybridization. U2 was probed to serve as a loading control.

phenotype we previously observed upon depletion of L35 r-protein (31).

### Aberrant pre-ribosomes lacking either L17 or L37 are retained in the nucle(ol)us

Ribosome maturation is initiated in the nucleolus, continues in the nucleoplasm, and is completed following export of the pre-40S and pre-60S ribosomal particles to the cytoplasm. Although efficient nucleocytoplasmic export of pre-40S particles requires minimal structural reorganization, pre-60S ribosomal particles undergo stringent structural and compositional rearrangements prior to export (6,7,63). To investigate the subcellular location of assembly intermediates in the absence of L17 and L37, we tracked the localization of GFP-tagged L25, a well-established 60S subunit reporter (64). Importantly, L25 is successfully assembled into pre-ribosomes in the absence of L17 or L37 (Figure 9C), and thus is a valid reporter in these mutants. As expected for an r-protein, we observed cytoplasmic localization of L25-eGFP in wild-type cells grown in galactose-containing medium (Figure 6A). However, *GAL-HA-RPL17* and *pGAL-RPL37* cells shifted from galactose- to glucose-containing medium exhibited increased intensity of L25-eGFP fluorescence, but not the 40S reporter S3-eGFP (Figure 6A). L25-eGFP signal in either L17- or L37-depleted cells largely colocalizes with the nucleolar Nop1-mRFP (53) (Figure 6B). This indicates the failure to form pre-60S ribosomal particles competent for transit to the nucleoplasm and subsequent nuclear export and maturation in the cytoplasm. Consistent with this, it has been reported that assembling pre-60S complexes are released to the nucleoplasm upon completion of 27SB pre-rRNA processing (53).

We conclude that upon depletion of either L17 or L37, the intranuclear transport of pre-60S complexes from the nucleolus to the nucleoplasm is blocked. Moreover, particles that are released from the nucleolus are not exported to the cytoplasm. Two possible scenarios might account for the latter observation. First, in the absence of L17 or L37, 66S pre-rRNPs might traffic to the nucleoplasm but are possibly not in a conformation competent for export through the nuclear pore. Another more specific scenario

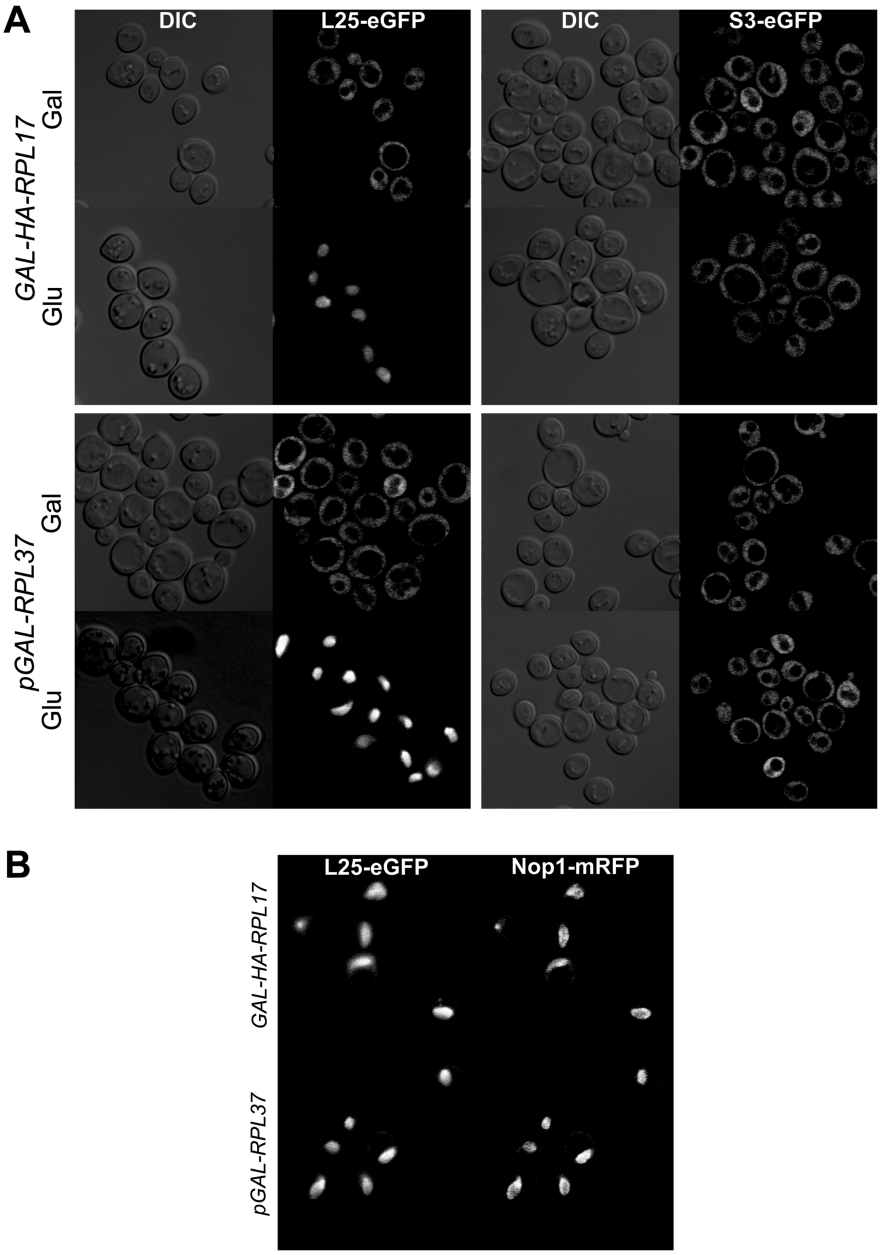


**Figure 6.** Depletion of L17 and L37 results in retention of pre-ribosomes in the nucleus. (**A**) *GAL-HA-RPL17* and *pGAL-RPL37* cells expressing either the 60S subunit reporter L25-eGFP or the 40S subunit reporter S3-eGFP were grown in galactose- or shifted to glucose-containing selective medium to an $OD_{610}$ of 0.4–0.6. Localization of GFP signal in wild type and mutant cells was detected using a Carl Zeiss LSM-510_META_UV_DuoScan inverted spectral confocal microscope. (**B**) Colocalization of L25-eGFP with the nucleolar marker Nop1-mRFP in *GAL-HA-RPL17* and *pGAL-RPL37* cells grown in galactose-containing media and shifted to glucose-containing media for 16 h.

is that L17 and L37 serve as platforms for the export machinery; hence in their absence, export is impaired. Indeed, some pre-60S export factors localize in close proximity to the polypeptide exit tunnel (65–67). Recently, cryo-EM analyses revealed that the export factor Arx1 interacts directly with r-proteins in this region, including L19, L25, L26 and L35 (68).

### L17 and L37 stably assemble into 27SB-containing pre-60S complexes in the nucleolus

The timing of assembly of r-proteins with nascent ribosomes has been broadly categorized into early and late maturation steps by pulse-labeling experiments (69). L17 and L37 were among those r-proteins that associate early with assembling ribosomes.

We took orthogonal approaches to investigate in further detail the timing of incorporation of L17 and L37 into pre-60S particles: (i) determining the localization of the GFP-tagged r-proteins in an *NMDΔ3100* dominant negative mutant defective for nucleocytoplasmic export of pre-60S ribosomal particles (58,70), (ii) assaying pre-rRNA intermediates that copurify with GFP-tagged L17 and L37 r-proteins by northern hybridization.

As shown in Figure 7A, fully functional GFP-tagged L17 and L37 accumulated in the nucleus upon overexpression of the dominant negative *NMD3Δ100* allele (9,1), similar to L25-eGFP, which assembles with pre-60S particles in the nucleolus. In addition, L17- and L37-GFP co-immunoprecipitated above-background levels of 27SB pre-rRNA and 7S pre-rRNA (Figure 7B). Consistently, TAP-tagged L17 and L37 copurified significant amounts of only $27SB_L$ and $27SB_S$ pre-rRNAs (Supplementary Figure S5A), and Nsa1-TAP containing complexes corresponding to 66SB pre-ribosomes (Figure 1B) significantly copurified L17 and L37 (Supplementary Figure S5B). $27SA_2$ pre-rRNA was also detected, albeit slightly above background, possibly indicating much less stable association with pre-ribosomes containing 27SA pre-rRNAs. Consistent with this observation, much lower levels of L17 and L37 copurified with Rrp5- and Npa2-TAP containing complexes corresponding to 90S-$66SA_2$ pre-ribosomes (Supplementary Figure S5B). GFP-tagged L17 and L37 copurified significant amounts of 25S, 5.8S and 5S rRNAs, as expected for a 60S r-protein (Figure 7B). Furthermore, 18S rRNA also copurified with L17- and L37-GFP, possibly indicating common nonspecific association of 40S with 60S subunits in buffers containing $Mg^{2+}$ or translating ribosomes (31). In contrast, background levels of 35S, 32S and 20S pre-rRNAs were detected. No (pre-)rRNAs were detected after affinity purification using untagged parent strain cell extracts (Figure 7B). Taken together, these observations indicate that L17 and L37 are most stably associated with pre-60S ribosomal particles containing 27SB pre-rRNAs, after $27SA_3$ pre-rRNA processing.

### The structure of ITS2 is largely unaffected in the absence of L17, L35 or L37

Mutational analysis of the ITS2 spacer sequence suggested that it plays an important role in the processing of 27S pre-rRNAs (71,72). More recently, Granneman *et al.* (73) showed that a subset of $A_3$ cluster proteins might regulate the structural conformation of ITS2 to allow processing of $27SA_3$ pre-rRNA to 27SB pre-rRNA. ITS2 is thought to undergo dynamic conformational changes to enable efficient pre-rRNA processing (72), between the ''hairpin'' structure (74) and the ''ring'' structure (75). Since L17, L35 and L37 are required for processing of 27SB pre-rRNA at the $C_2$ site in ITS2, we tested whether or not the structural conformation of ITS2 is affected upon depletion of these three r-proteins.

To survey the structure of ITS2 in wild-type and r-protein mutants, we employed *in vivo* chemical probing experiments using DMS. DMS methylates adenosines and cytosines unless protected by base-pairing or binding of a protein (76). In rare instances, guanosines and uracils can also be methylated by DMS *in vivo*, but the chemical basis of this has yet to be established (77). Modifications can then be detected by primer extension as stop sites preceding the methylated nucleotides. Because cells are most readily penetrated by DMS, it is the chemical of choice as a robust tool to interrogate RNA structures under *in vivo* conditions. As shown in Figure 8A, major primer extension stops were observed corresponding to most As and Cs predicted to be single-stranded in the hairpin structure of ITS2 (74) (Figure 8B). Consistently, nucleotides predicted to be base-paired or cross-linked to $A_3$–cluster proteins Nop15 and Cic1 (73) were largely protected. We observed no striking changes in nucleotide modification upon depletion of L17, L35 or L37. This is in stark contrast with the numerous changes in nucleotide modification in ITS2 observed upon depletion of the $A_3$ factor Cic1 (73) (Supplementary Figure S6). For additional comparison, we also assayed ITS2 structural changes in *nog2* conditional null strains that also exhibit a defect in 27SB pre-rRNA processing. Because the GTPase Nog2 is the last assembly factor to be recruited to enable 27SB pre-rRNA processing (78), we might predict that it causes a conformational change in ITS2 to allow the removal of this spacer sequence. However, we also found no striking change in nucleotide modification in ITS2 upon depletion of Nog2 (Figure 8A). We conclude that the absence of L17, L35 or L37 does not significantly affect the structure of ITS2 and that the block in processing of 27SB pre-rRNA is not primarily due to changes in ITS2 structure.

### L17, L35 and L37 are necessary for the stable association with pre-ribosomes of assembly factors specifically required for removal of ITS2 from 27SB pre-rRNA

To further understand the role of L17, L35 and L37 in 27SB pre-rRNA processing, we asked whether each r-protein is necessary for the recruitment of specific assembly factors or r-proteins into pre-rRNPs. TAP-tagged Nop7 was used to affinity-purify pre-60S assembly intermediates, when each of these r-proteins was either expressed or depleted. Nop7 is present in pre-rRNPs throughout the 60S subunit maturation pathway, and is not thought to be involved in 27SB pre-rRNA processing (51,79,80). Protein components were separated by SDS-PAGE, and assayed by silver staining and western blotting. SDS-PAGE profiles of wild-type and mutant pre-ribosomal proteins look similar, indicating that pre-rRNPs are largely intact and most assembly factors and r-proteins remain associated with aberrant assembly intermediates (Figure 9A). Specifically, the $A_3$ factors Nop7, Ytm1, Erb1, Cic1, Nop15 and Rlp7 [discussed in (44)] are unaffected in all three r-protein conditional null mutants. Furthermore, the SDS-PAGE profiles of L17-, L35- and L37-depleted strains are similar to those of assembly factor mutants that block processing of 27SB pre-rRNA (Figure 9A) (36,78). Many more changes in the pre-ribosomal protein population were observed in mutants in which an earlier pre-rRNA processing step is blocked (39,44) (Figure 9A), indicating that the effects that we see are specific.

The total population of different 66S pre-ribosomes that we purify contains up to ~45 r-proteins and ~75 assembly factors. Although these gels display several identifiable changes, the resolution is hence not adequate to detect all possible changes in protein composition. Thus, we tested for more specific changes by western blotting.

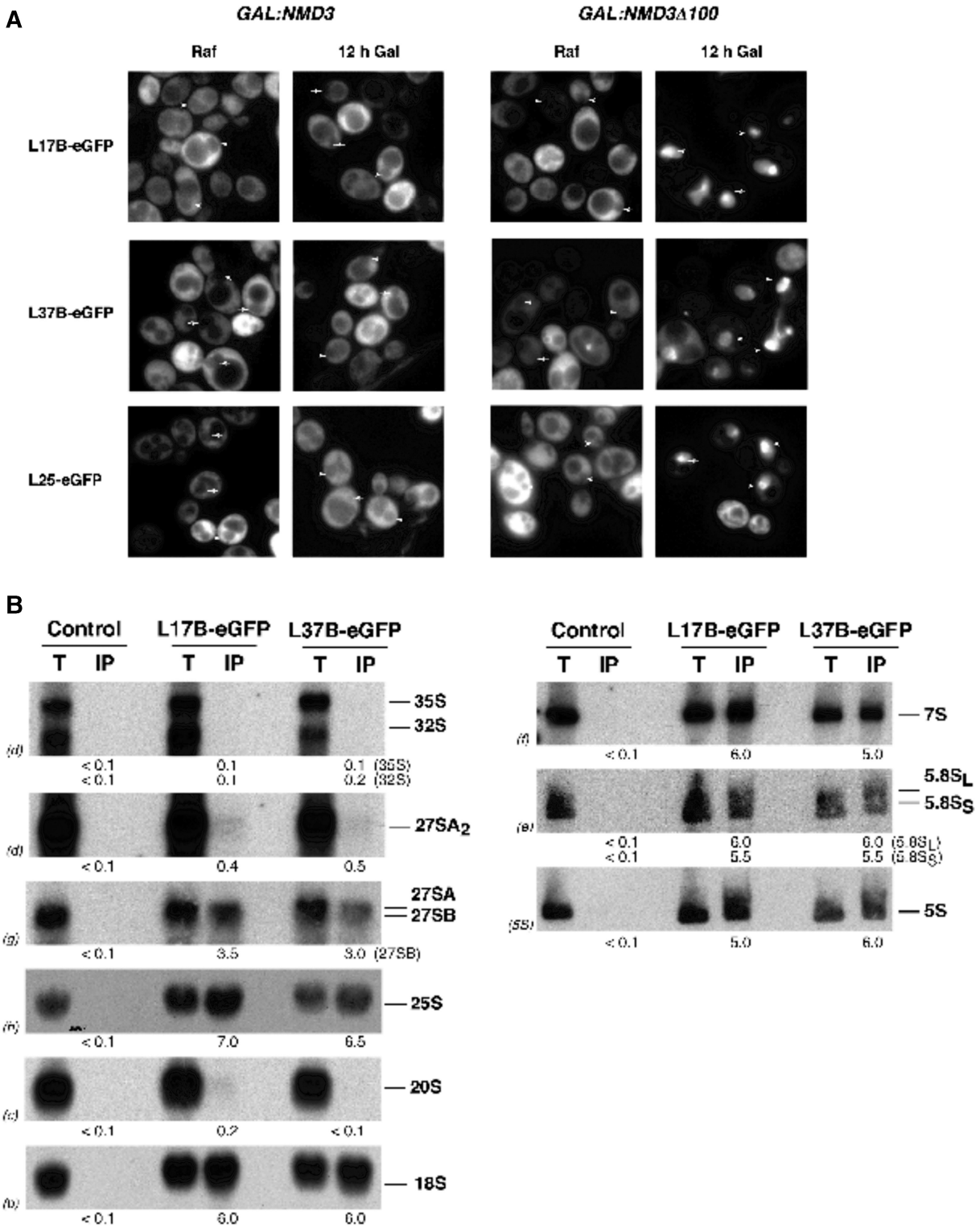


**Figure 7.** L17 and L37 associate with pre-ribosomal particles in the nucleolus. (**A**) Localization of L25-GFP, L17-GFP and L37-GFP was monitored upon induction of an *NMD3Δ100* dominant negative allele to inhibit pre-ribosome export. The pRS316-*GAL-NMD3Δ100* plasmid was transformed into *rpl25*, *rpl17* and *rpl37* null mutants expressing plasmid-derived L25-GFP, L17-GFP and L37-GFP, respectively. Transformants were grown in the presence of raffinose (Raf, SRaf-Leu-Ura medium). Galactose was then added to induce the expression of the Nmd3Δ100. The GFP signal was inspected by fluorescence microscopy after 24 h. Arrows point to nuclear fluorescence. (**B**) Pre-ribosomes containing L17-GFP and L37-GFP were affinity-purified from whole-cell extracts of strains expressing GFP-tagged L17 and L37 using GFP-Trap_ A beads. RNA from purified pre-ribosomal particles was assayed by northern hybridization. The untagged parent strain was used as the negative control. Signal intensities were quantified by phosphoimager scanning. Values indicate percentage of RNA recovered after purification.

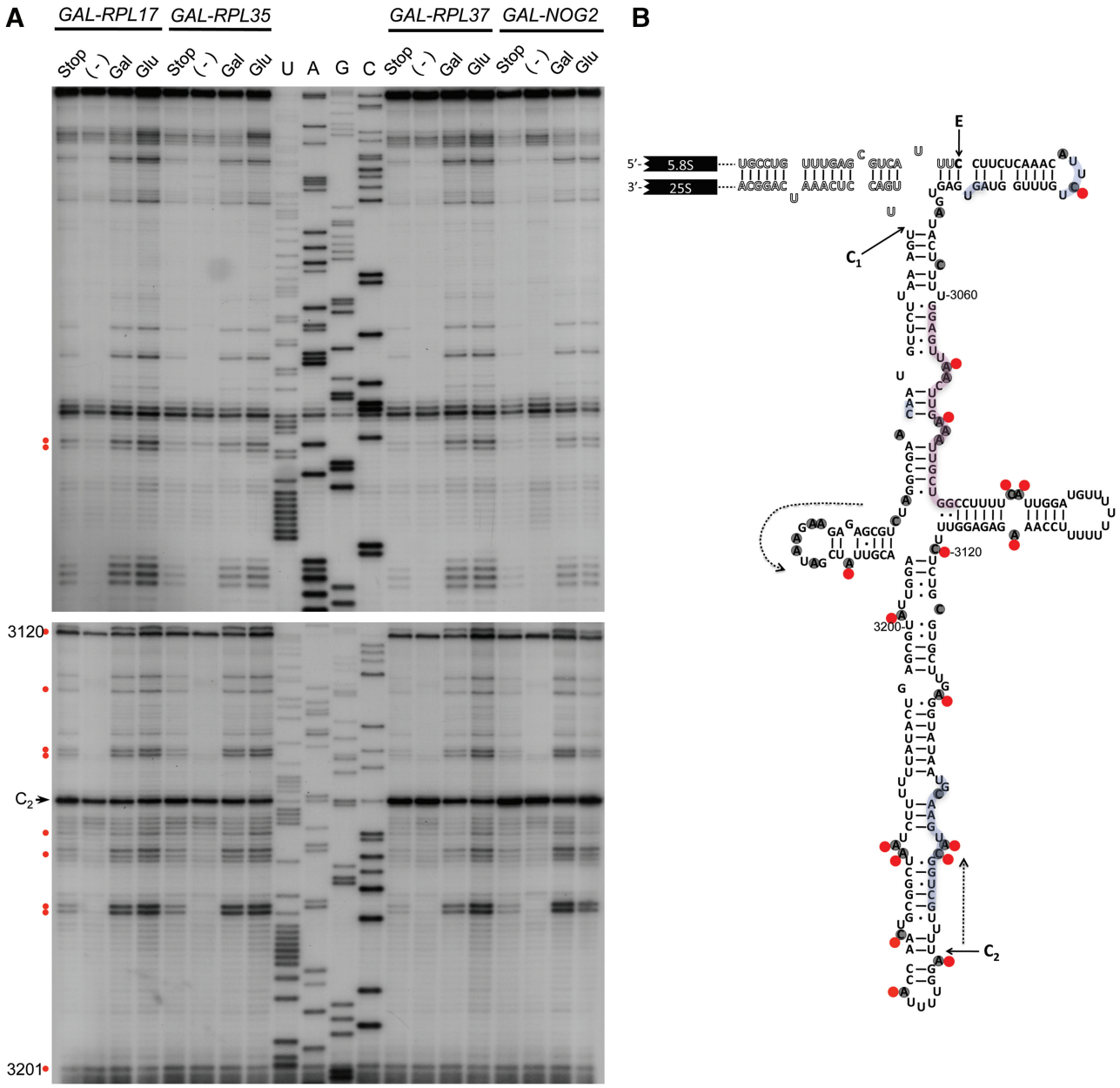


**Figure 8.** Depletion of L17, L35 or L37 does not significantly perturb the overall structure of ITS2. (**A**) *In vivo* dimethyl-sulfate (DMS) probing was performed on *GAL-HA-RPL17*, *GAL-HA-RPL35* and *pGAL-RPL37* strains grown in galactose or shifted from galactose to glucose for 16 h to deplete corresponding proteins. A *GAL-NOG2* strain shifted to glucose to block 27SB pre-rRNA processing, was used for comparison. Modifications were assayed by primer extension. Untreated RNA from wild-type strains was used as a control for naturally occurring primer extension stops. Stop controls indicate the efficiency of the quenching reaction after DMS treatment. Red dots indicate observed nucleotide modifications relative to untreated samples. (**B**) Positions of modified bases and the location of oligonucleotides used for primer extension are plotted on the hairpin structure of ITS2. Red dots indicate observed nucleotide modification while black shades indicate expected nucleotide modifications based on ITS2 secondary structure alone. Nucleotides cross-linked to $A_3$ factors Cic1 and Nop15 (73) are shaded in blue and magenta, respectively.

First, because L17, L35 and L37 are near each other in the structure of mature 60S subunits (41,42), we wondered whether they were mutually interdependent for assembly. When one r-protein is depleted, do the other two also fail to stably bind to pre-rRNPs? This might explain their common effect on 27SB pre-rRNA processing. Interestingly, we observed very modest effects on the other two proteins, if any, upon depleting each individual r-protein (Figure 9C). This suggests that each of these r-proteins is largely independently required for processing of 27SB pre-rRNA, and that their local rRNP environment is buffered against the absence of each protein.

We recently established the hierarchical recruitment pathway (referred to as the "B pathway") for proteins required for 27SB pre-rRNA processing (hereafter called "B factors") (78). Briefly, B factors (which include scaffolding and RNA-binding proteins Nip7, Mak11, Rpf2, Tif6, Rlp24 and Nsa2; the putative RNA methyltransferase Nop2; the DEAD box proteins/putative RNA helicases (DBP) Dbp10 and Spb4; and

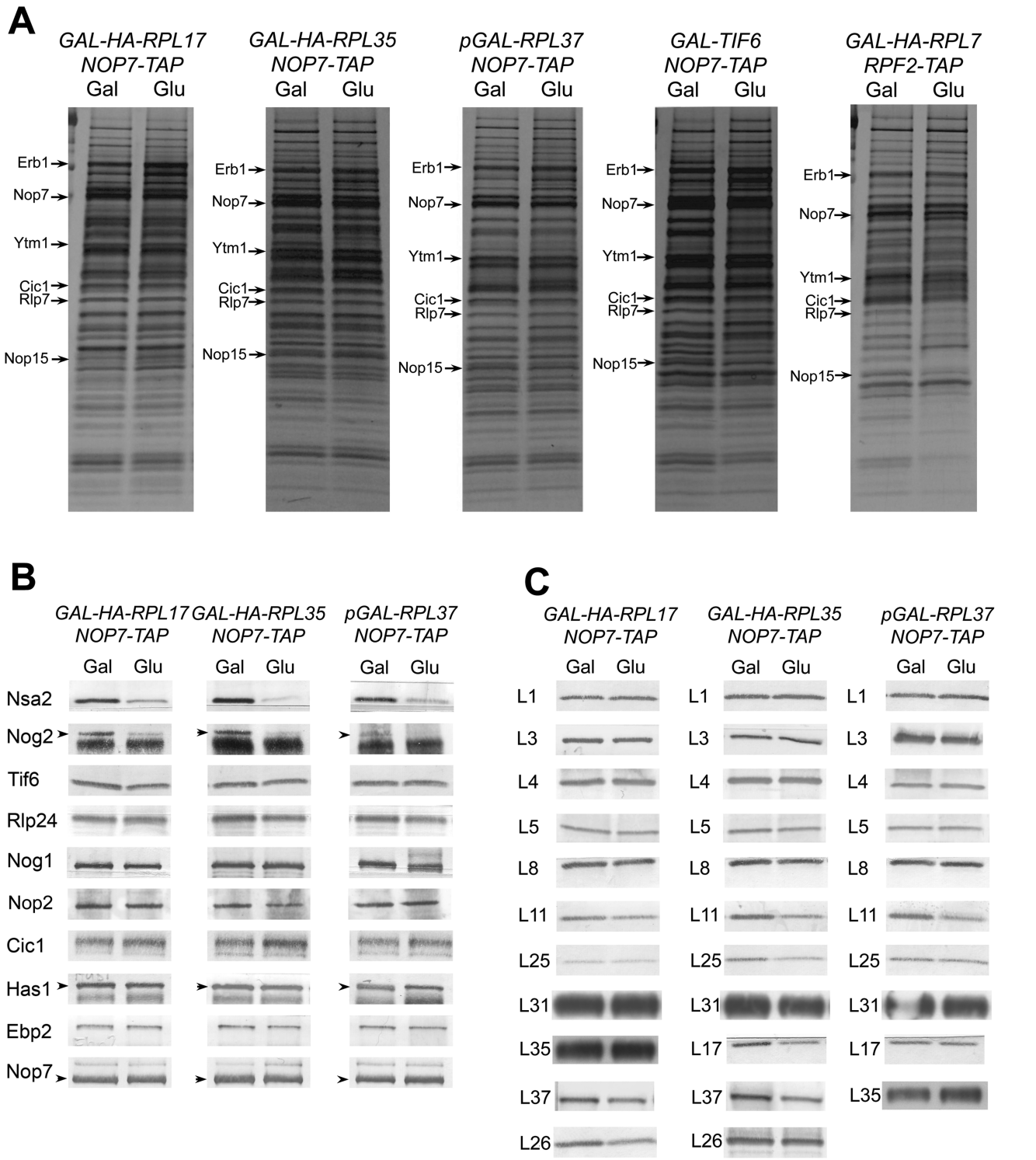


**Figure 9.** Effects of depleting L17, L35 or L37 on pre-ribosome composition. TAP-tagged assembly factors that copurify all pre-60S intermediates were used to affinity-purify pre-ribosomes from conditional mutant strains grown in galactose or shifted from galactose to glucose, to express or repress corresponding r-protein genes under *GAL1* promoter control, respectively. Protein constituents of purified pre-ribosomes were analyzed by SDS-PAGE followed by silver staining and western blotting. (**A**) Depletion of L17, L35 or L37 does not significantly alter the overall pre-ribosome profile. The *GAL-TIF6 RPF2-TAP* (affecting 27SB pre-rRNA processing) and *GAL-RPL7 RPF2-TAP* (affecting $27SA_3$ pre-rRNA processing) are shown as controls for phenotype specificity. (**B**) Two assembly factors required for processing of 27SB pre-rRNA are specifically diminished in mutant pre-ribosomes upon depletion of L17, L35 or L37. Western blotting was used to assay for specific changes in the amount of assembly factors present in wild type compared to mutant pre-ribosomes. (**C**) Levels of r-proteins are largely unaffected in L17, L35 or L37 mutants. The presence of r-proteins in purified pre-ribosomes was assayed by western blotting.

the GTPase Nog1) associate with pre-ribosomes via two parallel recruiting pathways independently required to recruit the GTPase Nog2. We therefore tested the effect of depleting L17, L35 or L37 on recruitment of members of the B pathway into preribosomes. In addition, we also assayed for the association of assembly factors acting on early 60S assembly steps, as well as a subset of r-proteins. The most remarkable observation is that, among the several proteins assayed in these mutants, only two proteins appear to be affected. Amounts of B factors Nsa2 and Nog2 were diminished in pre-ribosomes after depletion of L17, L35 or L37 (Figure 9B). In contrast, other proteins required for 27SB pre-rRNA processing such as the assembly factors Nop2, Tif6, Rlp24 and Nog1 (81–84) (Figure 9B), and r-proteins L5 and L11 (35) (Figure 9C), remain associated with pre-ribosomes. Consistent with a defect in processing of 27SB pre-rRNAs, assembly factors necessary for processing of upstream $27SA_3$ pre-rRNA intermediates [Has1 (85) and J. Dembowski, personal communication; Cic1 (44,86); Nop7 (44,79); Ebp2 (87)] (Figure 9B) are not affected when L17, L35 or L37 are depleted. Other r-proteins tested (L1, L4, L8, L25 and L31) (Figure 9C) are also not affected in these mutants. The effects upon depleting L17, L35 or L37 for 5, 10 and 16 h were qualitatively the same (Supplementary Figure S7). Interestingly, the unaffected proteins that function in 27SB pre-rRNA processing associate with the earliest assembly intermediates, either 90S or $66SA_2$ complexes, whereas Nsa2 and Nog2 join with assembling ribosomes after 27SB pre-rRNA is generated (62,88). Thus L17, L35 and L37 appear to be specifically required for a recruiting step immediately preceding removal of ITS2.

## DISCUSSION

### L17, L35 and L37 may link ITS1 and ITS2 processing events during early stages of 60S subunit maturation

The 5′-end of $5.8S_S$ rRNA, the major form of 5.8S rRNA in eukaryotes, is generated by the exonucleolytic trimming of $27SA_3$ pre-rRNA from the $A_3$ site to the $B_S$ site, forming $27SB_S$ pre-rRNA. This step is facilitated by six interdependent assembly factors with scaffolding functions, three exonucleases, and two RNA helicases [discussed in (39,44); J. Talkish and J. Dembowski, personal communication]. The scaffolding proteins are believed to properly structure $27SA_3$ pre-rRNA and provide a platform for recruitment of r-proteins, whereas the RNA helicases might facilitate conformational changes in pre-rRNPs to enable pre-rRNA processing (44,73) (J. Talkish and J. Dembowski, personal communication). In ribosome assembly mutants affecting the processing of $27SA_3$ pre-rRNAs, r-proteins L17, L26, L35 and L37 that predominantly bind to 5.8S/25S rRNA domain I fail to stably associate with preribosomes (39,44). These adjacent proteins surround the rim of the nascent polypeptide exit tunnel (Figure 2). Therefore, processing of $27SA_3$ pre-rRNA enables proper binding of L17, L26, L35 and L37 to 5.8S/25S domain I, which in turn stabilizes and maintains the resulting pre-rRNP structures (44). Here, we show that similar to L35 (31), L17 and L37 are necessary for the succeeding pre-rRNA processing step, the cleavage of 27SB pre-rRNA at the $C_2$ site in ITS2. The absence of L26 also affects 27SB pre-rRNA processing, albeit mildly (43). It is then conceivable that these four r-proteins may link communication between sequential nucleolytic reactions to remove the ITS1 and ITS2 spacer sequences upstream and downstream of the 5′- and 3′-ends of 5.8S rRNA, respectively. The region of 5.8S/25S domain I where L17, L35 and L37 interact might be particularly unstructured during early assembly steps, but become more structured upon proper binding of these r-proteins. This might be a signal to proceed to the subsequent removal of ITS2.

### L17, L35 and L37 are required for stable association of B factors Nsa2 and Nog2 with pre-ribosomes

What might account for the failure to cleave 27SB pre-rRNA at the $C_2$ site in ITS2? At least two possibilities may explain these pre-rRNA processing phenotypes: (i) the ITS2 spacer sequence where the processing reaction occurs may be misfolded and (ii) assembling pre-rRNPs may lack specific components required to enable catalysis of $C_2$ cleavage. We did not observe changes in ITS2 structure when L17, L35 or L37 are depleted, suggesting that structural changes are occurring elsewhere in 66SB pre-ribosomal complexes.

We began to look at the composition of metastable pre-ribosomes *en route* to degradation in L17, L35 or L37 mutants. This revealed that in the absence of any one of these three r-proteins, there are no apparent differences in the SDS-PAGE profile between wild-type and mutant pre-ribosomes. Consistent with the role of these r-proteins in 27SB pre-rRNA processing, assembly factors

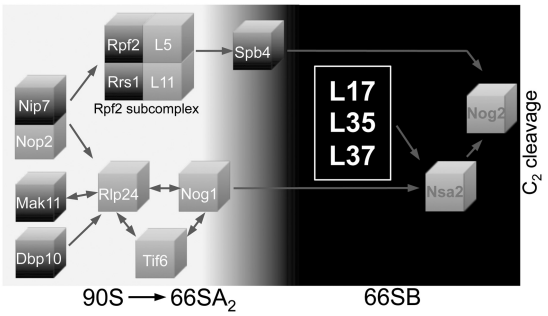


**Figure 10.** L17, L35 and L37 function at a recruiting step immediately preceding removal of ITS2. The hierarchical recruitment of protein factors required for 27SB pre-rRNA processing has recently been established. 12 of the 14 factors required for this processing step associate early with assembling ribosomes, with pre-rRNPs containing either 35S pre-rRNA (90S particles) or $27SA_2$ pre-rRNA ($66SA_2$ particles). Nsa2 and Nog2 associate with pre-60S complexes only after 27SB pre-rRNA is generated, immediately before cleavage at the $C_2$ site in ITS2. Stable association of L17, L35 and L37 with pre-ribosomes is indicated by the gradient from left to right (light grey being the weakest and black being the strongest). B factors tested for association with pre-ribosomes depleted of L17, L35 or L37 are shown in boxes. Among those tested, the only B factors affected by the absence of these r-proteins are Nsa2 and Nog2. The window during 27SB pre-rRNA processing in which L17, L35 and L37 are required is narrowed down right before recruitment of Nsa2 and Nog2.

required for early steps in assembly before 27SB pre-rRNA processing remained unaffected by their absence (Figure 9A and B). Among these are the $A_3$ factors (Ebp2, Has1, Nop7, Erb1, Ytm1, Nop15 and Rlp7) (50,52,78,86,88,89). Further analysis by western blotting confirmed that depletion of L17, L35 or L37 does not significantly affect the association of a subset of r-proteins (L1, L3, L4, L5, L8, L11, L25, L26 and L31) (Figure 9C) with assembling ribosomes. Interestingly, L4, L25, L26 and L31 are in the neighborhood surrounding the polypeptide exit tunnel (Figure 2). Furthermore, L17, L35 and L37 do not appear to be mutually interdependent for association with pre-ribosomes. These results suggest that the polypeptide exit tunnel region is particularly resilient to local disturbances. In support to this claim, 6 out of 11 nonessential proteins from the 60S r-subunit are in this region: L22 (60,91), L24 (60,92,93), L26 (43,60,91), L31 (60,94), L38 (47,60) and L39 (60,92,95) (Figure 2). These effects on r-protein binding could be supported by the fact that nascent polypeptides fold into secondary structures within the exit tunnel, suggesting the inherent conformational flexibility of this structural region (96,97).

The pre-rRNA processing defect in the absence of L17, L35 and L37 is similar to that seen when each of the 14 assembly factors required for 27SB pre-rRNA processing is depleted (36,61,81–84,88,98–103). These B factors are recruited into pre-ribosomes in a hierarchical manner, following two largely independent pathways that come together on Nog2 (78). Although the majority of B factors associate with early pre-rRNPs containing either 35S or $27SA_2$ pre-rRNAs, Nsa2 and Nog2 do not associate with pre-ribosomes until 27SB pre-rRNAs are generated, immediately before cleavage at the $C_2$ site in ITS2 is triggered (61,78,88). Both Spb4, which assembles with early preribosomes, and Nsa2, are necessary to recruit Nog2, and are positioned immediately upstream of Nog2 in the recruiting hierarchy for the B factors (Figure 10). Where are r-proteins necessary for removal of ITS2 from 27SB pre-rRNA positioned in the recruitment pathway? We found that assembly factors Nop2, Tif6, Rlp24, Nog1 (81–84) and r-proteins L5 and L11 (36) are unaffected in the absence of L17, L35 or L37. Thus, we can infer that Nip7, Rpf2, Rrs1, Dbp10 and Spb4 also remain associated with pre-ribosomes under these conditions. Notably, it is clear from our work that Nsa2 and Nog2 are the only B factors affected by the absence of L17, L35 or L37. These r-proteins could then be positioned close to the very end of the B pathway. Their stable association, together with the recruitment of Nsa2 and Nog2, triggers 27SB pre-rRNA processing. We are therefore able to home in on the period during ribosome biogenesis in which the presence of these three r-proteins is required (Figure 10). It is yet unclear whether the nascent polypeptide exit tunnel region is already in its final conformation during this step in the assembly process. However, it is possible that proper structuring of this 60S subunit neighborhood is essential for efficient cleavage at the $C_2$ site in ITS2. Key structural reorganizations are thought to take place during this processing step. 27SB pre-rRNA is the longest-lived pre-rRNA intermediate and its processing requires more DBPs/RNA helicases than any other step in 60S subunit assembly (78). As the last step, prior to release of pre-60S complexes from the nucleolus to the nucleoplasm where the particles are thought to subsequently undergo but a few rearrangements, 27SB pre-rRNA processing might be a structural checkpoint to ensure that exported pre-rRNPs already adopt a conformation that largely resembles mature 60S subunits. Consistent with this idea, it is postulated that nuclear pre-60S complexes *en route* to the cytoplasm are in a configuration analogous to "functionally inactive" ribosomes (7).

Our work suggests that L17, L35 or L37 are independently required to recruit the key B factors Nsa2 and Nog2 to facilitate maturation of 27SB pre-rRNA. A direct physical interaction often is responsible for recruitment of protein factors into preribosomes (36,39,44,83,104). For instance, the association of Nog1 into pre-ribosomes is facilitated by its direct interaction with Rlp24 (83). Similarly, protein–protein interactions among members of the Nop7 subcomplex (Nop7, Erb1 and Ytm1), the Rpf2 subcomplex (Rpf2, Rrs1, L5 and L11) and the Nop2-Nip7 heterodimer are required for their mutual recruitment into pre-rRNPs (36,78,104). Such may not be the case for the recruitment of Nsa2 and Nog2 into pre-ribosomes. Experiments to date fail to establish that they physically interact with L17, L35 or L37 (M. Gamalinda, unpublished). Hence, it is plausible that the presence of each of these r-proteins generates a pre-ribosome topology that enables binding of Nsa2 and Nog2, and the subsequent processing of 27SB pre-rRNA. L22 and L37e, the *H. marismortui* homologs of L17 and L37, respectively, are among the large subunit r-proteins with the highest percentage of their surface area in contact with rRNA (105). Together, L17, L35 and L37 interact with all domains of 5.8S/25S rRNAs, with numerous contact points in each domain (40–42) (Figure 2). In concert with assembly factors, the simultaneous interaction of these r-proteins with multiple rRNA regions may facilitate folding together of different rRNA domains, stabilize resulting pre-rRNP structures, and allow binding of Nsa2 and Nog2. The final loading of the GTPase Nog2 then may catalyze structural rearrangements that trigger processing at the $C_2$ site of ITS2. Alternatively or in addition, depletion of L17, L35 or L37 may affect the association of another protein we have not assayed, which directly recruits Nsa2 and Nog2. Finally, although other B factors remain stably associated with pre-rRNPs even in the absence of L17, L35 or L37, they may not be properly positioned to assume a pre-ribosome conformation competent to recruit Nsa2, Nog2 and/or the yet unknown endonuclease responsible for $C_2$ cleavage in ITS2.

The defect in processing of 27SB pre-rRNA that we observe upon depletion of L17, L35 or L37 could possibly be accounted for (i) entirely by the diminished amounts of Nog2 and Nsa2, (ii) by undetermined changes in pre-rRNP architecture and/or (iii) by unidentified changes in protein composition of pre-60S intermediates. Further analyses need to be carried out to pinpoint structural and pre-ribosomal changes in L17,

L35 and L37 conditional mutants that prevent 27SB pre-rRNA processing. Moreover, identifying where B factors interact with pre-rRNAs or pre-rRNPs would enable us to further understand how r-proteins together with *cis*- and *trans*-acting factors work together to enable processing of 27SB pre-rRNA at ITS2. To fully comprehend this processing step, it is also a big challenge to identify the elusive endonuclease for $C_2$ cleavage or to determine whether this cleavage could be a result of autocatalysis.

### The rate of turnover of pre-ribosomes in the absence of L17, L35 or L37 is slower than in r-protein mutants affecting earlier steps in pre-rRNA processing

Previously, our analysis of L7 and L8 mutants, which affect an early step of pre-rRNA processing, showed substantially rapid turnover of pre-rRNAs (39). In contrast, L17, L35 or L37 mutants exhibit more delayed turnover of unprocessed 27SB pre-rRNA (31) (Figure 5A). These defective pre-rRNA intermediates are presumably targeted for degradation by a surveillance machinery (106–108). At this point, it is not clear how pre-ribosomal particles are detected as being aberrant and what events trigger turnover in assembly mutants. Nevertheless, the general picture that emerges is that gross misfolding of rRNA may lead to a more rapid turnover of assembly intermediates, as seen in the L7 and L8 mutants. Pre-ribosome composition is affected to a far greater extent in the L7 or L8 mutants than in the L17, L35 or L37 mutants. When L7 or L8 are depleted from pre-ribosomes, the association of several proteins is affected: early- and late-assembling B factors, including Nsa2 and Nog2; $A_3$ factors including Nop15 and Cic1 that bind ITS2, and Erb1 and Nop7 that bind 25S rRNA domains I and III, respectively; r-proteins L17, L26, L35 and L37 that primarily bind 5.8S/25S rRNA domain I, in addition to other rRNA domains; and a number of r-proteins adjacent to L7 and L8 that that bind to 25S domain II and I, respectively (39). This suggests that L7 and L8 are necessary to establish the structure of several rRNA neighborhoods, nearby or farther away. The unstructured assembly intermediate in the absence of L7 or L8 may signal instantaneous turnover of pre-rRNAs, via the exosome or the exonuclease Rat1, which is present in preribosomes of L7 or L8 mutants (39). In stark contrast, upon depletion of L17, L35 or L37, thus far only Nsa2 and Nog2 are observed to be decreased in pre-ribosomes, whereas all the other r-proteins and assembly factors that fail to associate with pre-ribosomes in the absence of either L7 or L8 are still present. Strikingly, even adjacent r-proteins in the neighborhood surrounding the polypeptide exit tunnel are unaffected in L17, L35 and L37 mutants. This suggests that fewer pre-rRNA regions may be misfolded, accounting for the slower turnover of aberrant assembly intermediates in L17, L35 or L37 mutants. In this case, the unprocessed ITS2 might be the target for turnover by the exosome. Additionally, the $A_3$ factor Cic1, a known proteasome adaptor (109), which remains associated with ITS2 of unprocessed 27SB intermediates in the absence of L17, L35 or L37, might also signal turnover. Hence, correct timing of the removal of ITS2 might yet be another quality control checkpoint to ensure that aberrant 27SB-containing pre-rRNPs are eliminated.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR online: Supplementary Table 1, Supplementary Figures 1–7, Supplementary Method and Supplementary References [111–113].

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