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Functional analysis of *Saccharomyces cerevisiae* ribosomal protein Rpl3p in ribosome synthesis

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ABSTRACT

Ribosome synthesis in eukaryotes requires a multitude of *trans*-acting factors. These factors act at many steps as the pre-ribosomal particles travel from the nucleolus to the cytoplasm. In contrast to the well-studied *trans*-acting factors, little is known about the contribution of the ribosomal proteins to ribosome biogenesis. Herein, we have analysed the role of ribosomal protein Rpl3p in 60S ribosomal subunit biogenesis. *In vivo* depletion of Rpl3p results in a deficit in 60S ribosomal subunits and the appearance of half-mer polysomes. This phenotype is likely due to the instability of early and intermediate pre-ribosomal particles, as evidenced by the low steady-state levels of $27SA_3$, $27SB_S$ and $7S_{L/S}$ precursors. Furthermore, depletion of Rpl3p impairs the nucleocytoplasmic export of pre-60S ribosomal particles. Interestingly, flow cytometry analysis indicates that Rpl3p-depleted cells arrest in the G1 phase. Altogether, we suggest that upon depletion of Rpl3p, early assembly of 60S ribosomal subunits is aborted and subsequent steps during their maturation and export prevented.

INTRODUCTION

Ribosome biogenesis is a fundamental multistep process that, in eukaryotes, takes place largely within the nucleolus (1). Late steps in both 40S and 60S ribosomal subunit (r-subunit) synthesis occur in the nucleoplasm and after nuclear export of precursor particles in the cytoplasm (2,3). Ribosome synthesis is evolutionarily conserved throughout eukaryotes (4,5), and so far most of our understanding of this process has been obtained from studies with *Saccharomyces cerevisiae* (6,7). In the yeast nucleolus, three of the four rRNAs (18S, 5.8S and 25S) are transcribed as a single large primary transcript by RNA polymerase I and processed to the first detectable rRNA precursor (pre-rRNA), the so-called 35S pre-rRNA. The fourth rRNA (5S) is independently transcribed as a pre-rRNA (pre-5S) by RNA polymerase III. In the 35S pre-rRNA, the mature rRNA sequences are separated by two internal transcribed spacers (ITS1 and ITS2) and flanked by two external transcribed spacers (5′ ETS and 3′ ETS), which must be precisely and efficiently processed to ensure correct formation of mature rRNAs (Figure 1). Maturation of rRNAs is a well-defined pathway (Figure 1) and involves numerous *trans*-acting factors that are required for the processing and covalent rRNA modification reactions, such as small nucleolar RNA–protein (snoRNP) complexes, endonucleases and exonucleases, and different base methylases (6,8). Concomitantly to rRNA maturation, the pre-rRNAs assemble in an ordered manner with the 79 ribosomal proteins (r-proteins) and a large number of *trans*-acting factors that are generally referred to as r-subunit assembly factors (5,9) (for examples of *trans*-acting factors see http://www.medecine.unige.ch/~linder/proteins.html).

The process of r-subunit assembly is still poorly understood. An outline of this process was provided by sucrose density gradient analyses in the 1970s, which identified 90S, 66S and 43S pre-ribosomal particles (3,10,11). Recent advances employing proteomic approaches have revealed several distinct, successive pre-ribosomal particles and refined the model for the maturation of both 40S and 60S r-subunits [for a review (5,9,12)]. These proteomic approaches have also led to the identification of novel non-ribosomal proteins, increasing the number of *trans*-acting factors involved in ribosome biogenesis to over 180. Evidence towards an understanding of the function of many of these *trans*-acting factors has been obtained by using a complete repertoire of techniques, thus, addressing their temporal association with pre-ribosomal particles and revealing the pre-rRNA processing and nucleocytoplasmic export defects caused by their mutational inactivation or depletion [for a review, see (5)].

In contrast to the non-ribosomal proteins, the precise role of the r-proteins in ribosome biogenesis is still largely unexplored and most studies have been focused on their function during translation [for examples, see (13–16) and for a review, see (17)]. Moreover, and paradoxically, the specific presence of r-proteins in pre-ribosomal particles is

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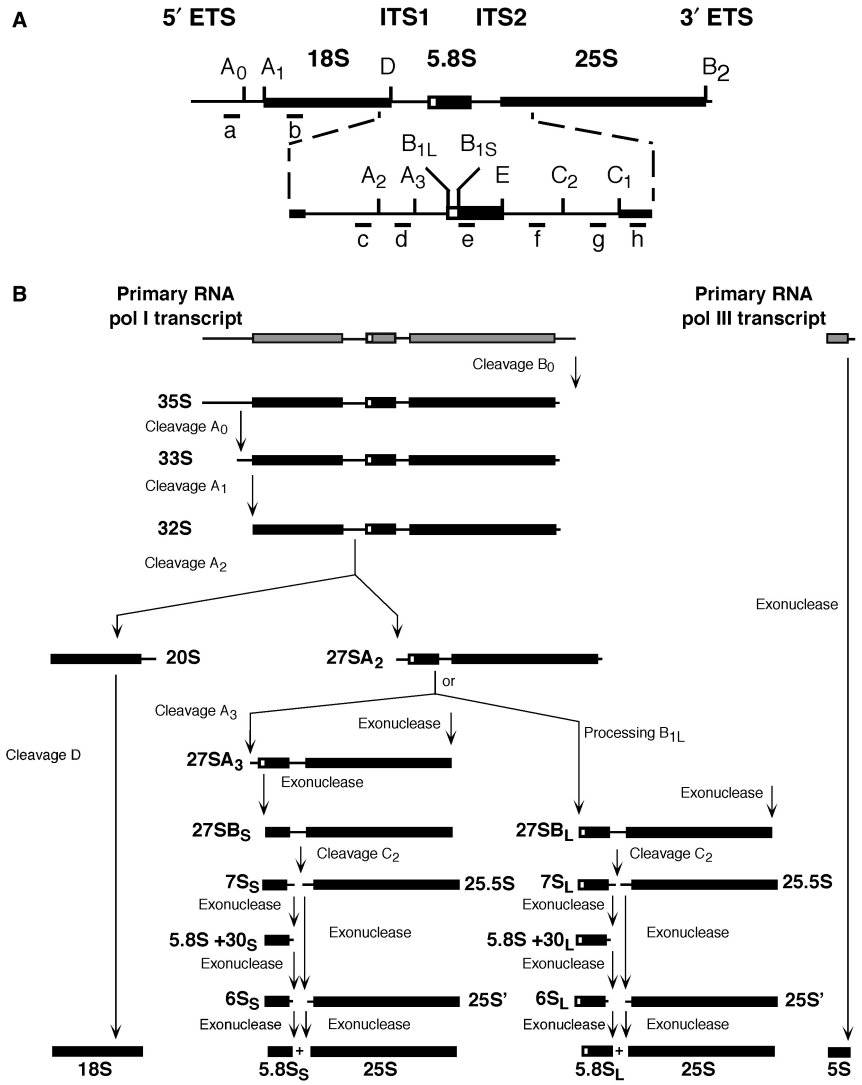

Figure 1. Pre-rRNA processing in *S. cerevisiae*. (**A**) Structure and processing sites of the 35S pre-rRNA. This precursor contains the sequences for the mature 18S, 5.8S and 25S rRNAs that are separated by two internal transcribed spacer sequences, ITS1 and ITS2, and flanked by two external transcribed spacer sequences, 5′ ETS and 3′ ETS. The mature rRNA species are shown as bars and the transcribed spacer sequences as lines. The processing sites and the various probes used are indicated. (**B**) Schematic representation of the pre-rRNA processing pathway of the 35S pre-rRNA and pre-5S rRNA. Cleavage and trimming reactions are indicated. The data presented in this study suggest that Rpl3p is required for stability of the 27S pre-rRNAs. For reviews on pre-rRNA processing and the known processing enzymes, see (6,8).

difficult to properly assign since r-proteins are common contaminants in purified complexes (18). A very recent report has systematically approached the role of individual 40S r-proteins in ribosome synthesis (19). This study revealed that most of the 33 r-proteins of the 40S r-subunit play distinct and essential roles in ribosome maturation and nucleocytoplasmic transport (19). However, an equivalent analysis of the 60S r-subunit proteins has not yet been reported. There are some examples indicating that mutation in or depletion of many 60S r-proteins cause deficits in 60S r-subunits (20–35), however, the contribution to ribosome biogenesis of only few 60S r-proteins has been analysed. So far, there is only detailed functional data available for Rpl5p, which is required for binding and stability of 5S rRNA (27,28), Rpl25p, which is required for efficient pre-rRNA processing at site C_2 (31) and Rpl10p, which is involved in recycling of Nmd3p and subsequent subunit joining (33,36,37).

Rpl3p is required for 60S r-subunit accumulation (20,21) and participates in the formation and proper activity of the peptidyltransferase centre (PTC) (16,38–41). In order to learn more about Rpl3p, we have investigated the effect of genetic depletion of Rpl3p on ribosome maturation and export from the nucleus to the cytoplasm. Our results indicate that Rpl3p is required for the normal accumulation of 60S r-subunits due to defects in pre-rRNA processing of $27SA_3$ and $27SB_{L/S}$ and export of pre-60S r-particles. This suggests that Rpl3p has an essential role in the assembly of early pre-60S r-particles and that aberrant pre-ribosomal particles deficient in Rpl3p are retained in the nucleus. Recently, it has been shown that depletion of human Rpl3p alters proper chromosome

segregation during mitosis (42), a hallmark of most cancer cells (43). In this study, we show that depletion of Rpl3p leads to a G1 arrest, however, it does not seem to interfere with proper chromosome segregation, measured as percentage of plasmid loss.

MATERIAL AND METHODS

Strains, media and genetic manipulations

All yeast strains used in this study are derivatives of strain W303 (*MAT***a**/*MAT*α *ade2-1*/*ade2-1 his3-11,15*/*his3-11,15 leu2-3,112*/*leu2-3,112 trp1-1*/*trp1-1 ura3-1*/*ura3-1 can1-100*/*can1-100*). JDY511 (*MAT*α *rpl3::*HIS3MX6) is a haploid *RPL3* disruptant that requires a plasmid-borne copy of *RPL3* for cell viability (44).

Growth and handling of yeast and standard media were performed by established procedures (45,46). Tetrad dissections were performed using a Singer MS micromanipulator. *Escherichia coli* DH5α strain was used for common cloning and propagation of plasmids (47).

Construction of a *GAL::RPL3* allele and *in vivo* depletion of Rpl3p

The *GAL::RPL3* strain was obtained after transformation of JDY511 [YCplac111-RPL3] with plasmid pZGA196 (a generous gift from G. Adam), which allows expression of *RPL3* under the control of the *GAL1* promoter (48), and subsequent segregation of YCplac111-RPL3. Growth behaviour on YPGal and YPD plates was further studied to test the faithful complementation and the shut-off of the *GAL::RPL3* construct under permissive and non-permissive conditions, respectively. For unknown reasons, the *GAL::RPL3* strain grows better in liquid YPGal medium containing 1 M sorbitol (YPGalS).

For *in vivo* depletion, the *GAL::RPL3* strain was grown in YPGalS medium at 30°C until mid-exponential phase (OD_{600} of 0.8). Cells were harvested, washed and used to inoculate cultures in YPD medium containing 1 M Sorbitol (YPDS). Cell growth was monitored over a period of 48 h, during which the cultures were regularly diluted into fresh YPDS medium to maintain exponential growth. As a control, the wild-type JDY511 [YCplac33-RPL3] strain was used. At different time points, cells were harvested and subsequently used for preparation of total protein and RNA and of cell extracts for polysome analysis.

Sucrose gradient centrifugation

Polysome preparation and analyses were performed as previously described (49) using an ISCO UA-6 system with continuous monitoring at A_{254}.

Protein and RNA extractions, western blotting, northern hybridization and primer extension analyses

Total yeast protein extracts were prepared and analysed by western blotting according to the standard procedures (47). The monoclonal anti-Rpl3p antibody was a gift from J.R. Warner (32).

RNA extraction, northern hybridization and primer extension analysis were carried out according to the standard procedures (50). In all experiments, RNA was extracted from samples corresponding to 10 OD_{600} units of exponentially grown cells and RNA corresponding to equal amounts of cells (ca. 5 μg for the wild-type strain) was loaded on gels or used for primer extension reactions. Sequences of oligonucleotides used for RNA hybridization and primer extension analyses have been described previously (51).

Fluorescence microscopy

To test pre-ribosomal particle export, the appropriate strains (see 'Results' section) were co-transformed with a pRS315 plasmid harbouring a RPL25-eGFP reporter (37) or a RPS2-eGFP reporter (52) and a pRS314 plasmid expressing the nucleolar marker DsRedNOP1 (37). Then, several transformants were grown to mid-log phase in selective liquid medium, washed, and resuspended in water. Acquisition was done in a Leica DMR microscope equipped with a DC camera following the instructions of the manufacturer. Digital images were processed with Adobe Photoshop 7.0.

Cell morphology was studied under the microscope with cells whose nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI).

Flow cytometry

Cells grown in logarithmic phase to an OD_{600} of 0.1 to 0.3 were harvested, fixed with 70% ethanol and DNA was stained with propidium iodide as previously described (53). Stained cells were analysed using a Becton Dickinson FACScan flow cytometer using CELL QUEST software packages to collect and analyse the data (BDIS, San José, CA).

RESULTS

Rpl3p is required for 60S r-subunit maturation

Rpl3p is an essential r-protein in yeast (54) that, in the 1980s, was shown to be required for normal accumulation of 60S r-subunits (20,21). Since then, the reports of Fried and co-workers remained the only information available on the role of this protein in ribosome biogenesis (20,21). To study in detail the function of Rpl3p in ribosome biogenesis, we first assessed steady-state levels of ribosomes upon its depletion. To this end, JDY511 [pZGA196] (*GAL::RPL3* strain) and JDY511 [YCplac33-RPL3] (*RPL3* strain) were grown in liquid YPGalS and shifted to liquid YPDS for different time points. In YPGalS, the growth rate of the *GAL::RPL3* strain was slightly slower than that of the wild-type *RPL3* control strain (doubling time of about 3 and 2 h, respectively), but it even decreased after transfer to YPDS (doubling times of 5.3, 7.6, 9.2 and more than 15 h after 6, 12, 18 and 24 h in YPDS, respectively). Western blot analysis revealed a marked reduction of Rpl3p in *GAL::RPL3* cells that coincided with the decrease in the growth rate in YPDS (data not shown).

Polysome extracts were prepared from cells harvested from the YPGalS cultures and at different times after the shift to YPDS. The *GAL::RPL3* strain showed profiles very similar to those of the *RPL3* strain when grown in YPGalS, although a slight deficit of free 60S versus 40S was reproducibly observed (Figure 2A and C). However, clear alterations in the profiles appeared after 6 h and became more pronounced at longer times in YPDS (Figure 2D and data not shown). The Rpl3p-depleted strain showed a strong decrease in the levels of free 60S r-subunits versus the levels of free 40S r-subunits and an overall decrease in the 80S peak and in polysomes (Figure 2D). In addition, there was an accumulation of half-mer polysomes (Figure 2D). Wild-type cells showed no alteration in the polysome profile when transferred to YPDS (Figure 2B). These results indicate that depletion of Rpl3p leads to a strong deficit in 60S r-subunits relative to 40S r-subunits.

Rpl3p is required for normal pre-rRNA processing

To characterize the basis of the net deficit in 60S r-subunits of the *GAL::RPL3* strain, we then analysed the effect of depletion of Rpl3p on pre-rRNA processing. Total RNA was isolated from *RPL3* and *GAL::RPL3* strains at various time points after transfer from liquid YPGalS to liquid YPDS, and steady-state levels of pre- and mature rRNA species were determined by northern blot and primer extension analyses. Different oligonucleotides hybridizing to defined regions of the 35S pre-RNA were used to monitor-specific processing intermediates (Figure 1A). As shown in Figure 3A, depletion of Rpl3p resulted in a marked decrease in 25S rRNA steady-state levels. This is likely due to an almost complete loss of the 27SB pre-rRNA species, which already becomes apparent at the shortest shift time point to YPDS. In addition, ongoing depletion of Rpl3p led to an accumulation of 35S pre-rRNA and aberrant 23S, 22S and 21S pre-rRNAs. These aberrant species extend from the 5′ end of the 35S pre-rRNA, site A_0 and site A_1 to site A_3, respectively. Hybridizations also identified another fragment, which extends from the 5′ end of the 35S pre-rRNA to site D (Figure 3A). Depletion of Rpl3p also resulted in a mild reduction in the levels of the 20S pre-rRNA and slight reduction in the levels of $27SA_2$ compared to those from the *GAL::RPL3* grown in YPGalS (Figure 3A).

The steady-state levels of low-molecular-weight rRNAs were also studied. As shown in Figure 3B, depletion of Rpl3p caused a strong decrease in the 7S pre-rRNA levels and a very slight reduction in the levels of mature 5.8S and 5S rRNAs, which was clearly noticeable only at the latest shift time points to YPDS. No differences were observed in the ratios of the long and short forms of the 5.8S rRNA.

To determine the levels of $27SA_3$ and distinguish between the $27SB_L$ and $27SB_S$ precursors, we assessed

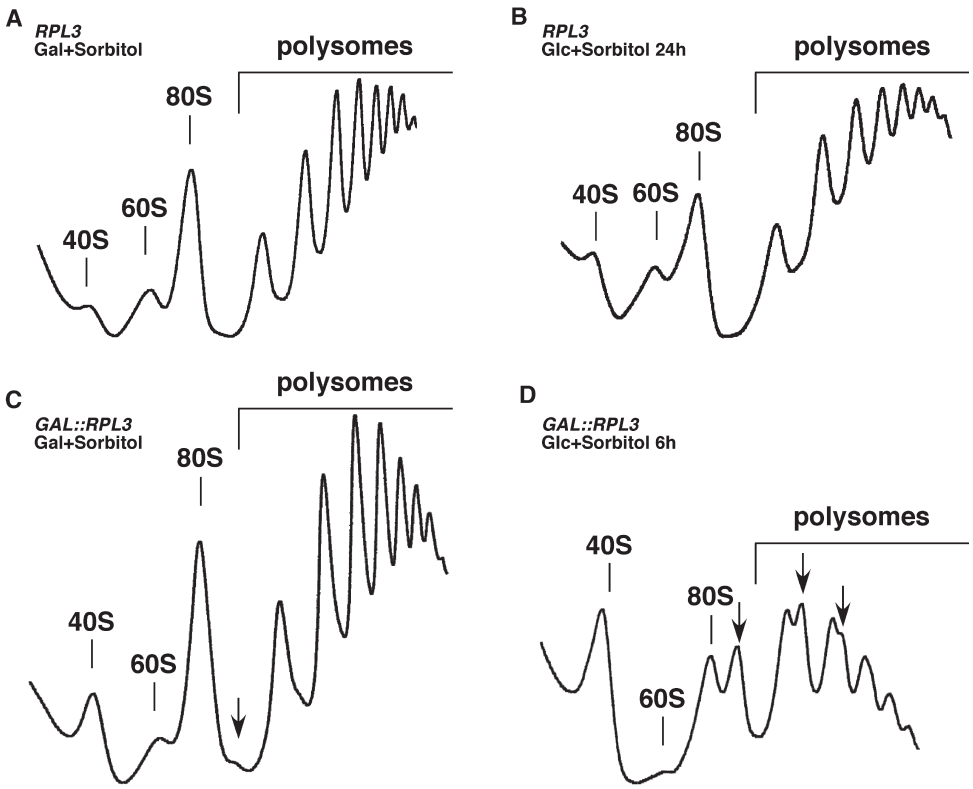

Figure 2. Depletion of Rpl3p results in a deficit in free 60S r-subunits and in the accumulation of half-mer polysomes. Strain JDY511 [YCplac33-RPL3] (*RPL3*) was grown in YPGalS at 30°C (**A**) or shifted to YPDS for 24 h (**B**). Strain JDY511 [pZGA196] (*GAL::RPL3*) was grown in YPGalS at 30°C (**C**) or shifted to YPDS for 6 h (**D**). Cells were harvested at an OD_{600} of 0.8, cell extracts were prepared and 10 A_{260} of each extract were resolved in 7–50% sucrose gradients. The A_{254} was continuously measured. Sedimentation is from left to right. The peaks of free 40S and 60S r-subunits, 80S free couples/monosomes and polysomes are indicated. Half-mers are labelled by arrows.

the levels of these precursors and of the $27SA_2$ pre-rRNA by primer extension using a probe that hybridizes to both the 27SB and 7S pre-rRNAs (probe f, see Figure 1B). As seen in Figure 4, the data are consistent with those from the northern hybridizations. Rpl3p depletion led to a slight decrease in $27SA_2$ pre-rRNA and a more drastic reduction in $27SA_3$ pre-rRNA. Interestingly, the intensity of the stop corresponding to site B_{1S} decreased substantially upon depletion of Rpl3p, whereas that of the stop at site B_{1L} decreased 6 h after transfer to glucose medium but increased to roughly normal levels at later time points. Similar results were observed when using a probe that hybridizes only to the 27SB pre-rRNAs (probe g; data not shown). Thus, these results indicate that $27SB_S$ was primarily affected upon depletion of Rpl3p.

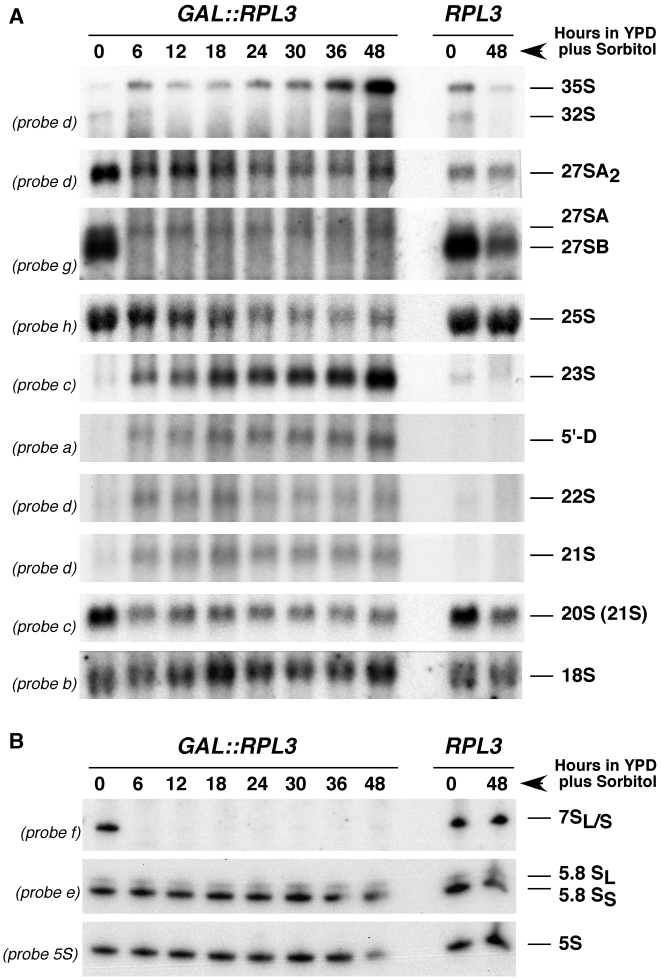

Figure 3. Effects of Rpl3p depletion on steady-state levels of pre-rRNAs and mature rRNAs. Strains JDY511 [YCplac33-RPL3] (*RPL3*) and JDY511 [pZGA196] *(GAL::RPL3)* were grown in YPGalS medium and then shifted to YPD medium. Cells were harvested at the indicated times and total RNA was extracted. (**A**) RNA corresponding to equal amounts of OD_{600} units of cells were resolved on a 1.2% agarose–formaldehyde gel, transferred onto a nylon membrane and hybridized consecutively with different probes. (**B**) RNA corresponding to equal amounts of OD_{600} units of cells was resolved on a 7% polyacrylamide–urea gel, transferred onto a nylon membrane and hybridized consecutively with different probes. Probe names are indicated between parentheses (see Figure 1A for their location in the 35S pre-rRNA).

Altogether, our results indicate that depletion of Rpl3p has a major impact on processing of $27SA_3$, which then also leads to underaccumulation of the $27SB_S$ pre-rRNA. In addition, Rpl3p depletion affects negatively ITS2 processing events; the low levels of $27SB_S$ may account for the diminution of $7S_S$ pre-rRNAs upon depletion, however, the alternative form $27SB_L$ continues to be synthesized but fails to be converted to $7S_L$ pre-rRNA. Finally, depletion of Rpl3p inhibits or delays processing at sites A_0–A_2, which leads to the accumulation of normal 35S pre-rRNA and aberrant 23S, 22S and 21S pre-rRNAs. The appearance of these aberrant rRNAs and the accumulation of the 5′-D fragment indicate that processing events in the 5′-ETS and ITS1 do not occur in the normal order following depletion of Rpl3p.

Rpl3p depletion impairs export of pre-60S r-particles from the nucleus to the cytoplasm

To determine whether the depletion of Rpl3p impairs nuclear export of pre-60S r-particles, we first analysed the localization of the 60S reporter construct Rpl25p-eGFP (37) in wild-type and *GAL::RPL3* strains.

Under permissive conditions (YPGalS medium), Rpl25p-eGFP was found predominantly in the cytoplasm in both strains. However, following a shift to non-permissive conditions (YPDS medium) for as short as 6 h, Rpl25p-eGFP accumulated in the nucleus in about 30% of the *GAL::RPL3* cells. This phenotype was more evident after 12 h in YPDS since around 70–90% of the cells showed a nuclear accumulation of Rpl25-eGFP (Figure 5). In many cells, we observed a very bright fluorescence signal for Rpl25-eGFP that was not restricted to the nucleolus, which was detected with the nucleolar marker DsRed-Nop1p. We did not observe nuclear accumulation of the Rpl25p-eGFP reporter in the wild-type *RPL3* control strain grown in YPDS (data not shown).

We conclude that normal and/or aberrant pre-60S r-particles accumulate in the nucleus upon depletion

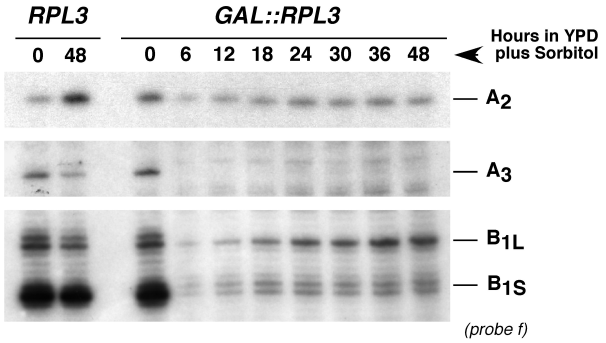

Figure 4. Effects of Rpl3p depletion on steady-state levels of 27S pre-rRNAs species. The same RNA samples described in the legend of the Figure 3 were used for primer extension analysis. Probe f (see Figure 1A for its location in the 35S pre-rRNA) was labelled and used for the reactions. Note that this probe allows detection of $27SA_2$ (as the stop at site A_2), $27SA_3$ (as the stop at site A_3), 27SB and 7S pre-rRNAs (as stops at sites B_{1L} and B_{1S}).

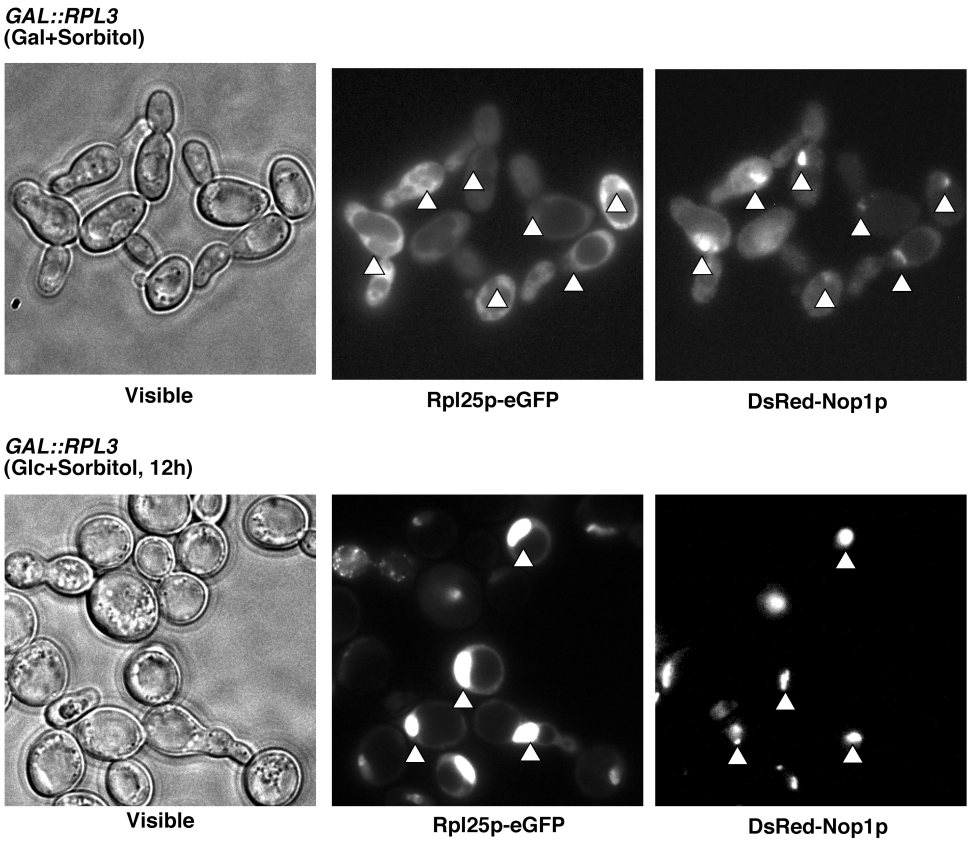

Figure 5. Depletion of Rpl3p leads to nuclear retention of the 60S r-subunit reporter Rpl25p-eGFP. The *GAL::RPL3* strain carrying the plasmids pRS315-Rpl25p-eGFP and pRS314-DsRed-Nop1p were grown in SGalS-Leu-Trp medium (Gal + Sorbitol) to early log phase and shifted for 12 h to SDS-Leu-Trp (Glc + Sorbitol). The subcellular localization of the Rpl25p-eGFP and DsRed-Nop1p was analysed by fluorescence microscopy. Triangles indicate the position of the nucleolus.

of Rpl3p. This phenomenon is specific for the large r-subunit, since no accumulation of pre-40S r-particles was observed when we studied the localization of the 40S r-subunit reporter Rps2p-eGFP (52) (data not shown)

Depletion of Rpl3p leads to defects in cell cycle and morphology

Our previous results (55) and unpublished observations (I.V.R., unpublished results) as well as results from Wozniak and co-workers (56) indicate that Rpl3p interacts functionally and physically with the WD-repeat Rrb1p protein, which has been suggested to act as the Rpl3p assembler onto pre-60S r-particles (55,56). Rrb1p has also been shown to be required for the metaphase/anaphase transition during the cell cycle and proper chromosome segregation (42). Rrb1p also functionally interacts with the origin recognition complex component Orc6p, involved in the initiation of DNA replication (42,57), and the yeast Pescadillo complex, which consists in yeast of Nop7p, Erb1p and Ytm1p and is required for both ribosome biogenesis and normal progression through the S phase of the cell cycle (42,58). Moreover, inactivation of human orthologues of Rrb1p, Nop7p, Erb1p/Bop1p, Orc6p and Rpl3p alters proper chromosome segregation (42). To study whether yeast Rpl3p is required for optimal progression through the cell cycle, we first examined cellular morphology of *GAL::RPL3* cells by light microscopy. A normal morphology was observed for most cells when grown in galactose medium (Figure 6A). However, 6 h after transfer to glucose medium, *GAL::RPL3* cells increased in size and a significant percentage (about 5%) showed an elongated shape and contained enlarged buds with pronounced apical growth. Apparently, these elongated cells contained duplicated, separated nuclei as shown by DAPI staining (Figure 6A).

Then, we performed fluorescence-activated cell sorting (FACS) analyses with yeast cultures of the *GAL::RPL3* and *RPL3* strains in early logarithmic phase. In asynchronous wild-type *RPL3* cells, we detected two peaks corresponding to cells with unreplicated (1C) and duplicated (2C) genomes, with the 1C peak being slightly higher than the one of 2C. A similar pattern was observed for the *GAL::RPL3* cells grown in galactose medium. However, 6 h after transfer to glucose medium, more *GAL::RPL3* cells remained 1C compared to the *RPL3* strain, most likely due to an arrest or delay in the transition through the G1 phase of the cell cycle (Figure 6B).

We conclude that depletion of Rpl3p causes a severe delay in the progression through the G1 phase of cell cycle.

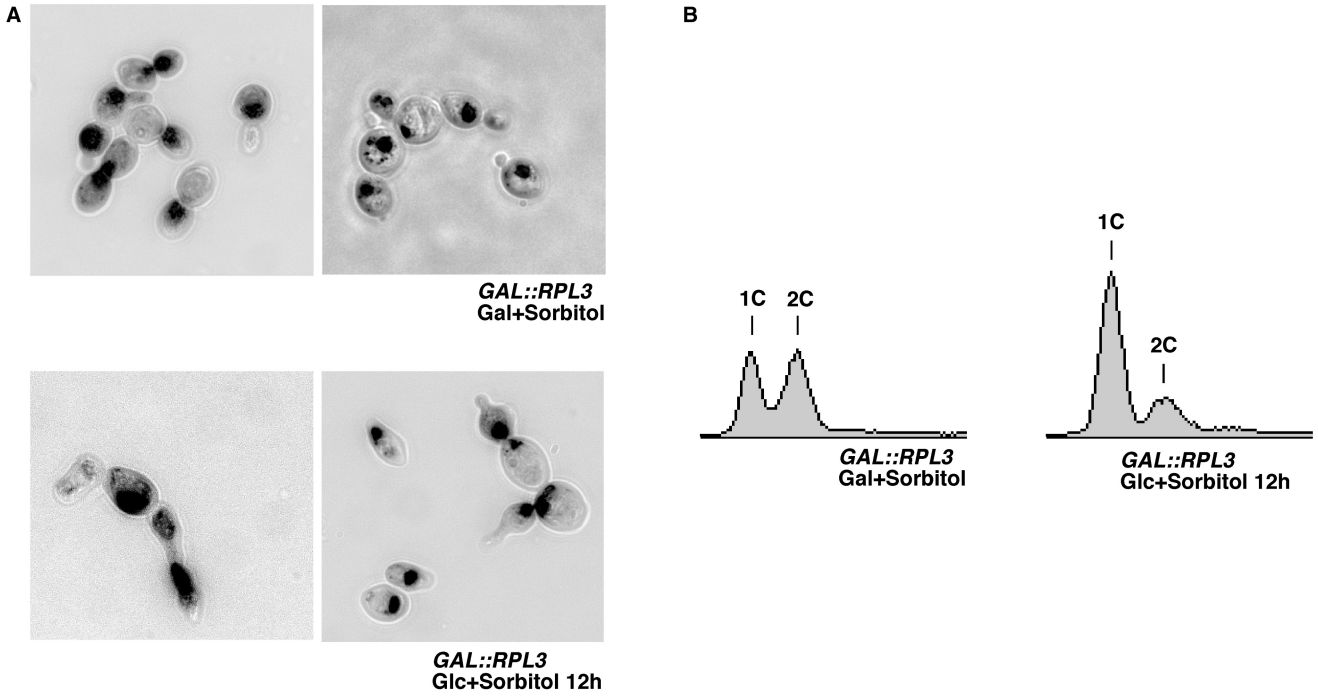

Figure 6. Depletion of Rpl3p leads to an arrest of the cell cycle at the G1 phase and an abnormal cell morphology. (**A**) Cell morphology of *GAL::RPL3* cells grown in YPGalS (Gal + Sorbitol) or shifted for 12 h to YPDS (Glc + Sorbitol). Cells were stained with DAPI for localization of nuclei and then visualized by fluorescence and phase contrast microscopy. Merged images are shown. (**B**) FACS analysis of unsynchronized *GAL::RPL3* cells grown in YPGalS or shifted for 12 h to YPDS at 30°C. 1C and 2C peaks correspond to cells with unreplicated and duplicated genomes, respectively.

DISCUSSION

In this work, we report the functional characterization of Rpl3p in ribosome biogenesis. Rpl3p, which is the largest r-protein (387 residues in *S. cerevisiae*; 43.7 kDa), is evolutionarily conserved in both sequence and structure in eukaryotes, eubacteria and archea (59,60). Examination of the structure of Rpl3p reveals that it contains two tightly packed globular domains and two extensions (61). As discussed by Dinman and co-workers (16,38,41), the globular domains are located on the solvent side of the 60S r-subunit and bind to the domain VIA of yeast 25S rRNA or bacterial 23S rRNA (59), very close to the site where the ribosome interacts with the elongation factors eEF1 and eEF2. The extensions anchor Rpl3p to the central core of the 25S rRNA. One of the extensions is at the N-terminus of the protein (residues 10 to 24) and the other is internal to the protein (residues 217 to 278). The latter extension comes very close to the PTC where it can stabilize the surrounding rRNA (59).

Rpl3p has been extensively studied with respect to its role in translation, more specifically as an important functional component of the PTC (16,38–41). In bacteria, Rpl3p is one of the few proteins essential for the PTC activity (62). In yeast, the first identified *rpl3* mutations conferred resistance to the PTC inhibitors trichodermin and anisomycin (63) and inability to maintain the M_1 killer virus (64). More recently, it has been shown that these mutations also affect the efficiency of programmed -1 ribosomal frameshifting due to a decrease in the PTC activity (16,40). In contrast to its characterization in translation, very little is known about the role of Rpl3p in ribosome biogenesis. To our knowledge, pre-rRNA processing has not been studied upon loss-of-function of bacterial Rpl3p. In yeast, the depletion of Rpl3p leads to a net deficit of 60S r-subunits [(21,54) and our results of Figure 2], and although pulse-chase experiments have been performed, they were not of enough resolution to assess the kinetics of pre-rRNA processing (21). In this paper, we describe the role of yeast Rpl3p in pre-rRNA processing. Northern blot and primer extension analyses indicate that there is a drastic reduction in the steady-state levels of almost all 27S pre-rRNAs and both 7S pre-rRNAs upon depletion of Rpl3p. As a consequence, there is an underaccumulation of mature 25S rRNAs. However, the levels of 5.8S and 5S rRNA were only mildly affected at late time points of depletion. This is in agreement with the common observation that 5.8S rRNA behaves more stable than 25S rRNA upon depletion of many *trans*-acting factors required for 60S r-subunit biogenesis [for examples, see (49,65)] and the published data indicating that 5S rRNA forms a stable RNP with the 60S r-protein Rpl5p (28). Intriguingly, primer extension analysis shows that the levels of $27SB_L$ pre-rRNA do not change significantly upon depletion of Rpl3p. Since it is likely that Rpl3p may not have a direct role in pre-rRNA processing reactions, we assume that the depletion of Rpl3p leads to abortive assembly of early pre-60S r-particles, which entails destabilization and degradation of the $27SA_3$ pre-rRNA and its immediate products 27SB

and 7S pre-rRNAs. The almost constant steady-state levels of $27SB_L$ pre-rRNA upon Rpl3p depletion might reflect changes in the relative degradation rate of aberrant pre-60S r-particles containing this precursor but deprived of Rpl3p. These aberrant pre-60S r-particles, which should contain the 60S r-protein Rpl25p properly assembled, might be defective for nucleocytoplasmic transport, as suggested by the retention of the Rpl25p-GFP in the nuclei of Rpl3p-depleted cells. This defect is apparently specific as export of small r-subunits was unaffected. Furthermore, our northern analyses clearly indicate that depletion of Rpl3p causes a decrease in the efficiency of processing at the early cleavage sites A_0, A_1 and A_2, thereby slightly affecting the levels of mature 18S rRNA and its 20S precursor. As a consequence, a 22S, a 21S and more abundantly a 23S aberrant pre-rRNA accumulated. This type of defect in 18S rRNA synthesis is a general feature of mutations that interfere with the synthesis of mature 25S and 5.8S rRNA (6). It has been proposed that these pre-rRNA processing defects arise from inefficient recycling of *trans*-acting factors that improperly disassemble from defective pre-60S r-particles (5,66). Finally, there is an accumulation of a 5′ ETS-D fragment, suggesting that the aberrant 23S pre-rRNA can be processed to site D upon depletion of Rpl3p. In general, point mutations in Rpl3p cause similar but much weaker pre-rRNA processing defects than its depletion (I.V.R., unpublished result). This finding suggests that the inability to incorporate Rpl3p has a more dramatic effect on the fate of pre-60S r-particles than the incorporation of functionally hampered Rpl3p mutant variants.

When and how is Rpl3p assembled? The ribosome assembly process is very difficult to assess experimentally and is not very well understood. In bacteria, it has been possible to reconstitute *in vitro* functional r-subunits from isolated mature rRNAs and purified r-proteins (67,68). These studies indicate that Rpl3p is amongst one of the first r-proteins that initiate *in vitro* assembly. In clear agreement with this fact, Rpl3p is present on the so-called p_150S precursor particles *in vivo* [for a review, see (69)]. In eukaryotes, since there is no *in vitro* ribosome self-assembly system from their components, the order of assembly of r-proteins into pre-ribosomal particles has not been characterized. *In vivo*, pulse-chase studies have suggested that yeast Rpl3p associates at a relatively early stage of the ribosomal maturation process (70). The purification of Rpl3p within early 66S pre-ribosomal particles E_0, E_1 and E_2 is in agreement with these results (71–74).

Rpl3p is one of several extension-containing r-proteins (75). Steric considerations require that these proteins bind rRNA at a stage prior to the formation of significant ternary structure. Steitz and co-workers have hypothesized that during assembly, the globular domains of bacterial Rpl3p bind first to sequences of domain VI of 23S rRNA, which adopt a structure similar to the final one present in the mature 50S r-subunit (59). This binding is strong and stabilizes the protein on the rRNA. Then, the extensions of Rpl3p, which depend on interactions with the surrounding rRNA to properly fold [for a real example, see (76)], bind sequentially to regions of internal rRNA domains to be accommodated inside the r-subunit and gain a stable structure (59,75). Since the overall structure of Rpl3p and its location in the large r-subunit is highly conserved between eubacteria, archaea and eukaryotes (60), we can imagine a similar mode of assembly for the yeast Rpl3p in early pre-60S r-particles. We have recently isolated nine independent recessive *rpl3* mutations, which are synthetically lethal with a subset of *trans*-acting factors involved in early steps in the synthesis of 60S r-subunits including the putative RNA helicase Dbp6p, the nucleolar protein Rsa3p and the putative Rpl3p assembler Rrb1p [(44,51,77) and I.V.R., unpublished results]. Further studies, using these above mutants as well as directed mutants where the extensions and the globular domains have been specifically altered, should help to dissect the contribution of the different Rpl3p domains to early 60S ribosome biogenesis events and to get insight into the mode of assembly of Rpl3p in pre-60S r-particles.

Finally, we herein describe that depletion of yeast Rpl3p leads to a G1 delay or arrest of the cell cycle, which is accompanied by a percentage of cells with abnormal cell morphology. In yeast, cell-cycle defects have been previously described for mutant or depleted strains in other ribosome biogenesis factors. In these cases, cell-cycle progression is impaired not only at the G1 phase but throughout the different stages of the cell cycle (42,57,78–84). The possible involvement of Rpl3p in cell cycle has been studied in other organisms; in zebrafish, while haplo-insufficiency in many r-proteins genes predispose to cancer, that in *RPL3* gives rise to similar tumour incidence as for a control line (85). On the other hand, transient depletion of human Rpl3p increases the percentage of abnormal mitosis and alters proper chromosome segregation (42). Interestingly, mutation in yeast Rrb1p arrests cell cycle at the G2/M phase by blocking mitosis and inducing chromosome instability, and transient depletion of GRWD, the human orthologue of Rrb1p, results in an increase of abnormal mitosis and an alteration in chromosome segregation (42). However, our initial experiments, using a centromeric-plasmid loss assay (86), suggest that *rpl3* mutation, at least the alleles we have tested, does not lead to defects in plasmid replication and maintenance (I.V.R., unpublished results). Further work is clearly needed to better understand the role of Rpl3p in ribosome biogenesis and clarify its putative link to cell-cycle progression.

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