Analysis of ribosome biogenesis factor-modules in yeast cells depleted from pre-ribosomes

Juliane Merl, Steffen Jakob, Katrin Ridinger, Thomas Hierlmeier, Rainer Deutzmann, Philipp Milkereit* and Herbert Tschochner*

Institut für Biochemie, Genetik und Mikrobiologie, University of Regensburg, Universitätsstr. 31, 93053 Regensburg, Germany

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ABSTRACT

Formation of eukaryotic ribosomes requires more than 150 biogenesis factors which transiently interact with the nascent ribosomal subunits. Previously, many pre-ribosomal intermediates could be distinguished by their protein composition and rRNA precursor (pre-rRNA) content. We purified complexes of ribosome biogenesis factors from yeast cells in which de novo synthesis of rRNA precursors was down-regulated by genetic means. We compared the protein composition of these largely pre-rRNA free assemblies with the one of analogous pre-ribosomal preparations by semi-quantitative mass spectrometry. The experimental setup minimizes the possibility that the analysed pre-rRNA free protein modules were derived from (partially) disrupted pre-ribosomal particles and provides thereby strong evidence for their pre-ribosome independent existence. In support of the validity of this approach (i) the predicted composition of the analysed pre-rRNA free protein modules were derived from (partially) disrupted pre-ribosomal particles and provides thereby strong evidence for their pre-ribosome independent existence. In support of the validity of this approach (i) the predicted composition of the analysed protein modules was in agreement with previously described rRNA-free complexes and (ii) in most of the cases we could identify new candidate members of reported protein modules. An unexpected outcome of these analyses was that free large ribosomal subunits are associated with a specific set of ribosome biogenesis factors in cells where neo-production of nascent ribosomes was blocked. The data presented strengthen the idea that assembly of eukaryotic pre-ribosomal particles can result from transient association of distinct building blocks.

INTRODUCTION

Eukaryotic ribosomes are composed of four ribosomal RNAs (rRNA) and about 80 ribosomal proteins. In addition to these structural ribosomal components, ribosome biosynthesis requires more than 150 non-ribosomal proteins and many non-coding, small RNAs. Transcription of the DNA coding for rRNA (rDNA) by RNA polymerase I (Pol-I) results in an rRNA precursor (pre-rRNA) which is bound by ribosomal proteins, processed by several endo- and exonucleases and folded into its final conformation. Processing of pre-rRNAs and assembly steps go along with multiple changes in the set of ribosome biogenesis factors associated with pre-rRNAs. Therefore, different ribosomal precursors can be distinguished by their content of pre-rRNAs and associated factors (1–7).

Assembly of about 40 ribosome biogenesis factors, U3 snoRNA, pre-rRNA and ribosomal proteins seems to occur already during synthesis of the primary rRNA transcript and results in the formation of the small subunit (SSU) processome, also referred to as 90S pre-ribosome (8–10). In yeast, SSU-processome components are involved in early pre-rRNA cleavages at processing sites A0 and A1 to remove the 5’external transcribed spacer (5’ETS) and in the cut at A2 within the internal transcribed spacer (ITS1) (8), leading to the two maturation branches in which biogenesis of the SSU and LSU (large subunit) further proceeds (11). Only a few of the SSU-processome associated proteins, like Enp1p, do not dissociate from nuclear SSU pre-rRNA processed at sites A0, A1 and A2. Therefore, Enp1p associates with nuclear RNPs containing the 35S pre-rRNA and SSU processome components as well as with SSU precursors which are exported to the cytoplasm and contain 20S pre-rRNA and factors involved in late steps of SSU biogenesis (1,12).
Among them are the serine kinase Rio2p, the putative endonuclease Nob1p and Ltv1p, all of which are required for removal of the 3’ region of 20S pre-rRNA leading to mature 18S rRNA containing SSUs (13–16).

Many of the more than 50 LSU biogenesis factors assemble transiently after cleavage of the ITS1 at site A2. A complex series of pre-rRNA processing events follows, in which interactions of many non-ribosomal proteins with the nascent pre-rRNA are required to generate finally the mature 5S, 5.8S and 25S rRNA containing LSU. First, 27SA2 pre-rRNA containing RNP are formed. Then, after maturation of the 5’ end of 5.8S rRNA precursors at site B1 involving Nop7p (17), 27SB1 containing particles are generated (2). 27SB pre-rRNA containing complexes include factors which are probably directly involved in cleavage of 27SB pre-rRNA at site C2 in the internal transcribed spacer region 2 (ITS2) resulting in the separation of 5.8S and 25S pre-rRNAs. After cleavage in the ITS2 proteins involved in further nuclear trimming of 5.8S and 25S pre-rRNA, among them Rix1p (18), and proteins which accompany or guide the LSU through the nuclear pore associate with the LSU precursors. Finally, before entry into mRNA translating polysomes the nascent LSUs undergo cytoplasmic maturation steps which include the release of several biogenesis factors, e.g. Arx1p, Tif6p and Nmd3p (19–21).

Indications for the rather complex protein composition of different pre-ribosomal particles came mainly from mass spectrometry based identification of ribosome biogenesis factors which co-purified from yeast cell extracts on affinity matrices (1–4,7,8,22–24). One systematic approach was to apply the tandem affinity purification procedure (TAP) on TAP tagged ribosome biogenesis factors (25) in buffer conditions which are thought to preserve some, but not all aspects of RNP architecture. Some smaller pre-rRNA free subcomplexes of ribosome biogenesis factors could be enriched from yeast cell extracts prepared by cell lysis using glass beads, were washed twice for 15 min with 2°C SSC at 30°C and loaded on denaturing agarose gels and analysed by northern blot. Hybridization was performed in 50% formamide; 5 × SSC; 0.5% SDS; 5 × Denhards solution at 30°C with the following 32P-labelled probes: # 205 (18S); 5’-CATGGCTTAATCTTTGAGAC-3’; # 212 (25S); 5’-CTCCGCTTATTGATATGC-3’. The blots were washed twice for 15 min with 2 × SSC at 30°C. Labelled rRNA signals were detected using a Phosphor Imager FLA3000 (Fujifilm). Data were quantified using MultiGauge V3.0 (Fujifilm).

Protein detection by western blot analysis
Expression levels of TAP-tagged biogenesis factors in yeast strains CG379 and YCC95 at 37°C were determined by western blot analysis. Same amounts of whole cell extracts, prepared by cell lysis using glass beads, were analysed using PAP visualization reagent (DakoCytomation, Z 0113) in a dilution of 1:3000 for detection of the TAP-Tag. For detection of rpS8 a rabbit anti-rpS8 antibody was diluted 1:2000 (G. Dieci). Protein signals were visualized by chemiluminescence.
using a Fluorescence Image Reader LAS3000 (Fujifilm). Data was quantified using MultiGauge V3.0 (Fujifilm).

**Fluorescence microscopy**

Temperature shifts of logarithmically growing yeast cells and in situ detection of tagged proteins were performed as described (35). Totally 0.1 × volume of 37% formaldehyde (methanol stabilized) was added to cultures and fixation of cells was performed for 1 h at 37°C. Spheroblasting of cells was done in 0.1 M potassium phosphate buffer pH 7.5 for 45 min at 30°C using 50 μg/ml zymolyase T100 (Seikagaku Corporation). Fixed spheroplasts were put on poly-L-lysine treated three-well diagnostic slides (Menzel–Glasier), blocked with 2% BSA in 1xTBS/0.1%NP40 and treated with the following antibodies in 1×TBS/0.1% BSA: rabbit anti-Protein A (Sigma, P-3775) in a dilution of 1:50 000 and mouse anti-Nop1p (Abcam, ab4575) in a dilution of 1:1000. For fluorescence detection the secondary antibodies Alexa Fluor 594 goat anti-rabbit (Molecular Probes, A-11012) and Alexa Fluor 488 goat anti-mouse (Molecular Probes, A-11017) were used in dilutions of 1:500 in 1%BSA: rabbit anti-Protein A (Sigma, P-3775) in a dilution of 1:1000. For fluorescence detection the secondary antibodies Alexa Fluor 594 goat anti-rabbit (Molecular Probes, A-11012) and Alexa Fluor 488 goat anti-mouse (Molecular Probes, A-11017) were used in dilutions of 1:500 in 1%BSA: rabbit anti-Protein A (Sigma, P-3775) in a dilution of 1:1000. For fluorescence detection the secondary antibodies Alexa Fluor 594 goat anti-rabbit (Molecular Probes, A-11012) and Alexa Fluor 488 goat anti-mouse (Molecular Probes, A-11017) were used in dilutions of 1:500 in 1%BSA: rabbit anti-Protein A (Sigma, P-3775) in a dilution of 1:1000.
DAPI in Moviol-solution [0.1 μg/ml Moviol (Hoechst) in 25% Glycerin/0.1 M Tris pH 8.5].

Images were captured with an AxioCam MR CCD camera mounted on an Axiovert 200M Zeiss microscope and processed with Axiovision V 4.7.1.0 and Adobe Photoshop.

Protein affinity purification experiments

Growth and temperature shifts of yeast strains (Table 1) were performed as indicated. Two to six litres of cell culture (400 ml culture for small scale RNA analysis) were harvested and whole cell extracts were prepared in 1 volume of lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, 0.15% NP-40, 1 mM PMSF, 2 mM benzamidine). An equal volume of glass beads (Ø 0.75–1 mm) was added and cells were lysed at 4°C in a bead mill (pulverisette 6/Fritsch, for small scale Vibrex/IKA). After centrifugation in a Ti45 rotor for 30’ with 35 000 rpm (small scale: 2 × 10’ in a table top at 14 000 rpm) the cleared lysate was incubated with 300 μl (small scale: 50 μl) of equilibrated IgG–Sepharose (Amersham) for 2 h at 4°C. Beads were washed five times with 2 ml and once with 10 ml of lysis buffer. Affinity purification experiments using Dynabeads® Pan Mouse IgG (Invitrogen, Cat. No.11041) were performed essentially the same as with IgG-Sepharose, using buffer A200 (20 mM Tris–HCl, pH 8, 200 mM KCl, 5 mM MgAc, 1 mM DTT, 0.5% Triton X-100, 0.1% Tween 20).

RNA was extracted from beads and extracts as described (36). For immunological detection of Protein A tagged proteins samples from beads and extracts were taken and analysed by western blot using PAP visualization reagent (DakoCytomation, Z 0113) in a dilution of 1:3000.

For comparative quantifications of proteins contained in affinity purified complexes TAP-tagged proteins bound to beads were eluted using AcTEVTM Protease (Invitrogen) for 2 h at 16°C and precipitated with methanol/chloroform.

Comparative quantification of proteins purified with TAP–tagged biogenesis factors

Protein samples were lyophilized, resuspended in 20 μl dissolution buffer (iTRAQTM labelling kit, Invitrogen) and reduced with 5 mM Tris–(2-carboxyethyl)phosphatse at 60°C for 1 h. Cysteins were blocked with 10 mM methylmethanethiosulfonate (MMTS) at room temperature for 10 min as described previously (37,38). After trypsin digest for 20 h at 37°C, tryptic peptides of the purifications of interest were labelled with different combinations of the four iTRAQ reagents according to the manufacturer (Invitrogen). Pairs of labelling reactions were combined and lyophilized.

The combined differently labelled peptides were dissolved for 2 h in 0.1% TFA and loaded on a nano-flow HPLC-system (Dionex) harbouring a C18-Pep-Mep column (LC-Packings). The peptides were separated by a gradient of 5% to 95% of buffer B (80% acetonitrile/0.05% TFA), fractions were mixed with five volumes of CHCA (alpha-cyano-4-hydroxy cinnamic acid; Sigma) matrix (2 mg/ml in 70% acetonitrile/0.1% TFA) and spotted on a MALDI-target. Spotted fractions were analysed by a 4700 series MALDI-TOF/TOF-system (Applied Biosystems). The six most intense peptide peaks per spot detected in the MS mode were further fragmented yielding the respective MSMS spectra. Measured m/z ratios were assigned to peptides and respective proteins by Mascot database search in a yeast protein database. Only proteins identified by at least two non-redundant peptides with a Confidence Interval >95% were included in the analysis; except for the Noc1–TAP analysis. Known contaminants in IgG-Sepharose affinity purification experiments (ribosomal proteins, translation factors, heat-shock proteins) were excluded from further quantitation analysis. Otherwise, the peak area for iTRAQ™ reporter ions were interpreted and corrected by the GPS-Explorer software (Applied Biosystems) and Excel (Microsoft). Average of all peptides of a given protein was calculated and outliers were deleted by manual evaluation. For determination of protein co-purification depending on rRNA de novo synthesis ratios of the signal intensities of the respective reporter ions were calculated. The ratio found for bait proteins was normalized to 1.

Sucrose gradient analysis

Cycloheximide was added to yeast cultures grown for 2 h at 37°C of OD600 0.5–0.8 to a final concentration of 100 μg/ml. After 15 min incubation at the given temperature and 15 min incubation on ice, the cells were harvested and lysed in lysis buffer (20 mM HEPES pH 7.5, 10 mM KCl, 5 mM MgCl2, 1 mM EGTA, 1 mM DTT, 100 μg/ml cycloheximide) using glass beads. An amount of 600 μg whole-cell extract was loaded on a 10–50% sucrose gradient and centrifugation was performed for 2.5 h at 39 000 rpm and 4°C in a SW40 rotor. Gradients were fractionated using a BioLogic LP chromatography system from Bio-Rad and analysed by western blot using PAP visualization reagent (DakoCytomation, Z 0113) in a dilution of 1:3000 for detection of the TAP-Tag. The distribution of ribosomal particles in the gradient was determined by analysing the blot with rabbit anti-rpS8 antibody in a dilution of 1:2000.

RESULTS

Analysis of expression levels and intracellular localization of ribosome biogenesis factors after shut down of rRNA de novo synthesis

We developed a general strategy to identify proteinaceous (sub-) complexes incorporated in pre-ribosomal RNPs which can form in vivo independent of the presence of (pre-) rRNA and might represent thereby functional building blocks of the RNPs.

To this end we used yeast genetics to conditionally inactivate Pol-I dependent rRNA synthesis and analysed the resulting effects on expression levels and intracellular localization of a selection of ribosome biogenesis factors.
We compared then the protein composition of assemblies of biogenesis factors affinity purified from cells with or without ongoing rRNA synthesis by semi-quantitative mass spectrometry.

First, we analysed how the steady state levels of rRNA precursors were affected after specific shut down of the Pol-I machinery in a conditional temperature-sensitive mutant of the essential Pol-I transcription factor Rrn3p. Therefore, (pre-) rRNA levels in the rrn3-8 mutant strain YCC95 (31) and in the corresponding RRN3 wild-type strain CG379 were analysed at permissive (24°C) and restrictive (37°C) temperature. RNA from equal number of cells was loaded and different rRNA precursors were affected after specific shut down of the rrn3-8 mutant strain CG379 were analysed at permissive (24°C) and restrictive (37°C) and in restrictive (3 h 37°C) by northern blot hybridization (Figure 1A). Pol-I transcripts a region of the rDNA-locus to yield the 35S rRNA precursor, which is processed in various endo- and exonucleolytic cleavage steps to finally result in the mature 18S, 5.8S and 25S rRNA. rRNA precursor levels of both large and small ribosomal subunit (respectively 27S and 20S pre-rRNA) were reduced after 3h incubation at 37°C to a residual amount of ~5% in the rrn3-8 mutant when compared to the level in wild-type cells (Figure 1A, compare lane 2 with lane 4).

Next, we created derivates of the strain YCC95 carrying the rrn3-8 allele, and of the corresponding RRN3 wild-type strain CG379. The coding sequence of the TAP-tag was integrated by homologous recombination in front of the stop codon of a selection of genes coding for different ribosome biogenesis factors. In this way, these genes remained under the control of their endogenous promoters but coded for C-terminal TAP-tagged fusion proteins. We chose for this approach proteins which have been described to be involved in specific biogenesis stages of either the small (Enp1p, Rio2p) or the large (Noc1p, Nop7p, Rix1p, Arx1p) ribosomal subunit.

Immunodetection of the ProteinA moiety of their TAP-tag by western blotting showed that in almost all cases the expression level of the tagged proteins was comparable in wild-type and rrn3-8 background at 37°C (Figure 1B, compare lanes 3 and 4, 5 and 6, 7 and 8, 11 and 12, 13 and 14). The protein level of Noc1p-TAP showed a slight reduction to ~40% (Figure 1B, lanes 9 and 10) after shift to the restrictive temperature in the rrn3-8 background in comparison to wild-type background. This result indicates that some proteinaceous constituents of pre-ribosomes are still present when rRNA de novo synthesis is shut down, although—in general—pre-rRNA-containing particles are strongly reduced.

Next we analysed the localization of the tagged proteins in both wild-type and rrn3-8 mutant background by immunocytochemistry. To detect subcellular compartments we used an antibody raised against the nucleolar protein Nop1p, and DAPI staining of the DNA for visualization of the nucleus (Figure 2). As expected (27,39), in the wild-type background Noc1p-TAP and Nop7p-TAP showed co-localization with the nucleolar marker protein Nop1p (Figure 2A). The staining for Rix1p-TAP overlapped with the DAPI signal, arguing for nucleoplasmic localization. A very faint cytoplasmic signal as it was reported in the literature was less evident (4). Arx1p-TAP was also detected in both the nucleoplasm and cytoplasm (4). The SSU biogenesis factor Enp1p-TAP showed both nuclear and weak cytoplasmic staining (1), whereas Rio2p-TAP showed strong cytoplasmic signals (14). When rRNA synthesis was shut down in the rrn3-8 mutant background by applying restrictive conditions (3h at 37°C), the signal detected by the anti Nop1p antibody appeared to be less defined when compared to the clear crescent shaped signals seen in the wild-type strain background (Figure 2B) and overlapped significantly with DAPI stained nucleoplasmic regions. This kind of intra-nuclear redistribution is most probably due to putative changes in the structure of the yeast nucleolus as a result of inhibition of Pol-I transcription (40). We observed a similar redistribution for Noc1p-TAP and Nop7p-TAP which showed co-staining with Nop1p in the wild-type strain background. On the other hand, shut down of rRNA de novo synthesis did apparently not lead to major nucleo-cytoplasmic redistribution of any of the
investigated proteins, except Enp1-TAP which showed a more pronounced nuclear accumulation under these conditions, also when factor distribution was investigated by semi-quantitative profile analysis (data not shown). Accordingly, the nucleo-cytoplasmic distribution of most of the herein analysed factors is not exclusively dependent on ongoing ribosome biogenesis.

Analysis of ribosome biogenesis factors association with (pre-) rRNA after shut down of rRNA de novo synthesis

Next, we wanted to know with which and with how much residual (pre-) rRNA the tagged ribosome biogenesis factors are still associated after rRNA de novo synthesis is shut down. Therefore, we affinity purified the tagged ribosome biogenesis factors from wild type and *rrn3-8* mutant cells after 3 h shift to restrictive conditions and analysed (pre-) rRNA contained in extracts and affinity purified fractions by northern blot hybridization. In parallel, the relative amounts of affinity purified, tagged ribosome biogenesis factors were determined by western blotting.

Pre-rRNA species which copurified with the different tagged ribosome biogenesis factors were as described from previous experiments (1,4,7,14,29). In wild-type background, Noc1p-TAP precipitated predominantly 27SA2 pre-rRNA, smaller quantities of 35S, 32S and 27SB pre-rRNA (Figure 3A, lane 6). Nop7p-TAP associated mainly with 27SB and 7S pre-rRNA. Early precursors like 35S and 32S pre-rRNA co-purified to a lower extent (Figure 3A, lane 10). Both Rix1p-TAP and Arx1p-TAP showed incorporation in pre-ribosomal particles containing 27SB and 7S pre-rRNA (Figure 3A, lanes 14 and 18). Both studied ribosome biogenesis factors of the small ribosomal subunit, Enp1p and Rio2p, showed association with 40S precursor particles containing the 20S pre-rRNA (Figure 3B, lanes 6 and 10). Only small amounts of 35S and 32S pre-rRNA were detected to co-purify with Enp1p-TAP. For all the analysed factors the co-purification efficiency of pre-rRNAs contained in precursors of the corresponding ribosomal subunit for whose maturation they are not required for was comparably low, indicating the reasonable specificity of the analysis (Figure 3A and B, lanes 6, 10, 14 and 18).

Western blot analysis indicated that for all studied tagged factors the efficiencies of their affinity purification were comparable no matter whether rDNA transcription was shut down or not. In contrast, the amount of co-purifying pre-rRNA was clearly reduced when rDNA transcription was impaired (Figure 3A and B, compare lanes 6 and 8, 10 and 12, 14 and 16, 18 and 20). The ratio of purified tagged protein versus co-purified pre-rRNA was strongly increased in this situation indicating that pre-rRNA free protein complexes could be enriched. Interestingly, in cells where rDNA transcription was not impaired, comparison of the amounts of purified Noc1p-TAP, Rix1p-TAP and Nop7p-TAP with the corresponding amounts of copurified pre-rRNA (wild-type, Figure 3A, lanes 6, 10 and 14) indicated that already under these conditions purified Noc1p-TAP and Rix1p-TAP fractions contained significant amounts of pre-rRNA-free protein (complexes). Accordingly, assemblies of ribosome biogenesis factors that are affinity purified under standard conditions contain varying amounts of pre-ribosomal RNPs and pre-rRNA free protein (complexes).

In contrast to early 60S biogenesis factors like Noc1p and Nop7p (data not shown), previous analyses suggested
that Arx1p and Rix1p associate with nascent ribosomal subunits containing mature 25S rRNA. However Arx1p and Rix1p seem to be largely excluded from 60S ribosomal subunits which are able to associate with 40S ribosomal subunits and initiate translation (4,41). Therefore, we compared the relative amounts of mature 25S rRNA and 18S rRNA which co-purify with Rix1p-TAP and Arx1p-TAP on an IgG-Dynabead matrix after in vivo inactivation of the rDNA transcription machinery (Figure 4A). When extracts were prepared from cells where rRNA transcription took place, the ratio of 25S rRNA to 18S rRNA retained on IgG-Dynabeads was six times higher in experiments with strain Y584 (Arx1p-TAP) and about two times higher in the experiment with strain Y583 (Rix1p-TAP) than with strain Y543 expressing no TAP tagged protein. Interestingly, when the same analysis was performed with cells where rRNA transcription was shut down, the ratio of 25S rRNA to 18S rRNA retained on IgG-Dynabeads was still about four times higher for the strain expressing Arx1p-TAP in comparison to the strain expressing no tagged protein (Figure 4A and B). In addition, sedimentation analysis of whole cellular extracts on sucrose gradients showed that after shut down of rRNA de novo synthesis the major population of Arx1p-TAP co-sediments with 60S ribosomal subunits (Figure 4C). Accordingly, in

![Figure 3. Co-purification of pre-rRNA with different TAP-tagged biogenesis factors of the (A) large and (B) small ribosomal subunit after shut down of Pol-I transcription. Northern hybridization analysis of precursor rRNA species of both ribosomal subunits was performed on RNA extracted from whole cell extracts (IN) and from affinity purified TAP-tagged (A) Noclp, Nop7p, Rix1p, Arx1p, (B) Rio2p and Enp1p (IP). Yeast strains carrying the RRN3 wild-type allele or the rrn3-8 allele were analysed at restrictive (3 h, 37°C) temperature. Different oligonucleotides ('Materials and methods' section) were used for detection of the different indicated (pre-) rRNA species. In parallel the amounts of tagged protein in input and affinity purified fractions were determined by western blotting using PAP visualization reagent. For each precipitation sample same signal intensities in IN and IP lanes reflect a purification recovery of 1%.

![Image](https://example.com/image.png)
agreement with previous analyses, Arx1p associates with LSUs containing mature 25S rRNA. Among them is, most likely, a population of nascent 60S ribosomal subunits, but Arx1p seems also to be attached to non-nascent free large ribosomal subunits which are not engaged in translation.

Changes in the protein composition of purified ribosome biogenesis factor assemblies after shut down of rRNA de novo synthesis

In summary, these analyses indicated that ribosome biogenesis factor assemblies purified from cells, in which rRNA de novo synthesis was shut down, were largely devoid of pre-rRNA and, except for Arx1p-TAP purified assemblies, also of mature rRNA molecules. Next, we compared by semi-quantitative mass spectrometry how the protein composition of these assemblies changes after in vivo depletion of nascent pre-rRNA.

One-step affinity purification of ProteinA tagged ribosome biogenesis factors from cells with or without ongoing rDNA transcription was performed using IgG–sepharose. Tryptic peptides from the purified assemblies of these two pools were separately labelled using two different iTRAQ reagents (38). Then, the two differentially labelled samples were combined, the mixture of peptides was fractionated by nano-flow reversed-phase-chromatography and analysed by MALDI-TOF/TOF (matrix assisted laser desorption ionization-time of flight) mass spectrometry.

In the fragmentation chamber of the mass spectrometer different reporter groups (114, 115, 116, 117 Da) are released from the specific iTRAQ labels and can be used to determine how much of the analysed peptide was linked to the respective iTRAQ reagent (Figure 5). Furthermore, the peptide itself is fragmented and analysis of the resulting fragment masses allows identification of the protein from which it was derived (38).

This methodology allowed us to compare the relative abundance of established ribosome biogenesis factors co-purifying with Noc1p-TAP, Nop7p-TAP, Rix1p-TAP or Arx1p-TAP from cells carrying either the temperature sensitive rrn3-8 or the corresponding wild-type RRN3 allele (Figure 6). As expected from the literature (1), Enp1p-TAP purified from RRN3 wild-type cells co-purified with a large set of proteins which are believed to be part of the SSU-processome, an assembly of factors which are required for nuclear steps of eukaryotic SSU maturation and which associate in vivo with an U3-snoRNP. Association of most of these factors with Enp1p-TAP was largely reduced (rrn-8:RRN3 ratio <0.2) when rDNA transcription was shut down, indicating that co-purification of Enp1p-TAP with SSU-processome components is mediated through pre-rRNA. On the other hand, co-purification of several other factors with Enp1p-TAP remained largely unaffected by inactivation of the rDNA transcription factor Rrn3p (rrn3-8:RRN3 ratio for Ltv1p 1.15, for Tsr1p 1.0,

Figure 4. Co-purification of mature 25S and 18S rRNA with TAP-tagged Rix1p and Arx1p after shut down of Pol-I transcription. (A) Northern hybridization analysis of mature rRNA species of both ribosomal subunits was performed on RNA extracted from whole cell extracts (IN) and from affinity purified TAP-tagged Rix1p and Arx1p (IP). Yeast strains carrying the RRN3 wild-type allele or the rrn3-8 allele were analysed at restrictive (3h, 37°C) temperature. Different oligonucleotides (Materials and methods’ section) were used for detection of the indicated 18S and 25S rRNA species. Same signal intensities in IN and IP lanes reflect a purification recovery of 1%. (B) The ratio of affinity precipitation efficiencies for 25S and 18S RNAs was calculated and normalized to the one found in the untagged wild-type yeast strain using MultiGauge V3.0 (Fujifilm). (C) Sedimentation behaviour of TAP-tagged Arx1p was analysed on sucrose density gradients with cellular extracts of strains carrying the RRN3 wild-type allele (rRNA synthesis +) or the rrn3-8 allele (rRNA synthesis −) after 3h shift to restrictive temperature. Distribution of ribosomal particles (40S, 60S, 80S, polysomes) in the gradient was determined by OD254 measurement (data not shown) and western blot analysis of the gradient fractions using an anti-rpS8 antibody. The amount of TAP-tagged Arx1p in each fraction and in the input-sample (IN) was also visualized by western blot analysis.
for Krr1p 0.53, for Hrr25p 0.73, for Rio2p 1.15).

Apparently, these proteins are part of a, most likely
proteinaceous, module which can interact with SSU pre-
cursors but whose in vivo existence does not require the
presence or ongoing production of pre-rRNA. This inter-
pretation is in general agreement with the recent observa-
tion, that an Enp1p-Ltv1p-rpS3 complex can be extracted
from pre-ribosomes in high salt conditions (28).

Comparison of the protein compositions of Rio2p-TAP
affinity-purifications from cells with or without ongoing
rRNA synthesis confirmed the existence of this protein
module. Association of Enp1p (rrn3-8:RRN3 ratio 0.97),
Ltv1p (rrn3-8:RRN3 ratio 1.3), Tsr1p (rrn3-8:RRN3 ratio
0.94), Krr1p (rrn3-8:RRN3 ratio 3.44), Hrr25
(rrn3-8:RRN3 ratio 0.81, based on one peptide), and addi-
tionally of Dim1p (rrn3-8:RRN3 ratio 0.82) with Rio2p-TAP
remained largely unaffected when rDNA
transcription was shut down. Interestingly, two other
ribosome biogenesis factors involved in pre-SSU matura-
tion, Nob1p and Pno1p/Dim2p co-purified as expected
from earlier experiments (1) with Rio2p-TAP in the
RRN3 wild-type strain, but their association was largely
reduced after inactivation of rrn3-8 indicating that the
Nob1p and Pno1p/Dim2p co-purification with
Rio2p-TAP is pre-rRNA mediated.

Using the same approach we analysed with which
proteins the LSU biogenesis factors Noc1p, Nop7p and
Rix1p associate in vivo in the absence of rRNA de novo
synthesis. Previous studies showed that in conditions
leading to in vivo or ex vivo disruption of pre-ribosomes,
Noc1p could be purified in a complex with Noc2p (27),
Nop7p in a complex with Erb1p and Ytm1p (29) and
Rix1p in a complex with Ipi1p and Ipi3p (18). The
experimental approach used in the present study
now indicated that these three protein complexes can form or persist in vivo independent of the presence and
de novo synthesis of pre-ribosomes (Figure 6, see also
Supplementary Figure S1). In addition, some significant
residual association of Rrp5p with the Noc1p/Noc2p
complex (rrn3-8:RRN3 ratio 0.44) and of the nucleolar
DEAD box helicase Drs1p with the Nop7p complex
(rrn3-8:RRN3 ratio 0.29) was observed when rRNA de
novo synthesis was shut down, suggesting that these
proteins are part of the corresponding protein modules.

Semi-quantitative mass spectrometry analysis of
Arx1p-TAP affinity purifications from extracts of cells
with or without ongoing rRNA synthesis showed that sig-
nificant amounts of Rei1p (rrn3-8:RRN3 ratio 0.81),
Alb1p (rrn3-8:RRN3 ratio 0.67) Lsg1p (rrn3-8:RRN3 ratio
0.5), Tif6p (rrn3-8:RRN3 ratio 0.63) and Nmd3p
(rrn3-8:RRN3 ratio 0.55) co-purify with Arx1p-TAP inde-
dependent of the presence of nascent large ribosomal
subunits. Our observation that Arx1p-TAP significantly
associated with mature 25S rRNA when rRNA de
novo synthesis was shut down (see above, Figure 4A) indicated
that it associates not only with nascent subunits but also
with free, non-nascent 60S ribosomal subunits.

Accordingly, copurification of Rei1p, Alb1p, Lsg1p,
Tif6p and Nmd3p with Arx1p-TAP in conditions where
neo-production of large ribosomal subunits is inhibited
might be due to their (pre-) ribosome independent associ-
ation with Arx1p or due to their Arx1p independent asso-
ciation with mature, free non-nascent 60S ribosomal
subunits.

DISCUSSION
A large variety of ribosome biogenesis factors is needed
for efficient production of eukaryotic ribosomes. Many of
them interact in a coordinated transient way with nascent
ribosomal subunits leading to the formation of specific
Figure 6. Semi-quantitative comparison of co-purifying ribosome biogenesis factors from cells with or without ongoing rRNA synthesis. The diagrams show the ratio of identified proteins co-purifying with TAP-tagged bait proteins from cells carrying the RRN3 wild-type and the rrn3-8 mutant allele. The bars indicate the average value of the calculated RRN3:rrn3-8 ratios for all identified peptides of the indicated protein. Error bars represent the standard deviation of these ratios ($P < 0.05$). The ratio of the bait protein (highlighted by a grey bar) is set to one. For co-purified proteins, ratios $>0.5$ were considered to reflect associations with bait proteins barely influenced by the absence of de novo rRNA synthesis. Association of proteins with intermediate ratios between 0.25 and 0.5 were classified to be stronger affected by the shutoff of rRNA synthesis. Nevertheless they are still significantly co-purified in the rrn3-8 mutant. Associations of proteins with ratios below 0.25 seem to be strongly dependent on rRNA de novo synthesis. Two independent purifications for the indicated bait proteins were performed and for each purification mass spectrometry analysis was repeated once. Quantitation of one representative experiment is shown.
pre-ribosomal intermediates. It was shown that in conditions leading to (partial) disruption of pre-ribosomes (3,17–30) or after differential centrifugation (26) (pre-) rRNA free protein modules of ribosome biogenesis factors can be isolated from yeast cellular extracts by affinity purification. The experimental approach chosen in this article, namely in vivo down-regulation of rRNA synthesis followed by one step affinity purification and comparative protein analysis of the purified assemblies by semi-quantitative mass spectrometry, turned out to reveal several new aspects about protein modules involved in eukaryotic ribosome maturation. First it allowed to formally analyse which protein modules can form or persist in vivo independent of the presence and de novo synthesis of pre-ribosomes. The strong argument provided in this article for pre-ribosome independent in vivo existence of protein modules (Rio2p/Enp1p-, Rix1p-, Noc1p- and Nop7p modules) involved in ribosome biogenesis implies that these modules represent functional entities which can transiently interact with nascent ribosomal subunits en bloc. Recently, Watkins and colleagues observed the accumulation of a 50S U3 sno-RNP which contained Rrp5p and Dbp4p and other unidentified factors in mammalian cells in which RNA polymerase I was inactivated (10). Altogether, these analyses indicate the existence of various pre-ribosomal independent ribosome biogenesis factors modules in eukaryotic cells.

The strategy applied in this article allowed usage of mild conditions (physiological MgCl2 and salt concentrations) during protein module isolation and semi-quantitative, comparative interpretation of the protein composition of pre-rRNA dependent and independent assemblies. Thereby, several new members of pre-rRNA free ribosome biogenesis factor modules could be identified. Our data suggest that Rio2p forms (a) module(s) with Enp1p, Ltv1p, Tsr1p, Krr1p, Hrr25p and Dim1p; that Rrp5 is a member of a Noc1-Noc2 module, and that Drs1p association with a Nop7p-Erb1p-Ytm1p module is enhanced by pre-rRNA, but does not strictly depend on it. The notion that Drs1p is part of Nop7p-Erb1p-Ytm1p modules is further supported by results of Woolford and colleagues (39). They found allele specific synthetic lethal phenotypes in nop7/drs1 double mutants and observed underproduction of 60S ribosomal subunits in drs1 mutant strains.

The appearance of pre-rRNA-free complexes containing both, putative early assembly biogenesis factors like Enp1p, and those which apparently bind late, like Hrr25p and Rio2p, does not necessarily mean that they have to assemble always as one complex with nascent subunits. The following scenarios are also possible. (i) Enp1p could bind at least at two different sites to the pre-ribosome. The formation of the two-binding sites is timely separated. The first occurring site binds Enp1p, but not late associating factors, whereas the other binding site interacts with a complex containing Enp1p, Rio2p and other late factors. Binding at different sites of the pre-ribosome was recently suggested for Prp43 (42). (ii) Enp1p assembles first on the pre-ribosome and is then replaced by a complex containing both the late factors and Enp1p, and (iii) Enp1 and late factors assemble independently, but are released together.

Interestingly, Nob1p and its putative interaction partner Pno1p/Dim2p (13,43), while consistently found in Rio2p containing pre-ribosomal assemblies (1), seem not to be part of the pre-rRNA free Rio2p protein modules (Figure 6). Beside Nob1p, a putative endonuclease containing a PIN domain (44), and Pno1p/Dim2p, Rio2p itself and several of the Rio2p module components are required for cytoplasmic 3′ processing of 18S pre-rRNA at site D (13,15,16,45,46). Apparently, the interaction of at least two discrete protein modules, the Nob1p-Pno1p/Dim2p module and the Rio2p module(s), with nascent 40S ribosomal subunits at most likely distinct sites seems to be necessary to allow efficient conversion of 3′ extended 18S rRNA into mature 18S rRNA by endonucleolytic cleavage at site D.

An in part unexpected outcome of these analyses was that free large ribosomal subunits are associated with a specific set of ribosome biogenesis factors, including Arx1p, in cells where neo-production of nascent ribosomes was blocked for prolonged times (3 h). We propose that Arx1p associated subunits represent free, non-nascent LSUs. De novo synthesis of ribosomes in yeast requires about 15 min and all analysed pre-rRNAs were turned over, either through degradation or productive processing, during the 3 h of rnr3-8 inactivation applied in the experimental setup (Figures 1A, 3A and B). Thereby, if 25S rRNA containing nascent subunits persisted during 3 h, they behaved in clear contrast to the other pre-ribosomal particles. A possible explanation in favour of this would be that release of Arx1p and other factors from nascent LSUs required active ongoing translation which might be disturbed by an unknown feedback mechanism when Pol-I activity is decreased in cells. On the other hand it was demonstrated by Nomura and colleagues that inactivation of the Pol-I machinery has no impact on ribosomal activity (47). In agreement with this, sucrose gradient analyses argue that small ribosomal subunits stay associated with polysomal fractions when Pol-I transcription is shut down (Figure 4C and our unpublished polysome profile data). Altogether we think it is worth to consider a regulatory role of Arx1p in both the synthesis and function of LSUs. Beside its suggested auxiliary role in nucleo-cytoplasmic translocation of pre-60S subunits (48–50) it might protect both nascent and non-nascent LSUs from degradation and/or regulate their interaction with 40S ribosomal subunits. Interestingly, the human homolog Ebp1, which was suggested to play a role in translational control (51–53), seems to lack the characteristics of yeast Arx1p mediating its interaction with nucleoporines and its efficient nucleo-cytoplasmic shuttling in in vitro assays (48). Possibly Arx1p plays a conserved role in eukaryotic translation while it gained additional non-essential function in nucleo-cytoplasmic transport of the LSU in certain organisms like the fast dividing S. cerevisiae.

In yeast cells lacking the ARX1 gene changes in pre-rRNA maturation or underaccumulation of de novo synthesized LSUs were not detected [(20), our own unpublished data], and polysome profiles revealing an
increase in both the free 40S and 60S subunits and the appearance of halfmers, could be indicative of a role of Arxlp in subunit joining [see for example (49), our unpublished data].

When de novo synthesis of rRNA was shut down several LSU biogenesis factors, as Nmd3p, Tif6p/eIF6, Reil1p, Alblp and Lsg1p, continued to co-purify with Arxl1p while others like Nug1p, Nog2p and Rrs1lp ceased in doing so (Figure 6).

Nmd3lp was already shown to interact with both nascent and non-nascent free LSUs (54,55), apparently several LSU biogenesis factors, as Nmd3p, Tif6p/eIF6, Reil1p, Alblp and Lsg1p, could be indicative of a role of increase in both the free 40S and 60S subunits and the appearance of halfmers, could be indicative of a role of increase in both the free 40S and 60S subunits and the appearance of halfmers, could be indicative of a role of increase in both the free 40S and 60S subunits and the appearance of halfmers, could be indicative of a role of increase in both the free 40S and 60S subunits and the appearance of halfmers, could be indicative of a role of increase in both the free 40S and 60S subunits and the appearance of halfmers, could be indicative of a role of increase in both the free 40S and 60S subunits and the appearance of halfmers, could be indicative of a role of increase in both the free 40S and 60S subunits and the appearance of halfmers, could be indicative of a role of increase in both the free 40S and 60S subunits and the appearance of halfmers, could be indicative of a role of increase in both the free 40S and 60S subunits and the appearance of halfmers, could be indicative of a role of increase in both the free 40S and 60S subunits and the appearance of halfmers.

Altogether, these data suggest that free 60S ribosomal subunits, neo-synthesized or released from 80S ribosomes during translation termination, are decorated with a common set of factors including Arxl1p, Tif6p/eIF6, Nmd3p and, eventually, in addition Reil1p, Alblp and Lsg1p. A dual function in both ribosome synthesis and regulation of LSU recycling during translation, as already suggested for mammalian eIF6/Tif6p (59, 60), would explain the unexpected association of these factors with mature LSUs.

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REFERENCES

1. Schäfer,T., Strauss,D., Petfalski,E., Tollervey,D. and Hurt,E. (2003) The path from nucleolar 90S to cytoplasmic 40S pre-ribosomes. EMBO J., 22, 1370–1380.
2. Saveanu,C., Namane,A., Gleizes,P., Lebreton,A., Rousselle,J., Noaillac-Depeyre,J., Gas,N., Jacquier,A. and Fromont-Racine,M. (2003) Sequential protein association with nascent 60S ribosomal particles. Mol. Cell. Biol., 23, 4449–4460.
3. Pérez-Fernández,J., Román,A., De Las Rivas,J., Bustelo,X.R. and Dosil,M. (2007) The 90S preribosome is a modular structure that is assembled through a hierarchical mechanism. Mol. Cell. Biol., 27, 5414–5429.
4. Nissan,T.A., Bassler,J., Petfalski,E., Tollervey,D. and Hurt,E. (2002) 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. EMBO J., 21, 5539–5547.
5. Milkereit,P., Kühn,H., Gas,N. and Tschochner,H. (2003) The pre-ribosomal network. Nucleic Acids Res., 31, 799–804.
6. Bernstein,K.A., Gallagher,J.E.G., Mitchell,B.M., Granneman,S. and Baserga,S.J. (2004) The small-subunit processome is a ribosome assembly intermediate. Eukaryotic Cell, 3, 1619–1626.
7. Harmpicharnchai,P., Jakovljevic,J., Horsey,E., Miles,T., Roman,J., Rout,M., Meagher,D., Imai,B., Guo,Y., Brame,C.J. et al. (2001) Composition and functional characterization of yeast 60S ribosome assembly intermediates. Mol. Cell, 8, 505–515.
8. Dragon,F., Gallagher,J.E.G., Compagnone-Post,P.A., Mitchell,B.M., Ponzewischer,K.A., Wehner,K.A., Wormsley,S., Settlage,R.E., Shabanowitz,J., Osheim,Y. et al. (2002) A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. Nature, 417, 967–970.
9. Wery,M., Ruidant,S., Schileoert,S., Leporé,N. and Lafontaine,D.L. (2009) The nuclear poly(A) polymerase and Exosome cofactor Trf5 is recruited cotranscriptionally to nucleolar surveillance. RNA, 15, 406–419.
10. Turner,A.J., Knox,A.P., Prieto,J., McStay,B. and Watkins,N.J. (2009) A novel small-subunit processome assembly intermediate that contains the U3 snoRNP, nucleolin, RPR5, and DBP4. Mol. Cell. Biol., 29, 3007–3107.
11. Trapman,J., Retèl,J. and Planta,R.J. (1975) Ribosomal precursor particles from yeast. Exp. Cell Res., 90, 95–104.
12. Chen,W., Bucaria,J., Band,D.A., Sutton,A. and Sternglanz,R. (2003) Emp1, a yeast protein associated with U3 and U14 snoRNAs, is required for pre-rRNA processing and 40S subunit synthesis. Nucleic Acids Res., 31, 690–699.
13. Fatica,A., Oelfinger,M., Dlakic,M. and Tollervey,D. (2003) Nolp1 is required for cleavage of the 3′ end of 18S rRNA. Mol. Cell. Biol., 23, 1798–1807.
14. Vanrobays,E., Geluge,J., Gleizes,P. and Caizergues-Ferrer,M. (2003) Late cytoplasmic maturation of the small ribosomal subunit requires RIO proteins in Saccharomyces cerevisiae. Mol. Cell. Biol., 23, 2083–2095.
15. Seiser,R.M., Sundberg,A.E., Wollam,B.J., Zobel-Thropp,P., Baldwin,K., Spector,M.D. and Lycan,D.E. (2006) Ltv1 is required for efficient nuclear export of the ribosomal small subunit in Saccharomyces cerevisiae. Genetics, 174, 679–691.
16. Geerlings,T.H., Faber,A.W., Bister,M.D., Vos,J.C. and Raué,H.A. (2003) Rix2p, an evolutionarily conserved, low abundant protein kinase essential for processing of 20S pre-rRNA in Saccharomyces cerevisiae. J. Biol. Chem., 278, 22537–22545.
17. Oelfinger,M., Leung,A., Lamond,A., Tollervey,D. and Luang,A. (2002) Yeast Pescadillo is required for multiple activities during 60S ribosomal subunit synthesis. RNA, 8, 626–636.
18. Gioloni,K., Nissan,T.A., Petfalski,E., Tollervey,D. and Hurt,E. (2004) Rea1, a dynin-related nuclear AAA-ATPase, is involved in late rRNA processing and nuclear export of 60S subunits. J. Biol. Chem., 279, 55411–55418.
19. Hedges,J., West,M. and Johnson,A.W. (2005) Release of the recycling adapter, Nmd3p, from the 60S ribosomal subunit requires Rpl10p and the cytoplasmic GTPase Lsg1p. EMBO J., 24, 567–579.
20. Lebreton,A., Saveanu,C., Decourty,L., Rain,J., Jacquier,A. and Fromont-Racine,M. (2006) A functional network involved in the recycling of nucleocytoplasmic pre-60S factors. J. Cell Biol., 173, 349–360.
21. Senger,B., Lafontaine,D.L., Grindorage,J.S., Gadola,S., Camasses,A., Sanni,A., Garnier,J.M., Breitenbach,M., Hurt,E. and Fasiolo,F. (2001) The nucleolar Tif6p and Ef1lp are required for late cytoplasmic step of ribosome synthesis. Mol. Cell., 8, 1363–1373.
22. Gaivin,A., Aloy,P., Grandi,P., Krause,R., Boesche,M., Marzioch,M., Rau,C., Jensen,L.J., Bastuck,S., Dümpelfeld,B. et al. (2006) Proteomic survey reveals modularity of the yeast cell machinery. Nature, 440, 631–639.
23. Krogan,N.J., Cagney,G., Yu,H., Zhong,G., Guo,X., Ignatchenko,A., Li,J., Pu,S., Datta,N., Tikuisis,A.P. et al. (2006) Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. Nature, 440, 657–643.
24. Grandi,P., Rybin,V., Bassler,J., Petfalski,E., Strauss,D., Marzioch,M., Schüe,S., Kuster,B., Tschochner,H., Tollervey,D. et al. (2002) 90S pre-ribosomes include the 35S pre-rRNA, the...
U3 snoRNP, and 40S subunit processing factors but predominantly lack 60S synthesis factors. Mol. Cell., 10, 105–115.
25. Rigaut,G., Shevchenko,A., Rutz,B., Wilm,M., Mann,M. and Séraphin,B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. Nat. Biotechnol., 17, 1030–1032.
26. Krogan,N.J., Peng,W., Carone,M., Robinson,M.D., Haw,R., Zhong,G., Guo,X., Zhang,X., Canadien,V., Richards,D.P. et al. (2004) High-definition macromolecular composition of yeast RNA-processing complexes. Mol. Cell, 13, 225–239.
27. Milkereit,P., Gadal,O., Podtelejnikov,A., Trumel,S., Gas,N., Petfalski,E., Tollervey,D., Mann,M., Hurt,E. and Tschochner,H. (2001) Maturation and intranuclear transport of pre-60S ribosomes requires Noc proteins. Cell, 105, 499–509.
28. Schäfer,T., Maco,B., Petfalski,E., Tollervey,D., Böttcher,B., Aebi,U. and Hurt,E. (2006) Hrr25-dependent phosphorylation state regulates organization of the pre-40S subunit. Nature, 441, 651–655.
29. Miles,T.D., Jakovljevic,J., Horsey,E.W., Harnpicharnchai,P., Tang,L. and Woolford,J.L. (2005) Ytm1, Nop7, and Erb1 form a complex necessary for maturation of yeast 60S preribosomes. Mol. Cell. Biol., 25, 10419–10432.
30. Lebreton,A., Rousselle,J., Lenormand,P., Namane,A., Jacquier,A., Fromont-Racine,M. and Saveanu,C. (2008) 60S ribosomal subunit assembly dynamics defined by semi-quantitative mass spectrometry of purified complexes. Nucleic Acids Res., 36, 4988–4999.
31. Cadwell,C., Yoon,H.J., Zehbarjadian,Y. and Carbonj,J. (1997) The yeast nuclear protein Cbf5p is involved in rRNA biosynthesis and interacts genetically with the RNA polymerase I transcription factor RRN3. Mol. Cell. Biol., 17, 6175–6183.
32. Puig,O., Caspary,F., Rigaut,G., Rutz,B., Bouveret,E., Bragado-Nilson,E., Wilm,M. and Séraphin,B. (2001) The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods, 24, 218–229.
33. Knop,M., Siegers,K., Pereira,G., Zacharie,W., Winsor,B., Nasmyth,K. and Schiebel,E. (1999) Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. Yeast, 15, 963–972.
34. Milkereit,P., Strauss,D., Bassler,J., Gadal,O., Kühn,H., Schütz,S., Gas,N., Lebrun,J., Hurt,E. and Tschochner,H. (2003) A Noc complex specifically involved in the formation and nuclear export of ribosomal 40S subunits. J. Biol. Chem., 278, 4072–4081.
35. Léger-Silvestre,I., Milkereit,P., Ferreira-Cerca,S., Saveanu,C., Rousselle,J., Choesnel,V., Guinefoileau,C., Gas,N. and Gleizes,P. (2004) The ribosomal protein Rp51p is required for nuclear export of the 40S subunit precursors in yeast. EMBO J., 23, 2336–2347.
36. Ferreira-Cerca,S., Püll,G., Gleizes,P., Tschochner,H. and Milkereit,P. (2005) Roles of eukaryotic ribosomal proteins in maturation and transport of pre-18S rRNA and ribosome function. Mol. Cell, 20, 263–275.
37. Chen,X. and Andrews,P.C. (2009) Quantitative proteomics analysis of pancreatic zymogen granule membrane proteins. Meth. Mol. Biol., 528, 327–338.
38. Ross,P.L., Huang,Y.N., Marchese,J.N., Williamson,B., Parker,K., Hattan,S., Khainovski,N., Pillai,S., Dey,S., Daniels,S. et al. (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell. Proteom., 3, 1154–1169.
39. Adams,C.C., Jakovljevic,J., Roman,J., Harnpicharnchai,P. and Woolford,J.L. (2002) Saccharomyces cerevisiae nuclear protein Nop7p is necessary for biogenesis of 60S ribosomal subunits. RNA, 8, 150–165.
40. Trumel,S., Léger-Silvestre,I., Gleizes,P.E., Teulière,F. and Gas,N. (2000) Assembly and functional organization of the nucleolus: ultrastructural analysis of Saccharomyces cerevisiae mutants. Mol. Biol. Cell., 11, 2175–2189.
41. Hung,N. and Johnson,A.W. (2006) Nuclear recycling of the pre-60S ribosomal subunit-associated factor Arxl depends on Rex1 in Saccharomyces cerevisiae. Mol. Cell. Biol., 26, 3718–3727.
42. Bohnsack,M.T., Martin,R., Granneman,S., Ruprecht,M., Schleiff,E. and Tollervey,D. (2009) Prp43 bound at different sites on the pre-rRNA performs distinct functions in ribosome synthesis. Mol. Cell., 36, 583–592.
43. Tone,Y. and Toh-E.A. (2002) Nob1p is required for biogenesis of the 26S proteasome and degraded upon its maturation in Saccharomyces cerevisiae. Genes Dev., 16, 3142–3157.
44. Fatica,A., Tollervey,D. and Đaklić,M. (2004) PIN domain of Nob1p is required for D-site cleavage in 20S pre-rRNA. RNA, 10, 1698–1701.
45. Gelperin,D., Horton,L., Beckman,J., Henndorff,S. and Lemmon,S.K. (2001) Bms1p, a novel GTP-binding protein, and the related Tsr1p are required for distinct steps of 40S ribosome biogenesis in yeast. RNA, 7, 1268–1283.
46. Vanrobaeys,E., Géligue,G., Caizergues-Ferrer,M. and Lafontaine,D.L.I. (2004) Dim2p, a KH-domain protein required for small ribosomal subunit synthesis. RNA, 10, 645–656.
47. Wittekind,M., Kolb,J.M., Dodd,J., Yamagishi,M., Mémé,S., Buhrer,J.M. and Nomura,M. (1990) Conditional expression of RPA190, the gene encoding the large subunit of yeast RNA polymerase I: effects of decreased rRNA synthesis on ribosomal protein synthesis. Mol. Cell. Biol., 10, 2049–2059.
48. Brüdtachisch,B., Kowalski,E., Bange,G., Yao,W., Sekimoto,T., Baumgärtel,V., Boese,G., Bassler,J., Wild,K. et al. (2007) Arxl functions as an unorthodox nuclear export receptor for the 60S preribosomal subunit. Mol. Cell, 27, 767–779.
49. Hung,N., Lo,K., Patel,S.S., Helmke,K. and Johnson,A.W. (2008) Arxl is a nuclear export receptor for the 60S ribosomal subunit in yeast. Mol. Cell Biol., 19, 735–744.
50. Lo,K. and Johnson,A.W. (2009) Reengineering ribosome export. Mol. Biol. Cell, 20, 1545–1554.
51. Monic,T.P., Perrin,A.J., Birtley,J.R., Sweeney,T.R., Karakasiliotis,L., Chaudhry,Y., Roberts,L.O., Matthews,S., Goodfellow,I.G. and Curry,S. (2007) Structural insights into the transcriptional and translational roles of Ebp1. EMBO J., 26, 3936–3944.
52. Pilipenko,E.V., Pestova,T.V., Kolupaeva,Y.G., Khitrina,E.V., Poperchenyaya,A.N., Agol,V.I. and Hellen,C.U. (2000) A cell cycle-dependent protein serves as a template-specific translation initiation factor. Genes Dev., 14, 2028–2045.
53. Squatrito,M., Mancino,M., Sala,L. and Draetta,G.F. (2006) Ebp1 is a dsRNA-binding protein associated with ribosomes that modulates eIF2alpha phosphorylation. Biochem. Biophys. Res. Commun., 344, 859–868.
54. Hedges,J., Chen,Y., West,M., Bussiere,C. and Johnson,A.W. (2006) Mapping the functional domains of yeast NMD3, the nuclear export adapter for the 60S ribosomal subunit. J. Biol. Chem., 281, 36579–36587.
55. Ho,J.H., Kallstrom,G. and Johnson,A.W. (2000) Nascent 60S ribosomal subunits enter the free pool bound by Nmd3p. RNA, 6, 1625–1634.
56. Gadal,O., Strauss,D., Kessl,J., Trumpower,B., Tollervey,D. and Hurt,E. (2001) Nuclear export of 60S ribosomal subunits depends on Xpo1p and requires a nuclear export sequence-containing factor, Nmd3p, that associates with the large subunit protein Rpl10p. Mol. Cell. Biol., 21, 3405–3415.
57. Benelli,D., Marzi,S., Mancone,C., Alonzi,T., la Teana,A. and Draetta,G.F. (2006) Ebp1 is rate-limiting in translation, growth and transformation. Nature, 445, 684–688.
58. Gandin,V., Miluzio,A., Barbieri,A.M., Beugnet,A., Kiyokawa,H., Marchisio,P.C. and Biffo,S. (2009) Eukaryotic initiation factor 6 is rate-limiting in translation, growth and transformation. Nature, 455, 684–688.