Here we report the functional characterization of Pwp2, an evolutionary conserved component of the 90 S pre-ribosome. Conditional depletion of the Pwp2 protein in yeast specifically impairs pre-rRNA processing at sites A0, A1, and A2, leading to a strong decrease in 18 S rRNA and 40 S ribosomal subunit levels. Pre-ribosomal particle sedimentation analysis indicated that these defects are caused by a block in the formation of 90 S pre-ribosomes. We demonstrate that in Pwp2-depleted cells the U3 small nucleolar ribonucleoprotein is not able to interact with the 35 S pre-rRNA and accumulates as a free complex. Similarly, other 90 S particle components such as Imp3 and Imp4 do not associate with the pre-rRNA precursor in the absence of Pwp2. In addition, we have found that after blocking U3 ribonucleoprotein assembly, Pwp2 predominantly accumulates as a complex in association with five proteins: Dip2, Utp6, Utp13, Utp18, and Utp21. Immunoprecipitation and gradient sedimentation analysis revealed that this Pwp2 small subcomplex is capable of interacting directly with the 35 S pre-rRNA 5′ end. Taken together, these results indicate that Pwp2 forms part of a stable particle subunit independent of the U3 small nucleolar ribonucleoprotein that is essential for the initial assembly steps of the 90 S pre-ribosome.

It has been calculated that a yeast cell produces ~40 new ribosomes per second to keep its metabolic and growth status (1). This biosynthetic process imposes a costly metabolic demand, since it involves the biosynthesis and assembly of ~80 ribosomal proteins, four rRNAs (25, 18, 5.8, and 5 S), large numbers of proteins involved in rRNA maturation, and multiple snoRNPs (2–5). Of all of these biosynthetic processes, the generation of 25, 18, and 5.8 S rRNAs is perhaps the most challenging for cells. This process initiates in the nucleolus with the transcription by RNA polymerase I of the 35 S pre-rRNA precursor, a 7,000-nucleotide-long polycistronic transcript that contains the sequences of the 18, 5.8, and 25 S rRNAs embedded in non coding regions (for a scheme, see Fig. 1). The 35 S rRNA is rapidly cleaved at sites A0 (giving rise to the 33 S pre-rRNA), A1 (generating the 23 S pre-rRNA), and A2 (producing the 20 S and the 27 S A2 pre-rRNAs; Fig. 1). The 20 and 27 S A2 precursors mature further following two separate branches. In the first one, the 20 S pre-rRNA is exported to the cytosol where it is cleaved at site D to produce the 18 S rRNA which, in turn, will form part of the 40 S ribosomal subunit (Fig. 1). In the other processing branch, the 27 S A2 pre-rRNA remains in the nucleolus where it is processed following two alternative routes to give rise to the 5.8 and 25 S rRNA products (Fig. 1). These two rRNAs, together with the 5 S rRNA independently transcribed by RNA polymerase III outside the nucleolus, will be the final RNA components of the 60 S ribosomal subunit (2, 3, 5). These two processing pathways are not mutually dependent, as demonstrated by the isolation of mutants affecting exclusively the production of either 18 or 25 S rRNAs. In addition to the cleavage steps, the pre-rRNAs undergo a number of covalent modifications such as base methylation and isomerization (6, 7).

All rRNA maturation steps require the implication of at least 170 proteins and about as many snoRNPs that assemble with the pre-rRNAs in discrete multiprotein/RNA complexes known as pre-ribosomal particles (3, 4, 8). These molecules are derived from transcription of specific loci catalyzed by either RNA polymerase II (proteins) or III (snoRNAs). By analogy to the ribosomal subunits, pre-ribosomal complexes have been classically referred to as 90, 66, and 43 S according to their specific sedimentation profile on sucrose gradients (9, 10). The 90 S particles contain assembly/processing factors that mediate the early cleavage of the 35 S pre-rRNA precursor at sites A0, A1, and A2. The 43 and 66 S particles harbor downstream pre-rRNAs involved in the production of 40 and 60 S ribosomal subunits, respectively. Despite significant progress made using genetic approaches during the last decade, the complexity of the pre-ribosomal particles precluded until very recently their structural and biochemical characterization. This impediment has been tackled recently through the use of proteomics, a high throughput approach that allowed the identification of a plethora of specific 90, 60, and 40 S components and protein subcomplexes (3, 4, 8, 11). The preliminary characterization of some of these new proteins in yeast has shown that their elimination usually leads to a lethal phenotype derived from the block of specific rRNA maturation branches, suggesting that the architecture of the different pre-ribosomal particles requires a multidimensional network of intermolecular interactions for maintaining both their stability and rRNA matura-
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Fig. 1. Schematic representation of the endonucleolytic cleavage steps leading to the generation of the mature rRNA species from a common 35 S rRNA nucleolar precursor. A, the 35 S pre-rRNA precursor contains the nucleotide sequences for the 18, 5.8, and 25 S rRNAs. These sequences are flanked by 5’ and 3’ ETS and separated by two ITS. B, under normal biosynthetic conditions, the 35 S pre-rRNA transcribed by DNA polymerase I is cleaved at site A0, producing the 33 S pre-rRNA species. This molecule is then cleaved rapidly at site A1 to generate the 32 S pre-rRNA, which, in turn, is cleaved at site A2 to produce the 20 and 27 S A2 pre-rRNAs. The 20 S pre-rRNA is exported to the cytoplasm where it undergoes dimethylation by Dim1 and further cleavage at site D to generate the mature 18 S rRNA. 27 S A2 is processed following two alternative routes. In one case, the precursor is cleaved at site A3, generating the 27 S A3. This product is then processed by exonuclease digestion at site B1 to generate the 27 S B1 species. In the other case, 27 S A2 is trimmed by an unknown mechanism to generate the 27 S B2 intermediate. 27 S B2 and 27 S B3 are processed by similar steps to render 5.8 and 25 S mature rRNAs. First, an endonucleolytic cleavage at site C1 generates the 7 S and the 26 S pre-rRNAs. The 7 S pre-rRNA is then digested 3’ to 5’ to generate a transient 6 S species that, upon further maturation, gives rise to the 5.8 S rRNAs. The 26 S pre-rRNA is digested 5’ to 3’ to give rise to the 25 S pre-rRNA that, in turn, finally matures to generate the 25 S rRNA. More detailed information about this maturation process can be found in Refs. 2, 3, and 5.

In this work, we have started to apply this experimental approach to one of the recently identified components of the 90 S pre-ribosomal particle, the yeast periodic tryptophan protein 2 (Pwp2). Pwp2 is a 923-amino acid-long protein mainly composed by a repetitive arrangement of 8 WD domains, a motif distribution consistent with a β propeller tridimensional structure (12). In addition, it contains a highly conserved coiled-coil domain of unknown function at the C terminus. Pwp2 was discovered in humans in 1996 due to its close proximity to AIRE, a gene locus involved in progressive myoclonus epilepsy (13, 14). At the same time, its yeast homologue was isolated during the characterization of the Saccharomyces cerevisiae chromosome III. Since then, the sequencing of different genomes revealed that PWP2 has been conserved as a single copy gene in all eukaryotes. Although this high structural and phylogenetic conservation points toward an essential function for this protein, its specific role in the cell has remained unsettled until recently. Initially, Pwp2 was described as an essential protein in yeast with a subcellular distribution in cytoplasmic clusters (15). Moreover, it was observed that yeast deficient in Pwp2 generated chains of cells that could not separate due to defects in septum hydrolysis. Based on these observations, it was assumed that Pwp2 was a cytoskeletal-related protein involved in the hydrolysis of the cell wall at the septum after cell division (15). In contrast to this, Pwp2 was recently identified as a nucleolar protein involved in 18 S rRNA biogenesis that forms part of the 90 S pre-ribosomal particle and of a small 90 S pre-ribose subcomplex (11, 16, 17). Independently to those studies, we focused on Pwp2 during our search for regulators of cell morphogenesis in yeast. During such characterization, we found that Pwp2 is involved in ribosomal biogenesis.

Notwithstanding these advances, there are still important questions to be addressed to understand all rRNA biogenesis steps. Among those, perhaps the most challenging one is unveiling the specific role of each of the identified proteins in the complex functional network of pre-rRNA processing. Such characterization will need the individual functional analysis of each pre-ribosomal component, the monitoring of its dynamic behavior (assembly, disassembly, protein levels, and posttranslational modifications) during the full process of rRNA biogenesis, as well as the understanding of the relative position of each molecule in relation to the other proteins and RNAs of the particle. In this context, another important question is to reveal the building blocks contributing to the tridimensional structure of each pre-ribosomal particle and to define whether the numerous protein subcomplexes identified by systematic proteomic analysis correspond to functional particle subunits. Given their inherent complexity, the resolution of these pending questions will probably require a multifaceted approach combining genetic, biochemical, and proteomic techniques.

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### EXPERIMENTAL PROCEDURES

**Yeast Strains and Genetic Methods—Conditional mutant strains for Pwp2 (YMD12, MPP10 (YMD27), and NOP58 (YMD59) under the control of the repressible GAL1 promoter were generated by the one-step PCR strategy (18). This strategy resulted in the in-frame fusion of either KANMX-GAL1-TRP1-GAL1-TRP1-GAL1 HA cassettes upstream of the ATG of the appropriate gene. These strains are referred to in the main text as GAL:Ha-pwp2, GAL:Ha-nop58, and GAL:Ha-nop58.**

**Epitope-tagged gene strains (YMD19, YMD43, YMD44, YMD57, YMD66, and YMD68) were also generated by PCR and one-step insertion by homologous recombination in the genome. In these strains, the epitope-tagged versions are the only source of the proteins in question, and their expression is driven from their cognate promoters.**

**Genetic manipulations were done using standard procedures.** Correct integrations were corroborated by PCR. The genotypes of all the yeast strains used in this study are listed in Table I. Growth of yeast strains was done in either galactose (YPGal, 0.4% yeast extract, 0.8% peptone, 0.1% adenine, 2% galactose)- or glucose (YPD, 0.4% yeast extract, 0.8% peptone, 0.1% adenine, 2% dextrose)-containing medium.

### Western Blot Analysis—Preparation of total cellular lysates for direct immunoblotting was performed as described previously (19). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose filters using a Trans-BlotSTM apparatus (Bio-Rad). After incubations with the appropriate antibodies, immunoreactive bands were detected using a Trans-BlotTM apparatus (Bio-Rad). After incubations with antibodies at 4 °C, immunoblots were performed as described previously (19). Proteins and RNA samples from the same immunoprecipitations were sequentially incubated with different antibodies or radiolabeled oligonucleotide probes.

### RNA Preparation and Northern Blot Analysis—RNAs derived from yeast cells, immunoprecipitates, or gradient fractions were isolated using the hot-phenol procedure (20). Analysis of RNA integrity and quantitation of 25/18 S ratios were performed on an Agilent Bioanalyzer™. Total RNAs were separated in either 1.2% agarose-formaldehyde or 8% acrylamide, 8 μm gels and transferred to Hybond-N+ membranes (Amersham Biosciences). The following oligonucleotides were used for analyzing pre-rRNA intermediaries (see Fig. 3): CACGCCTTCCGCGCCGT-3′, derived from 0.5 ml of 50 mM sodium acetate, 10 ml EDTA (pH 5.2), 1% SDS, and processed for RNA extraction. After ethanol precipitation, RNAs were resuspended in 20 μl of RNA dot-blot buffer (7.5 mM sodium citrate, 75 mM NaCl, 50% formamide, 0.5% formaldehyde). After denaturation at 68 °C for 15 min, RNAs were immobilized onto Hybond-N+ nylon membranes using a Milliblot-D system (Millipore) and UV-cross-linked before hybridization. In some cases (i.e. Fig. 5), membranes containing either protein or RNA samples from the same immunoprecipitations were sequentially incubated with different antibodies or radiolabeled oligonucleotide probes.

### Sucrose Gradient Analysis—Polyacrylamide analysis by sucrose gradient centrifugation was documented as described (22). In brief, cell cultures (200 ml) were grown to an_\text{opt}_ between 0.8 and 1.0. Immediately before harvesting, cycloheximide (Sigma) was added to a final concentration of 0.1 mg/ml-1. Cells were lysed in 700 μl of PP lysis buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 30 mM MgCl2, 0.1 mM dithiothreitol, 0.2% Triton X-100) supplemented with a mixture of protease inhibitors (Complete™, Roche Applied Science). Prechilled lysates were incubated with antibodies at 4 °C for 2 h under gentle rotation. Monoclonal antibodies used were anti-Nop1 (EnCor Biotechnology), anti-MYC (Roche Applied Science), and anti-HA (Covance).

### Purification of the Pwp2 Complex and Mass Spectrometry Analysis

The 25–30 S Pwp2 complex was purified from the YMD59 strain (see Table I) using a large scale co-immunoprecipitation approach. To this end, yeast cultures (2 liters) were grown to an_\text{opt}_ of 0.8–1.0. Cells were harvested in 5 ml of cold IP buffer and washed with a bead beater (Bioseps). The lysates obtained were centrifuged for 20 min at 20,000 × g at 4 °C and supernatants transferred to 70Ti ultracentrifuge tubes (Beckman). After a centrifugation at 100,000 × g for 2 h at 4 °C, the supernatants were incubated for 2 h at 4 °C with 200 μl of an anti-MYC 9E10 affinity matrix (Covance). This mix was transferred to a dispos.
able chromatography column (Bio-Rad) and washed with 40 ml of IP buffer. Bound material was eluted in SDS-PAGE loading buffer and loaded in an 8% SDS-polyacrylamide gel. After electrophoresis, gels were silver-stained and gel sections with the proteins of interest sliced out. Samples were subjected to in-gel trypsin digestion and the resulting fragments analyzed in a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Ultraflex, Bruker). Protein identification was performed by searches on the Swiss-Prot data base using the Mascot algorithm. These procedures were performed in the Genomics and Proteomics Unit of the Centro de Investigacion del Cancer of Salamanca.

RESULTS

Pwp2 Depletion in Yeast Cells Causes Defects in Bud Morphogenesis and rRNA Biogenesis—Since Pwp2 function is essential for S. ceriseiae (15), we decided to generate an inducible yeast strain in which the endogenous PWP2 locus was replaced by a GAL::HA-pwp22 construct. Due to the presence of the GAL1 promoter in this new allele, this yeast strain can now express a HA-tagged version of Pwp2 in a galactose-dependent manner. In order to perform microarray experiments confirming that the shift of this yeast strain from a galactose to a glucose-rich medium induced a strong repression of the GAL::HA-pwp22 allele in a time-dependent manner (Fig. 2A, inset). These cells also underwent a dramatic decrease in their growth rates under such conditions (Fig. 2A), confirming that the GAL::HA-pwp22 construct is a truly conditionally null allele of the PWP2 gene.

To characterize the defects responsible for the lethal phenotype of yeast lacking Pwp2, we first examined processes related to cytoskeletal organization and cell division. Microscopic analysis of GAL::HA-pwp22 cells growing in galactose medium indicated that they have a normal morphology (Fig. 2B, left panel). However, in the presence of glucose medium, GAL::HA-pwp22 cells increase significantly in size and display large vacuoles in the cytosol (Fig. 2B, right panel). A significant percentage of cells (~20%) show also abnormal cell division, as evidenced by the presence of enlarged buds with pronounced apical growth that do not separate from the mother cell even after long periods of time (Fig. 2B, right panel). A small percentage of cells (2%) showed two or even three elongated buds that were kept interconnected along the same polarized axis, suggesting that they have arisen as a consequence of dysfunctions in cytokinesis rather than in cell separation. These phenotypes are different from those reported before for a conditionally null strain of PWP2 (15). In that case, it was reported that Pwp2 depletions induces mostly chains of three to eight connected cells, a defect attributed to dysfunctions in the hydrolysis of the septum. Whether these phenotypic differences are due to different genetic backgrounds or to other causes remains to be determined. To shed light into the causes of this cell division defect, we decided to perform microarray experiments using samples of GAL::HA-pwp22 cells grown in galactose and glucose medium. During the characterization of the total RNA obtained for these experiments, we unexpectedly found that cells lacking Pwp2 have a major defect on the biogenesis of the 18 S rRNA. Accordingly, quantitative analysis of the rRNA content in GAL::HA-pwp22 cells showed that the 25/18 S ratio increases dramatically upon Pwp2 depletion (Fig. 2C). This observation was further confirmed by Northern blot analysis of total RNAs using probes specific for different mature rRNAs species. As seen in Fig. 2D, the loss of Pwp2 expression correlates with a progressive reduction of the 18 S rRNA species, while it does not affect the levels of 25 S, 5.8 S, and 5 S rRNAs.

To see the impact of this defect on the production of ribosomes, we evaluated the steady-state levels of these organelles in the presence or absence of Pwp2 in yeast cells. GAL::HA-pwp22 cells show a normal polysomal profile when grown in galactose medium, including normal levels of free ribosomal 40 and 60 S subunits, monosomes (80 S particles), and polysomes (Fig. 2E, right top panel). However, when shifted to glucose medium, these cells show a progressive reduction of free 40 S particles (Fig. 2E, lower panels), the ribosomal subunits that contain the 18 S rRNA. Interestingly, we also observed that the polysome content was significantly reduced in Pwp2-deficient cells despite the presence of relatively high amounts of apparently intact 80 S monosomes (Fig. 2E, lower panels). In agreement with the lack of effect on the 25 and 5.8 S rRNA contents (see Fig. 2D), the free pool of 60 S ribosomal subunits increases in GAL::HA-pwp22 cells grown in glucose and, at later time points, acquire levels even higher than those found at the 80 S peak (Fig. 2E, lower panels). The specific effect of Pwp2 on the biogenesis of the 40 S ribosomal subunit was further confirmed by densitometric quantitation of total ribosomal subunits separated under conditions of polysome runoff and low Mg2+. The A260/40 S ratios for the wild type and GAL::HA-pwp22 cells when grown in the presence of glucose and galactose are 1.2 and 1.6, respectively (Fig. 2F, first and second panels). This ratio increases up to 3.8 in GAL::HA-pwp22 cells growing in glucose (Fig. 2F, right panel). Wild type cells show no alterations in 40 S biosynthesis when transferred to glucose medium (Fig. 2, E and F), further confirming that those effects are due to the specific loss of Pwp2 expression rather than to an indirect effect derived from the culture conditions used. These results indicate that Pwp2 depletion leads to a specific reduction of 18 S rRNA levels and consequently to a 40 S ribosomal subunit deficit, which blocks normal ribosomal production. During the elaboration of this work, two groups independently identified Pwp2 as a component of the 90 S pre-ribosomal particle (16, 17). In agreement with this, we could demonstrate using both co-immunoprecipitation and gradient centrifugation experiments that Pwp2 is an integral part of the 90 S pre-ribosomal particle (see below). These results, coupled to the published observations, point toward an implication of Pwp2 in the early steps of ribosomal biogenesis that lead to the generation of the 40 S subunit. In addition, we have found that the depletion of Pwp2 protein leads to bud morphogenesis defects in yeast cells. The characterization of these defects and their relationship to the deficit in ribosome biogenesis is currently under investigation and will be the subject of a separate study. The analysis of the implication of Pwp2 in the initial steps of pre-rRNA processing is reported below.

Pwp2 Is Required for Pre-rRNA Cleavage at Sites A0, A1, and A2—The elimination of 40 S ribosome units observed in Fig. 2 is consistent with Pwp2 having a specific role in the initial steps of the processing of the 35 S pre-rRNA, the polycistronic rRNA precursor that contains the 18, 25, and 5.8 S rRNAs. This processing takes place through two basically independent maturation branches in yeast (see Fig. 1) (2, 3, 5). To investigate the specific role of Pwp2 in rRNA biogenesis, we performed Northern blot analysis to monitor the effect of the loss of Pwp2 protein in all those maturation events. To this end, we cultured our conditionally null GAL::HA-pwp22 yeast strain in glucose medium, and after the indicated periods of time, we isolated total RNAs. As comparative control, we obtained RNAs from GAL::HA-pwp22 cells before the shift from the galactose to the glucose rich medium. To eliminate indirect effects derived from the growth in glucose medium, we also isolated in parallel RNAs from wild type cells growing for the indicated periods of time in glucose medium. Total RNAs were then subjected to

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2 M. Dosil and X. R. Bustelo, unpublished observations.
FIG. 2. Pwp2 protein depletion affects cell morphology, 18 S rRNA levels, and 40 S ribosomal subunit production. A, growth curves of wild type (diamonds) and conditionally null mutant GAL::HA-pwp2 (triangles) cells after transfer from galactose- to glucose-containing medium for the indicated periods of time. Inset, time-dependent depletion of Pwp2 after the shift of the GAL::HA-pwp2 strain to glucose medium. GAL::HA-pwp2 cells were harvested and lysed after the indicated hours of culture in glucose-containing medium. Equivalent amounts of total cellular lysates (40 μg) were separated electrophoretically, transferred onto nitrocellulose filters, and subjected to anti-HA immunoblot analysis. Equal loading of samples was corroborated by Ponceau red (Sigma) staining of the immobilized proteins before the immunoblotting.

B, morphology of Pwp2 depleted cells. GAL::HA-pwp2 cells were photographed when growing in YPGal (left panel) or after being transferred to YPD for 30 h (right panel).

C, relative content of 25 and 18 S rRNAs in Pwp2 depleted cells. Total RNAs were prepared from GAL::HA-pwp2 cells following transfer to glucose-containing YPD medium for the indicated periods of time. The relative contents of the 25 and 18 S rRNAs were quantitated using the Agilent Bionalyzer™ apparatus according to the specification of the commercial supplier.

D, Northern blot analysis of total RNAs obtained from wild type (PWP2) cells grown in YPD for 24 h (first lane on the left) and GAL::HA-pwp2 cells grown either in YPGal (time point 0) or YPD for the indicated times. Filters were hybridized to oligonucleotide probe 3 (complementary to the 25 S rRNA; second panel from top), probe 2 (complementary to the 18 S rRNA; second panel from top), probe 7 (complementary to the 5.8 S rRNAs; third panel from top), and a probe specific for 5 S rRNA (lower panel). The location of each probe within the rRNA molecules is shown in Fig. 3A. E, polysome analysis of the wild type PWP2 strain cultured in YPD (top left panel) and the GAL::HA-pwp2 strain cultured in either YPGal (top right panel) or YPD (bottom panels) for 18 h. Extracts (5 A260 units) prepared in low Mg2+/H1001 and in the absence of cycloheximide were resolved in low Mg2+/H1001 linear sucrose gradients and quantitated as indicated above. Gal, galactose; Glu, glucose.

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Fig. 2.
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Fig. 3. Pwp2 depletion affects pre-rRNA processing at sites \( A_0 \), \( A_1 \), and \( A_2 \). A schematic representation of the 35 S pre-rRNA showing the positions of the oligonucleotide probes (1–8) utilized in the Northern blot experiments. B–E, steady-state levels of pre-rRNA precursors in the presence or absence of Pwp2. Wild type (\( PWP2 \)) and conditionally null \( GAL::HA-pwp2 \) strains were grown in YP-Gal and shifted to YPD. At the indicated times, total RNA was extracted and examined by Northern blot analysis using probes 4 (\( B \), 5 (\( C \), 6 (\( D \), and 1a (\( E \)). In some cases (\( E \)), we used also a conditionally null mutant strain for MPP10 (\( GAL::HA-mpp10 \)) cells as a positive control. The migration of pre-rRNA precursors is indicated by either arrows or arrowheads on the right side of each panel.

Northern blot analysis using probes specific for each cleavage step of the 35 S precursor (Fig. 3A). Hybridization of RNAs with probe 4, complementary to a region between sites D and \( A_2 \) of the ITS1 (Fig. 3A), revealed that the accumulation of the 20 S pre-rRNA is totally impaired in Pwp2 deficient cells (Fig. 3B, arrow). Probing the same RNAs with oligonucleotide 5, which is complementary to ITS1 sequences between sites \( A_2 \) and \( A_3 \) (Fig. 3A), evidenced also an additional defect in the production of the 27 S \( A_2 \) pre-rRNA (Fig. 3C, arrow). Unexpectedly, these Northern blots did not detect the presence of a 23 S pre-rRNA species (Fig. 3, B and C), an aberrant by-product usually observed when defective cleavage occur at \( A_0 \), \( A_1 \), and \( A_2 \) sites (Fig. 1). Due to this, we decided to modify our technique of analysis, loading larger quantities of total RNA and running them much longer in denaturing agarose gels. In addition, we used as positive control total RNAs obtained from \( GAL::HA-mpp10 \), a yeast strain conditionally deficient for a protein (Mpp10) previously shown to interfere with the cleavage of the 35 S pre-rRNA at those sites (23). Under these new conditions, we could detect the expected 23 S by-product in both Pwp2 and Mpp10 depleted cells after hybridizing the total RNAs to an oligonucleotide probe located upstream of the \( A_0 \) cleavage site (Fig. 3, A and E). Accumulation of a 23 S pre-rRNA species after the glucose switch was also detected with oligonucleotide 5 (located between sites \( A_2 \) and \( A_3 \) (Fig. 3A)) but not with oligonucleotide 6 (located downstream of cleavage site \( A_3 \); Fig. 3A), indicating that the aberrant 23 S rRNA molecule extends from the 5′ end to the \( A_3 \) site of the 35 S pre-rRNA. Regardless of the method of analysis, all probes used in these experiments revealed an accumulation of the 35 S primary transcript in \( GAL::HA-pwp2 \) cells grown in glucose (Fig. 3, B–D, arrowheads), further suggesting that all the early cleavage steps from \( A_0 \) to \( A_2 \) are indeed inhibited in cells depleted of Pwp2. In contrast to these results, the hybridization of total RNAs derived from these experiments with probe 8, an oligonucleotide complementary to ITS2 sequences located between sites \( E \) and \( C_2 \) (Fig. 3A), revealed normal levels of the 27 S B pre-rRNA in the absence of Pwp2 (Fig. 3D, arrow). This result further strengthens our previous observations indicating that Pwp2 is not essential for the processing branch that generates the mature 25 and 5.8 S rRNAs (see Fig. 1). Since genetic depletion of the U3 or U14 snoRNAs inhibits processing of 18 S rRNA (2), we finally investigated whether the depletion of Pwp2 could affect the accumulation of these snoRNAs. Northern blot analysis indicated that the expression of U3, U14, and other small RNAs remains unchanged in the absence of Pwp2 protein (Fig. 3F). Taken together, these results indicate that Pwp2 plays a direct role in the early processing of pre-rRNAs at the \( A_0 \), \( A_1 \), and \( A_2 \) sites, a maturation step that is essential for 18 S rRNA generation.

Pwp2 Is Needed for the Assembly of U3-snoRNP into 90 S Pre-ribosomal Particles—Since Pwp2 lacks any obvious catalytic domain, we hypothesized that its role in pre-RNA processing at \( A_0 \), \( A_1 \), and \( A_2 \) sites was probably related with the overall formation and/or composition of the 90 S pre-ribosomal particle rather than with the endonucleolytic cleavage events at the 35 S precursor 5′ region. To test this hypothesis, we performed sucrose gradients to analyze the effect of Pwp2 depletion and/or overexpression on the integrity of the 90 S particle. After ultracentrifugation, the fractions obtained were subjected to either Western or Northern blot analysis to monitor the sedimentation profile of Pwp2, previously described components of the 90 S pre-ribosomal particle (Nop1 protein, U3 snoRNA, 35 S), and 27 S pre-rRNAs.
As expected (24), we found that the 35 S and 27 S pre-rRNAs sediment in wild type cells at the ~90 and ~60 S regions of the gradient, respectively (Fig. 4A, second panel from top). The U3 snoRNA is predominantly present in large particles, presumably 90 S pre-ribosomes, which co-sediment with the 35 S pre-rRNA (Fig. 4A, third panel from top). A small portion of the U3 snoRNA is also detected at the upper fractions of the gradient (Fig. 4A, third panel from top, fractions 1–4), the region expected to contain the 10–12 S free U3 monoparticle (25). The sedimentation profile of Nop1, a common protein component of C/D box snoRNP (2, 26–28), resembles that obtained with U3 snoRNA (Fig. 4A, lower panel). In the same gradients, the main fraction of Pwp2 co-sediments with 90 S pre-ribosomes. A lower, but reproducible, proportion of Pwp2 sediments also at the 25–30 S region of the gradient (Fig. 4A, fourth panel from top, fraction 5). Instead, only trace amounts of Pwp2 were found co-sedimenting with the free U3 snoRNP (Fig. 4A, compare third and fourth panels from top). The sedimentation of all these molecules in the expected positions demonstrated that our ultracentrifugation experiments allowed the good separation of pre-ribosomal particles and its components. Moreover, they are consistent with Pwp2 being a bona fide component of the 90 S pre-ribosome and suggest that it might form part of a 25–30 S complex different from free U3 snoRNP.

When cells overexpressing Pwp2 were analyzed, we found that this protein becomes misslocalized at the upper fractions of the gradient (Fig. 4B, fourth panel from top). Despite this, the distributions of 35 S and 27 S pre-rRNAs are similar to those seen in wild type pwp2-HA cells (compare Fig. 4A, second panels from top). Likewise, U3 snoRNA and Nop1 were also detected at the expected peaks (10–12 and 90 S, respectively). However, we noticed an enrichment of both U3 snoRNP and Nop1 in the 10–12 S fraction that is concomitant with a reduction of their levels in the 90 S fraction (compare Fig. 4, A and B, third and fifth panels from top, respectively). This result suggests that the overexpression of Pwp2 induces, despite its delocalization, only a mild negative effect on the assembly of the 90 S pre-ribosomal particle. This is consistent with the normal viability of the GAL::HA-pwp2 strain when grown in galactose.

In contrast to the above observations, the elimination of Pwp2 in yeast cells does induce pronounced changes in the sedimentation profiles of different components of the 90 S pre-ribosomal particle. The most dramatic change is observed with the U3 snoRNA, since it loses its normal co-localization with the 35 S pre-rRNA and accumulates exclusively in gradient fractions corresponding to the size of uncomplexed U3 snoRNP (Fig. 4C, third panel from top). Less dramatic changes are seen in the case of the 35 S pre-rRNA which sediments at 70–80 S (Fig. 4C, second panel from top, fractions 8 and 9), a size slightly smaller than the usual 90 S observed in wild type cells (Fig. 4A, second panel from top, fractions 10–12). The depletion of Pwp2 also affects the distribution of Nop1, since this protein tends now to accumulate preferentially at the region of the free U3 snoRNP particle (Fig. 4C, lower panel). However, unlike free U3 snoRNP, there is still a significant proportion of Nop1 present in fractions of higher sedimentation.
coefficient (Fig. 4C, lower panel, see fractions 7–9), suggesting that the interaction of other C/D box snoRNPs with the 35 S pre-rRNA is not affected by the absence of Pwp2. The loss of Pwp2 has no major effect on the sedimentation profile of the 27 S pre-RNA intermediate (Fig. 4C, second panel from top), a result consistent with our previous observations indicating that Pwp2 does not play any role in the generation of 23 and 28 S rRNAs (see Figs. 2C,D and 3). Altogether, these results indicate that the docking of the U3 snoRNP onto the 35 S pre-rRNA is not an autonomous event, requiring instead the collaboration of Pwp2 and/or Pwp2-associated pre-ribosomal factors.

Effects of Pwp2 on Different Components of the 90 S Pre-ribosomal Particle—To evaluate whether the small U3-containing complexes accumulating in Pwp2-depleted cells represent the fully matured U3 snoRNP, we monitored by co-immunoprecipitation experiments the presence in those complexes of Rrp9 and Nop1 proteins. Rrp9, the yeast homologue of hU3–55K, is a U3-specific binding protein that associates with the U3 snoRNA monoparticle in late maturation steps in the nucleolus (29–31). Nop1, the yeast homologue of fibrillarin, is a pan-specific C/D box snoRNA-binding protein that interacts with the U3 snoRNA precursor and the other core proteins (Nop56, Nop58, Snu13) during early assembly steps taking place in the yeast nucleolar bodies (2, 26–28, 32). To detect Rrp9, we modified its locus in both wild type and conditionally null GAL::HA-pwp2 strains grown in either YPGal or YPD for 18 h. In each case, Nop1 immunoprecipitates were subjected to Western blotting with anti-MYC and anti-Nop1 antibodies, respectively (Fig. 5A and E). Co-immunoprecipitation analysis with RNA species was performed by RNA dot-blot using oligonucleotide probe 1a (third panel from top in A, C, D, and E, named A5 RNA DB), oligonucleotide probe 8 (bottom panel in A, C, D, and E, named EC2 RNA DB), and an oligonucleotide probe for U3 (second panel from bottom in A, C, D, and E; labeled as U3 RNA BD). Probe 1a can hybridize to the full-length 35 S pre-rRNA, to the cleaved 5′/H11032 fragment, and to any aberrant 23 S species (see Figs. 1 and 3A). Probe 8 can hybridize to both the 35 and the 27 S pre-rRNAs (see Figs. 1 and 3A). B, association of Nop1 with pre-rRNAs and U3 snoRNA in Pwp2-depleted cells. In this case, the co-immunoprecipitation analyses were performed in the negative control strain (GAL::HA-nop58 grown in YPD; first lane), in a control wild type YMD19 strain (pwp2-HA; second lane), and in the conditionally null GAL::HA-pwp2 strain grown in either YPGal or YPD for 18 h. In each case, Nop1 immunoprecipitates were subjected to immunoblot and RNA dot-blot analysis as described above (A and C–E). In all cases, the name of the screened molecules is indicated at the left of each panel. The analytical technique used is indicated on the right of each panel. The antibodies used in the immunoprecipitations are indicated at the bottom of each panel. IP, immunoprecipitation; DB, dot blot.
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third panel from top). The interactions of all these molecules with Rrp9 are specific, since no associations are observed in anti-MYC immunoprecipitations derived from lysates containing untagged Rrp9 (Fig. 5A, lane on the left). Nop1 was also found to interact with U3 snoRNP in a Pwp2-independent manner (Fig. 5B, third panel from top). However, unlike Rrp9, this protein is still able to associate with the 35 and 27 S pre-rRNAs in the absence of Pwp2 (Fig. 5B, second and fourth panels from top, respectively). This is probably due to the fact that Nop1 can form complexes with other C/D box snoRNAs, while Rrp9 is specific for U3 (2, 26–28, 31, 33). Consistent with this, the interactions of Nop1 with U3, 35 S RNAs and the 27 S precursor do not occur when the assembly of C/D box snoRNPs is blocked by the elimination Nop58 (Fig. 5B, lane on the left), an essential structural element of the snoRNP core (19, 26). According to these results, the maturation and integrity of U3 and other C/D box snoRNPs is not affected by the lack of Pwp2. Thus, Pwp2 must be mostly required for the binding of the U3 snoRNP particle to the 35 S pre-rRNA precursor.

To study the importance of Pwp2 for the assembly of other protein factors onto the 35 S pre-rRNA precursor, we decided to check its influence on the status of the Mpp10/Imp3/Imp4, a protein subcomplex that participates in the cleavage of this precursor at sites A0, A1, and A2 (34). Although the function of this complex remains largely unknown, recent data indicate that this multiprotein complex appears to interact directly with the U3 snoRNA and the 35 S pre-rRNA via specific RNA binding domains present in Imp3 and Imp4, respectively (34, 35). We explored the effect of Pwp2 depletion on the interactions of Imp3 and Imp4 with the 35 S pre-rRNA, Nop1, and U3 snoRNA using the same co-immunoprecipitation strategy utilized above for Rrp9. As shown in Fig. 5, C and D, the interactions of Imp3 and Imp4 with the 35 S pre-rRNA, the U3 snoRNA, and Nop1 are totally dependent on the expression of Pwp2. Interestingly, the stability of Imp3 and Imp4 is not affected by the depletion of Pwp2 (Fig. 5, C and D, upper panels). Since Imp3 and Imp4 are highly unstable when not associated with Mpp10 (34), this result suggests that the Mpp10/Imp3/Imp4 complex remains stable in the absence of Pwp2. The overexpression of Pwp2 only has minor effects on the interactions analyzed (Fig. 5, C and D, third lanes).

As a control for the specificity of these experiments, we finally tested the effect of Pwp2 on the behavior of Nop7, a protein involved in 25 S biogenesis (36). As shown in Fig. 5E, this protein strongly associates with both Nop1 and the 27 S pre-rRNA. By contrast, it shows only marginal interactions with U3 or the 5’ ETS of the 35 S pre-rRNA (Fig. 3E, third and fourth panels from top). Upon Pwp2 depletion, the interactions of Nop7 with Nop1 and 27 S precursors still occur with very high affinities (Fig. 5E, lower panel), further demonstrating that Pwp2 is not required for the processing branch leading to the 25 and 5.8 S RNAs. In summary, we conclude from these experiments that Pwp2 is essential for the binding of the U3 snoRNP and the Mpp10/Imp3/Imp4 subcomplex to the 35 S pre-rRNA. In addition, we can also infer from our data that Pwp2 is not important for the stability of the Mpp10/Imp3/Imp4 subcomplex or for the maturation and assembly of snoRNPs of the C/D box family.

Depletion of Nop58 Leads to the Accumulation of a Pwp2 Small Subcomplex, Which Interacts Directly with the 35 S Pre-rRNA—The sedimentation profile of Pwp2 in sucrose gradients indicated that, in addition to its association with the 90 S preribosomal particle, this protein forms part of a smaller 25–30 S subcomplex. This subcomplex might correspond to a small Pwp2 hexameric complex identified in two large scale proteomic studies that has not been functionally characterized (11, 16). To shed light into the nature of this Pwp2 subcomplex, we decided to test its stability when the formation of the 90 S particle is blocked. To make possible these experiments, we generated pwp2-MYC strains in either a wild type background or in a conditionally null mutant background for Nop58 (Gal::HA-nop58). In galactose-containing medium, this mutant strain overexpresses Nop58 and is viable. However, when shifted to glucose-containing medium, Nop58 expression is repressed, leading to abnormal U3 snoRNA assembly and to a rapid stop in cell division (19, 26). Immunofluorescence experiments indicated that the depletion of Nop58 does not interfere with the nucleolar localization of Pwp2. Using co-immunoprecipitation coupled with RNA dot-blot analyses, we found that Pwp2 can form stable complexes in vivo with Nop1, 35 S/A0 pre-rRNAs, and U3 snoRNA both in wild type (pwp2-MYC grown in glucose) and Gal::HA-nop58/pwp2-MYC (grown in galactose) cells (Fig. 6A, second, third, fourth and fifth panels from top, respectively). The interaction of Pwp2 with both U3 and Nop1 is highly reduced in Gal::HA-Nop58/pwp2-MYC cells growing in glucose (Fig. 6A, third and fourth panels from top), suggesting that Pwp2 is stably associated with some pre-rRNA sequences in the absence of U3 snoRNA. In contrast, none of the RNA species co-immunoprecipitating with Pwp2 hybridized with a probe specific for 35/27 S pre-rRNAs (Fig. 6A, lower panel). Northern blot analysis evidenced that Pwp2 co-immunoprecipitated with the U3 snoRNA and pre-rRNA species corresponding in size to the 5’ ETS-A0 cleaved fragment (Fig. 6B). Instead, no intact 35 or 23 S pre-rRNA species were found in these experiments.

The absence of association of Pwp2 with the 35 S pre-rRNA could be due to the exclusive binding of this protein to the 5’ ETS-A0 region (Figs. 1 and 3A) or, alternatively, to the degradation of the 35 S molecules during the immunoprecipitation experiments. To discriminate between these two possibilities, we performed sucrose gradient centrifugations of yeast cell lysates and analyzed the pre-rRNA species co-sedimenting with Pwp2. Under conditions of overexpression, Nop58 is mostly delocalized at the upper fractions of the gradient (Fig. 6C, lower panel). Despite this, the sedimentation profiles of both 35 and 27 S pre-rRNAs remain similar to those observed in wild type cells (Fig. 6C, upper panel; compare with the profile shown in Fig. 4, A and B). The U3 snoRNA and Nop1 were also detected at the expected 90 and 10–12 S regions of the gradient, although their relative levels at 90 S pre-ribosomal particles were slightly diminished, showing a clear accumulation of free U3 snoRNA (Fig. 6C, fourth and sixth panels from top, respectively). Much more apparent are the effects of Nop58 overexpression on Pwp2, since a considerable proportion of this protein shifts toward the upper part of the gradient (Fig. 6C, fifth panel from top, fractions 4–6). The region of accumulation of Pwp2 in Nop58 overexpressing cells matches the described small peak of Pwp2 sedimentation previously found in wild type cells (see Fig. 4A, fourth panel from top). Instead, it is clearly distinct from the sedimentation region of free U3 snoRNPs (Fig. 6C, fourth panel from top, fractions 1–5). When Gal::HA-nop58 cells were grown in glucose for 14 h, the cellular Nop58 content undergoes the expected reduction (Fig. 6D, lower panel). Under these conditions, U3 snoRNA and Nop1 levels are also significantly lowered, although they still show their normal sedimentation profiles (Fig. 6D, fourth and sixth panel, respectively). By contrast, Pwp2 was detected mostly at the ~25–30 S fraction, with little amount found at 90 S (Fig.
Fig. 6. Pwp2 is part of a 25–30 S subcomplex that interacts with 5′ ETS sequences of the 35 S pre-rRNA. A. association of Pwp2 with Nop1 and pre-rRNA species in Nop58-depleted cells. PWP2 and pwp2-MYC cells were harvested after growing in YPD. GAL::HA-nop58/pwp2-MYC cells were harvested after growing in either YPGal orYPD for 14 h. Cell extracts from those samples were immunoprecipitated with anti-MYC immunoprecipitates and subjected to immunoblot analysis with anti-MYC (top panel) and anti-Nop1 (second panel from top) antibodies. The pre-rRNA sequences associated with the anti-MYC immunoprecipitates were identified by RNA dot-blot analysis using either the oligonucleotide probes specific for A0–A1 fragment (see Fig. 3A). B. Northern blot analysis of the pre-rRNA species associating with Pwp2. Wild type (PWP2) and GAL::HA-nop58/pwp2-MYC cells were grown in galactose (YPGal) and shifted to glucose (YPD) medium. At the indicated times, cell extracts were prepared and immunoprecipitated with anti-MYC antibodies. After the final wash, each immunoprecipitate was divided in two halves. One half was subjected to immunoblot analysis with anti-MYC antibodies to detect Pwp2 (upper panel). The other half was used to extract the associated RNA that was subsequently analyzed by Northern blot analysis using oligonucleotide probes specific for either U3 (lower panel) or the 5′ ETS-A0 fragment (second panel from top; see Fig. 3A for probe localization within the pre-rRNA). C and D, sedimentation profile of Pwp2 and other 90 S pre-ribosomal components in the presence or absence of Nop58. GAL::HA-nop58/pwp2-MYC cells growing in either YPGal (C) or in YPD for 14 h (D) were harvested, lysed, and fractionated on 7–50% linear sucrose gradients. Pwp2, Nop1, and Nop58 proteins were detected by Western blot using anti-MYC (fifth panels from top), anti-Nop1 (sixth panels from top), and anti-HA antibodies, respectively (lower panels). The sedimentation profiles of the 35 and 27 S pre-rRNAs were revealed by Northern blotting with probe 8 (C and D, upper panels). The 5′ ETS-A0 fragment was detected by Northern blot with probe 1a in both denaturing agarose gels (C and D, second panels from top) and polyacrylamide gels (C and D, third panels from top). The presence of U3 snoRNA (C and D, fourth panels from top) was analyzed by Northern blot, as described in legend to Fig. 4. The localization of each oligonucleotide probe can be seen in Fig. 3A. The name of the screened molecules is indicated at the left of each panel. The analytical technique used is indicated at the right of each panel. The fraction number is indicated at the bottom of the panels.

Characterization of the 25–30 S Pwp2-containing Protein Complex—To determine the composition of the Pwp2 multiprotein complex, we purified stable Pwp2-binding proteins using anti-MYC affinity chromatography. These immunopurifications were performed using cell extracts from GAL::HA-nop58/pwp2-MYC cells grown in glucose for 14 h, conditions under which most of Pwp2 is associated with the 25–30 S complex (see Fig. 6D). The analysis by mass spectrometry of the immunoprecipitates obtained revealed that Pwp2 binds in stoichiometric amounts to Dip2, Utp21, Utp13, Utp18, and Utp6 (Fig. 7), five proteins previously identified as components of the 90 S pre-ribosome (16, 17). This complex is identical to a small 90 S
pre-ribosomal subcomplexes previously identified in two large scale proteomic studies performed in wild type cells (11, 16). Altogether these results indicate that Pwp2, Dip2, Utp21, Utp13, Utp18, and Utp6 constitute a subunit of the 90 S pre-ribosomal particle capable of interacting directly with the 5′ ETS of the 35 S pre-rRNA.

**DISCUSSION**

In this work we report the functional characterization of Pwp2, a WD/β propeller family protein involved in rRNA biogenesis. Using a combination of genetic and biochemical approaches, we have confirmed recent observations indicating that Pwp2 is a nucleolar protein essential for proper 18 S rRNA biosynthesis (16, 17). The close relationship of Pwp2 with the early steps of 18 S rRNA biogenesis was further demonstrated by our experiments showing that the depletion of this protein leads to abnormal processing of the 35 S pre-rRNA precursor at A₀, A₁, and A₂ sites. The deleterious effects of the Pwp2 deficiency impinge exclusively in the 18 S rRNA biosynthetic branch, since the generation of the 25 and 5.8 S rRNAs is not affected. Thus, it seems that Pwp2 is needed in the 90 S particle to allow the proper endonucleolytic cleavages of the 35 S precursor at A₀, A₁, and A₂ sites, but once these steps are accomplished, it is released from the cleaved pre-rRNA to get recycled back onto new nascent 35 S molecules. Our observations indicating that Pwp2 remains associated with the cleaved 5′ ETS-A₀ fragment under certain conditions and that it does not associate with pre-rRNA intermediates of the 25/5.8S biosynthetic branch give further support to the exclusive implication of Pwp2 in the events leading to 35 S pre-rRNA cleavage at A₀, A₁, and A₂ sites. Further dissection of the involvement of Pwp2 in this process indicated that this protein is important for the binding of U3 snoRNP and the Mpp10/Imp3/Imp4 complex to the 5′ end of the 35 S pre-rRNA precursor. However, and in agreement with its lack of association with free U3 snoRNPs, Pwp2 does not interfere with the normal assembly and/or maturation of any of those two complexes prior to their assembly onto 35 S pre-rRNA transcripts.

The assembly of high molecular weight macromolecular complexes in cells is usually based on the serial and repetitive incorporation of proteins with reduced structural variability. One of the best examples of such assembly is the F-actin cytoskeleton whose polymerization is based on the incorporation of G-actin monomers. This simple mechanism is not possible in the case of the 90 S pre-ribosomal particle, since it is composed of more than 50 proteins of different structures and, in some instances, coming from different intranucleolar localizations. In this context, it is difficult to envision how the ordered assembly of the particle may occur, since it is unlikely that each of its protein components would have the sufficient steresospatial information to allow the subsequent incorporation of other components. One possible solution to this architectural problem is to reduce the structural complexity of the 90 S particle by loading its constituents as pre-formed, multi-protein subunits. If this is true, it is reasonable to think that these subunits could be identified in vivo as individual biochemical entities. To see whether this was the case for Pwp2, we utilized a biochemical stratagem based on the blockage of 90 S particle formation via mutations that disrupt U3 snoRNP formation. We hypothesized that, under these conditions, we could visualize any stable subunit components containing Pwp2 without being obscured by the presence of the rest of 90 S particle components. This strategy revealed that Pwp2 does form a stable complex with other proteins, leading to the generation of a multiprotein subunit with a sedimentation profile in sucrose gradients of ~25–30 S. Using mass spectrometry analysis, we could demonstrate that this particle is composed of Pwp2 and five additional proteins: Dip2, Utp6, Utp13, Utp18, and Utp21. Although the specific function of those proteins is still unknown, it is worth noting that all of them contain either WD (Pwp2, Dip2, Utp13, Utp18, Utp21) or HAT (Utp6) domains, two structural motifs often present in RNA processing factors (8, 37). Moreover, all these proteins have been identified before as integral components of the 90 S pre-ribosomal particle and, in the case of Dip2, Utp6, and Utp13, shown to be essential for 18 S rRNA production (17). These data confirm their likely implication on the 18 S rRNA biogenesis pathway. Two independent observations indicate that this Pwp2 subunit is not an artifact generated under our experimental conditions of Nop58 depletion. First, we have shown using gradient sedimentation analysis that the Pwp2 25–30 S particle can be also detected at low levels in wild type cells. Second, this hexameric complex has been detected in wild type cell lysates using tandem affinity purification in combination with size exclusion chromatography or pre-fractionation by high speed centrifugation (11, 16). These results strongly suggest that the 90 S pre-ribosomal particle is composed of several stable subunits, two of which are the U3 snoRNP and the Pwp2 25–30 S complex.

Our biochemical analyses revealed that a considerable proportion of the Pwp2 25–30 S subunit found in Nop58-depleted cells remains associated to the 5′ ETS-A₀ fragment after its cleavage from the 35 S pre-rRNA. Using the same experimental strategy, we could not detect U3 snoRNA or Nop1 associated with Pwp2, indicating that the binding of the Pwp2 complex to the cleaved 5′ ETS-A₀ fragment does not require U3 snoRNP under these conditions. In wild type cells the Pwp2 25–30 S is present in a relatively small proportion and the 5′ ETS-A₀ fragment is not usually detectable due to its rapid degradation after cleavage (38, 39). It is therefore possible that the 25–30 S Pwp2 complex forms a transient intermediate with the 5′ ETS-A₀ that, under wild type conditions, is rapidly destroyed upon recycling of the Pwp2 complex onto new nascent 35 S rRNA transcripts. This may be a distinctive property of the
Pwp2 complex because the cleaved 5’ ETS-A$_0$ fragment does not stick to the U3 snoRNP even when this particle accumulates at high levels in cells lacking Pwp2. According to these data, it seems that the recycling dynamics of the Pwp2 complex and U3 snoRNP are quite distinct after the cleavage step. The observations that the Pwp2 complex can bind to the 5’ ETS-A$_0$ fragment allow us to make also some inferences on the physical interaction between the Pwp2 complex and the 5’ end of the 35 S pre-rRNA, a property only attributed before to U3 snoRNP and the Mpp10/Imp3/Imp4 complex. This interaction, unlike that found with the cleaved 5’ ETS-A$_0$ fragment, cannot occur in the absence of U3 snoRNP as judged by the absence of co-sedimentation of Pwp2 with the 35 S pre-rRNA when the expression of Nop58 has been shut down. This apparent paradox can be explained if we assume that the binding of U3 snoRNP and its associated Mpp10/Imp3/Imp4 complex to the 35 S pre-rRNA induces a conformational change in the pre-rRNA that allows its subsequent recognition and binding by the Pwp2 complex. Alternatively, it is possible that the binding of U3 snoRNP/Mpp10/Imp3/Imp4 to the Pwp2 complex would expose its RNA binding domain by inducing the release of the bound 5’ ETS-A$_0$ fragment present from the previous cleavage event. In any event, it is important to take into consideration that Pwp2 is also essential for the binding of U3 snoRNP and Mpp10/Imp4 to the 35 S pre-rRNA, suggesting that the initial step in the biogenesis of the 18 S rRNA is the concerted and mutually dependent binding of this multiprotein complexes to the polycistronic pre-rRNA precursor.

By performing pre-ribosomal particle analysis in individual conditional mutants we have shown that the assembly of the early 90 S pre-ribosome is a stepwise process and that some of its subunits, such as the U3 snoRNP and the Pwp2 subcomplex, are independently assembled. It may be interesting to perform similar analysis to those shown here with other uncharacterized 90 S components to reveal additional building blocks of this pre-ribosomal particle. Taking the Pwp2 subcomplex as a reference, we may anticipate that some of these blocks will be discrete pre-ribosomal subcomplexes already identified by proteomic techniques. However, large scale protein purification schemes may not be revealing all particle subcomplexes. A good example of this is the U3 snoRNP monoparticle, whose components (Nop1, Nop58, Nop56, Snu13, and Rpr9) were identified several years ago both in yeast and animal cells (33, 40). This small ribonucleoprotein complex has not been discretely found in systematic pre-rRNA processing proteomic analysis performed in wild type cells, even when its specific component (Rpr9) was tagged and used as bait (11, 16). This could be due to the fact that the free U3 snoRNP is normally present in low amounts or that it is unstable under certain protein tagging or purification schemes. The analysis of individual mutant strains (e.g. U3 snoRNP in Pwp2-depleted cells), which accumulate particle subunits due to assembly defects might overcome such problems. Application of proteomic analysis to such specific mutant strains in combination with detailed biochemical characterization of subunit components should improve our understanding of pre-ribosomal particle structure, assembly, and function.

In addition to its implication on rRNA biogenesis, we have found that the deletion of the PWP2 gene leads to abnormal bud morphogenesis and defective cell cycle progression (Fig. 2). At this moment, it is difficult to discriminate whether the latter problems are due to an indirect effect derived from abnormal ribosome function or, alternatively, a direct implication of Pwp2 and/or associated proteins in those cellular functions. Intriguingly, recent proteomic analyses have revealed the physical association of ribosome biogenesis factors with multi-protein complexes involved in other unrelated cellular functions (41, 42). Moreover, previous reports have shown that the mutation of some of these factors also affects in different aspects of the yeast cell cycle. Thus, it has been described that Nop15/YNL110c/NFK participates in cytokinesis (43), Yph1/Nop7 and Noc9 in DNA replication (44, 45), and Sda1 in progression through G1 (46, 47). Interestingly, all these proteins have been specifically involved in 60 S but not in 40 S ribosomal subunit biogenesis. Continuing efforts in the characterization of Pwp2 will contribute to shed light into the actual participation of this protein in pre-rRNA processing and other biological processes.

Acknowledgments—We thank M. Blázquez, S. Ogueta, and E. Fermián for the expert technical assistance provided. We thank M. Tamame and members of her laboratory for protocols and reagents and for critically reading the manuscript. We also appreciate the advice of R. Daga in gradient fractionation experiments and the comments of J. de la Cruz and J. P. Ballesta.

REFERENCES

1. Warner, J. R. (1999) Trends Biochem. Sci. 24, 437–440
2. Venema, J., and Tollervey, D. (1999) Annu. Rev. Genet. 33, 261–311
3. Fatica, A., and Tollervey, D. (2002) Curr. Opin. Cell Biol. 14, 313–318
4. Tschochner, H., and Hurt, E. (2000) Trends Cell Biol. 10, 255–263
5. Kressler, D., Linder, P., and de La Cruz, J. (1999) Mol. Cell. Biol. 19, 7979–7912
6. Krüger, T. (2001) EMBO J. 20, 3617–3622
7. Decatur, W. A., and Fournier, M. J. (2003) J. Biol. Chem. 278, 695–698
8. Fromont-Racine, M., Senger, B., Saveanu, C., and Fasiolo, F. (2003) Gene (Amst.) 313, 17–42
9. Uden, S. A., and Warner, J. R. (1972) J. Mol. Biol. 65, 227–242
10. Trapman, J., Retel, J., and Planta, R. J. (1975) Exp. Cell Res. 90, 95–104
11. Krogan, N. J., Peng, W. T., Caygney, G., Robisonn, M. D., Haw, R., Zhong, G., Guo, X., Zhang, X., Cannadie, V., Richards, D. P., Beattie, B. K., Lalev, A., Zhang, W., Davierwala, A. P., Maiman, J. B., Starostine, A., Tikitusii, A. P., Griguil, J., Datta, N., Bray, J. E., Hughes, T. R., Emili, A., and Greenblatt, J. F. (2004) Mol. Cell 13, 225–239
12. Li, D., and Roberts, R. (2001) Cell Mol. Life Sci. 58, 2085–2097
13. Yamakawa, K., Gao, D. Q., and Korenberg, J. R. (1996) Cytogeten. Cell Genet. 74, 140–145
14. Lalioti, M. D., Chen, H., Rossier, C., Shaafaitian, R., Reed, J. D., and Antonarakis, S. E. (1996) Genomics 33, 321–327
15. Shaafaitian, R., Payton, M. A., and Reed, J. D. (1996) Gen. Mol. Biol. 252, 101–114
16. Grandi, P., Rybin, V., Bassler, J., Petfalski, E., Straus, D., Marziouch, M., Schafer, T., Kuster, B., Tschochner, H., Tollervey, D., Gavvin, A. C., and Hurt, E. (2002) Mol. Cell 9, 105–115
17. Dragon, F., Gallagher, J. E., Compagnone-Post, P. A., Mitchell, B. M., Porwancher, K., Wehner, K., Wilm, M., Rosbash, M., Branlant, C., and Luhrmann, R. (2000) Science 281, 1401–1404
18. Lafontaine, D. L., and Tollervey, D. (1999) RNA (N. Y.) 5, 455–467
19. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1984) Current Protocols in Molecular Biology, Vol. 2, John Wiley & Sons, Inc., New York
20. Kressler, D., de la Cruz, J., Rojo, M., and Linder, P. (1997) Mol. Cell. Biol. 17, 7283–7294
21. Fei, M., Cigan, A. M., Paddock, C. J., Harashina, S., and Hinnebusch, A. G. (1991) Mol. Biol. Cell 2, 3203–3216
36. Oeffinger, M., Leung, A., Lamond, A., Tollervey, D., and Lueng, A. (2002) RNA (N. Y.) 8, 626–636
37. Preker, P. J., and Keller, W. (1998) Trends Biochem. Sci 23, 15–16
38. Allmang, C., Pfeil, E., Podtelejnikov, A., Mann, M., Tollervey, D., and Mitchell, P. (1999) Genes Dev. 13, 2148–2158
39. de la Cruz, J., Kressler, D., Tollervey, D., and Linder, P. (1998) EMBO J. 17, 1128–1146
40. Pluk, H., Soffner, J., Luhmann, R., and van Venrooij, W. J. (1998) Mol. Cell. Biol. 18, 488–498
41. Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S. L., Millar, A., Taylor, P., Bennett, K., Boutiler, K., Yang, L., Wolting, C., Donaldson, I., Schandorff, S., Shewnarane, J., Va, M., Taggart, J., Goudreault, M., Muskat, B., Alfarano, C., Dewar, D., Lin, Z., Michalickova, K., Willems, A. R., Sassi, H., Nielsen, P. A., Rasmussen, K. J., Andersen, J. R., Johansen, L. E., Hansen, L. H., Jespersen, H., Podtelejnikov, A., Nielsen, E., Crawford, J., Poulsen, V., Sorensen, B. D., Matthiesen, J., Hendrickson, R. C., Gleeson, F., Pawson, T., Moran, M. F., Durocher, D., Mann, M., Hogue, C. W., Figeys, D., and Tyers, M. (2002) Nature 415, 180–183
42. Gavin, A. C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruciat, C. M., Remor, M., Hofert, C., Schelder, M., Brajenovic, M., Ruffner, H., Mertz, A., Klein, K., Hudak, M., Dickson, D., Rudi, T., Gnau, V., Bauch, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M. A., Copley, R. R., Edelmann, A., Querfurth, E., Rybin, V., Drewes, G., Raida, M., Bouwmeester, T., Bork, P., Seraphin, B., Kuster, B., Neubauer, G., and Superti-Furga, G. (2002) Nature 415, 141–147
43. Oeffinger, M., and Tollervey, D. (2003) EMBO J. 22, 6573–6583
44. Du, Y. C., and Stillman, B. (2002) Cell 109, 835–848
45. Zhang, Y., Yu, Z., Fu, X., and Liang, C. (2002) Cell 109, 849–860
46. Zimmerman, Z. A., and Kellogg, D. R. (2001) Mol. Biol. Cell 12, 201–219
47. Buscemi, G., Saracino, F., Masnada, B., and Carbone, M. L. (2000) J. Cell Sci. 113, 1199–1211