# Pol5 is an essential ribosome biogenesis factor required for 60S ribosomal subunit maturation in *Saccharomyces cerevisiae*

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## ABSTRACT

**In *Saccharomyces cerevisiae*, more than 250 *trans*-acting factors are involved in the maturation of 40S and 60S ribosomal subunits. The expression of most of these factors is transcriptionally coregulated to ensure correct ribosome production under a wide variety of environmental and intracellular conditions. Here, we identified the essential nucleolar Pol5 protein as a novel *trans*-acting factor required for the synthesis of 60S ribosomal subunits. Pol5 weakly and/or transiently associates with early to medium pre-60S ribosomal particles. Depletion of and temperature-sensitive mutations in Pol5 result in a deficiency of 60S ribosomal subunits and accumulation of half-mer polysomes. Both processing of 27SB pre-rRNA to mature 25S rRNA and release of pre-60S ribosomal particles from the nucle(ol)us to the cytoplasm are impaired in the Pol5-depleted strain. Moreover, we identified the genes encoding ribosomal proteins uL23 and eL27A as multicopy suppressors of the slow growth of a temperature-sensitive *pol5* mutant. These results suggest that Pol5 could function in ensuring the correct folding of 25S rRNA domain III; thus, favoring the correct assembly of these two ribosomal proteins at their respective binding sites into medium pre-60S ribosomal particles. Pol5 is homologous to the human tumor suppressor Myb-binding protein 1A (MYBBP1A). However, expression of MYBBP1A failed to complement the lethal phenotype of a *pol5* null mutant strain though interfered with 60S ribosomal subunit biogenesis.**

**Keywords: ribosome biogenesis; pre-rRNA processing; MYBBP1A; ribosomal proteins uL23 and eL27; nucleolus**

## INTRODUCTION

Ribosomes are ubiquitous organelles that perform the translation of the mRNAs. In all organisms, ribosomes are composed of two ribosomal subunits (r-subunits), the large subunit being about twice the molecular mass of the small one. Both r-subunits are built from ribosomal RNAs (rRNAs) and ribosomal proteins (r-proteins). In the yeast *Saccharomyces cerevisiae*, the large or 60S r-subunit is composed of three rRNA species (25S, 5.8S, and 5S) and 46 r-proteins, whereas the small or 40S r-subunit contains the 18S rRNA and 33 r-proteins (for reviews, see Melnikov et al. 2012; Wilson and Doudna Cate 2012). The complex network of interactions connecting the rRNAs with the individual r-proteins and between rRNA segments or between different r-proteins has been revealed at atomic resolution by the crystal structures of the 80S ribosome and the two r-subunits (for reviews, see Jenner et al. 2012; Klinge et al. 2012; Yusupova and Yusupov 2014).

The synthesis of ribosomes is a major cellular activity. In yeast, it has been estimated that more than 2000 ribosomes are produced per minute, thus, the content of an exponentially growing cell is about 200,000 ribosomes (Warner 1999). Due to the huge investment of resources

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devoted to ribosome production, ribosome biogenesis is a tightly regulated process that involves the coordinated maturation of the precursors rRNAs (pre-rRNAs) and their concomitant assembly together with the r-proteins (for reviews, see Fromont-Racine et al. 2003; Woolford and Baserga 2013; Kressler et al. 2017). Biogenesis of ribosomes is also a highly dynamic process, taking place both in the nuclear and cytoplasmic cellular compartments. In the yeast nucleolus, about half of the 100–200 tandem rDNA copies are transcribed as two primary transcript units; the large one, which is synthesized by RNA polymerase I (RNAP I), encompasses the mature sequences of 18S, 5.8S, and 25S rRNAs, while the precursor of 5S is independently transcribed by RNAP III. Maturation of rRNAs is a well-defined pathway (Supplemental Fig. S1) and involves processing and covalent rRNA modification reactions (for review, see Fernández-Pevida et al. 2015). Pre-rRNA maturation takes place inside preribosomal particles, which are formed by the association of many *trans*-acting assembly factors with the pre-rRNAs and the r-proteins (for reviews, see Woolford and Baserga 2013; Kressler et al. 2017). The latter ones assemble in a hierarchical manner; thus, distinct r-proteins, called primary binding proteins, stably bind directly to the nascent pre-rRNAs, while secondary and ternary binding proteins associate later and always after the previous binding of one or more primary r-proteins; the last r-proteins assemble in the cytoplasm (for review, see de la Cruz et al. 2015). *Trans*-acting factors transiently bind to the particles in a distinctive spatiotemporal manner and ensure the speed, directionality and accuracy of ribosome biogenesis, as they perform specific functions not only during the maturation of the pre-rRNAs but also during the progressive folding of the pre-rRNAs, the assembly of the r-proteins, the acquisition of nuclear export competence, nuclear export, and the final cytoplasmic maturation steps of the preribosomal particles to mature r-subunits (Kressler et al. 1999, 2010, 2017; Venema and Tollervey 1999; Woolford and Baserga 2013; Nerurkar et al. 2015; Peña et al. 2017; Bassler and Hurt 2019). The purification of preribosomal particles by in vivo affinity-purification methods has allowed the isolation, characterization and compositional analysis of several intermediates along the ribosome maturation pathway (for reviews, see Fromont-Racine et al. 2003; Kressler et al. 2010; Woolford and Baserga 2013; Bassler and Hurt 2019). More recently, progress in cryo-electron microscopy (cryo-EM) has revealed the structure at a near-atomic resolution of some of these affinity-purified preribosomal particles, including the precise localization of the *trans*-acting factors that are stably associated with them (for reviews, see Bassler and Hurt 2019; Klinge and Woolford 2019). More importantly, the structures of different particles along the r-subunit assembly pathway provide insights into how *trans*-acting factors, pre-rRNAs, and r-proteins mechanistically participate during the rearrangements and remodeling events of preribosomal particles throughout their subsequent maturation steps (for reviews, see Konikkat and Woolford 2017; Kressler et al. 2017; Peña et al. 2017; Barandun et al. 2018; Bassler and Hurt 2019; Klinge and Woolford 2019).

Practically all protein *trans*-acting factors involved in ribosome biogenesis, more than 250, have been identified and functionally characterized in yeast. A large number of these factors were identified by classical and modern molecular genetics, including the systematic functional analysis of yeast open reading frames, or a consequence of being nucleolar proteins (e.g., see Kressler et al. 1999; Venema and Tollervey 1999; Henras et al. 2008). Many other factors were identified as components of affinity-purified preribosomal particles either isolated by specific or high-throughput approaches (e.g., see Bassler et al. 2001; Harnpicharnchai et al. 2001; Grandi et al. 2002; Nissan et al. 2002; Schäfer et al. 2003; Gavin et al. 2006). Additional factors have been described as the result of integrative and predictive approaches for identifying sets of coregulated genes, as they belong to the so-called "rRNA and ribosome biosynthesis" (RRB) regulon, also known as the "ribosome biogenesis" (RiBi) regulon (Wade et al. 2001, 2006; Jorgensen et al. 2004), by computational network-guided genetics (Li et al. 2009) or directly by studying the pre-rRNA processing defects of yeast mutants by a microarray study (Peng et al. 2003). Nowadays, new *trans*-acting factors are still being discovered as typified by the dedicated chaperones for r-proteins (Pillet et al. 2017).

In this work, we functionally characterize the protein Pol5 (YEL055Cp), thereby, revealing that it plays a role in ribosome biogenesis. Pol5 was initially identified as an essential nucleolar protein, which harbors a DNA polymerase activity in vitro that is fully dispensable in vivo (Shimizu et al. 2002). Instead of being required for DNA replication, it has been suggested that Pol5 is required for transcription of the large pre-rRNA by RNAP I (Shimizu et al. 2002). Consistently, Pol5 weakly copurifies with the so-called UtpA/t-UTP subcomplex (Gallagher et al. 2004; Krogan et al. 2004), which is believed to be the protein module that binds first to the large pre-rRNA during the formation of early 90S preribosomal particles. UtpA association to the large pre-rRNA seems to occur cotranscriptionally and this is required for both efficient pre-rRNA transcription and recruitment of other subcomplexes to these nascent particles (Gallagher et al. 2004; Pérez-Fernández et al. 2007, 2011; Chaker-Margot et al. 2015; Zhang et al. 2016; Gallagher 2019). Our data, however, indicate that Pol5 is an RRB/RiBi factor required for maturation of 60S r-subunits. Thermosensitive (ts) *pol5* mutations and depletion of Pol5 result in a 60S r-subunit shortage that is due to both defective 27S pre-rRNA processing leading to reduced levels of 25S mature rRNAs and impairment of nuclear export of pre-60S r-particles. In agreement with

these phenotypes, affinity purification of a fully functional GFP-tagged Pol5 construct yields mainly 27SB pre-RNAs, therefore, suggesting that Pol5 associates with early/medium pre-60S r-particles. In addition, we also show that overexpression of specific 60S r-proteins (uL23 and eL27A) suppresses the growth defect of selected ts *pol5* mutants. Strikingly, Pol5 shares some similarity to mammalian Myb-binding protein 1A (MYBBP1A), which is a tumor suppressor that represses the activity of selected transcription factors involved in the control of several relevant biological processes such as cellular division, proliferation and apoptosis (e.g., Mori et al. 2012; Felipe-Abrio et al. 2019). MYBBP1A, as Pol5, also participates in rRNA transcription by RNAP I and pre-rRNA processing reactions in the pathway for mature 28S/5.8S rRNA production (Hochstatter et al. 2012; Tafforeau et al. 2013). However, whether Pol5 and MYBBP1A are functional orthologs is still unclear; in this regard, our data show that human MYBBP1A cannot complement a yeast *pol5* null mutant strain.

## RESULTS

### Pol5 is a member of the RRB/RiBi regulon

Genes encoding proteins involved in different aspects of yeast RNA metabolism are known to have similar transcription patterns in cells progressing through different physiological programs (e.g., cell cycle, sporulation, and diauxie) or subjected to a variety of insults (e.g., osmotic or heat shock stresses, and drug treatments such as rapamycin) (Causton et al. 2001; Wade et al. 2001, 2006; Jorgensen et al. 2004). Collectively, all these genes (~250) are classified as members of the RRB/RiBi regulon, which does not include any of the 138 r-protein genes (for reviews, see Broach 2012; Bosio et al. 2017). Despite its possible role in ribosome biogenesis (see Introduction), *POL5* was not ranked among the best approximately 200 genes belonging to the RRB/RiBi regulon and was described as lacking RRPE and PAC consensus sequences in its promoter (see Jorgensen et al. 2004; Wade et al. 2006). These elements are characteristic of the promoter regions of the RRB/RiBi genes. They are located at positions between 50 and 200 bp upstream of their respective translational start sites and play a role in their transcriptional regulation (for reviews, see Bosio et al. 2011, 2017). To determine whether the expression of *POL5* is coregulated with that of *bona-fide* members of the RRB/RiBi regulon, we performed two independent analyses: (i) first, we checked for RRPE and PAC consensus sequences within the promoter region of *POL5*. As depicted in Figure 1A, the *POL5* promoter indeed contains a degenerate RRPE box in the −150 bp position with only one deviation in its first nucleotide

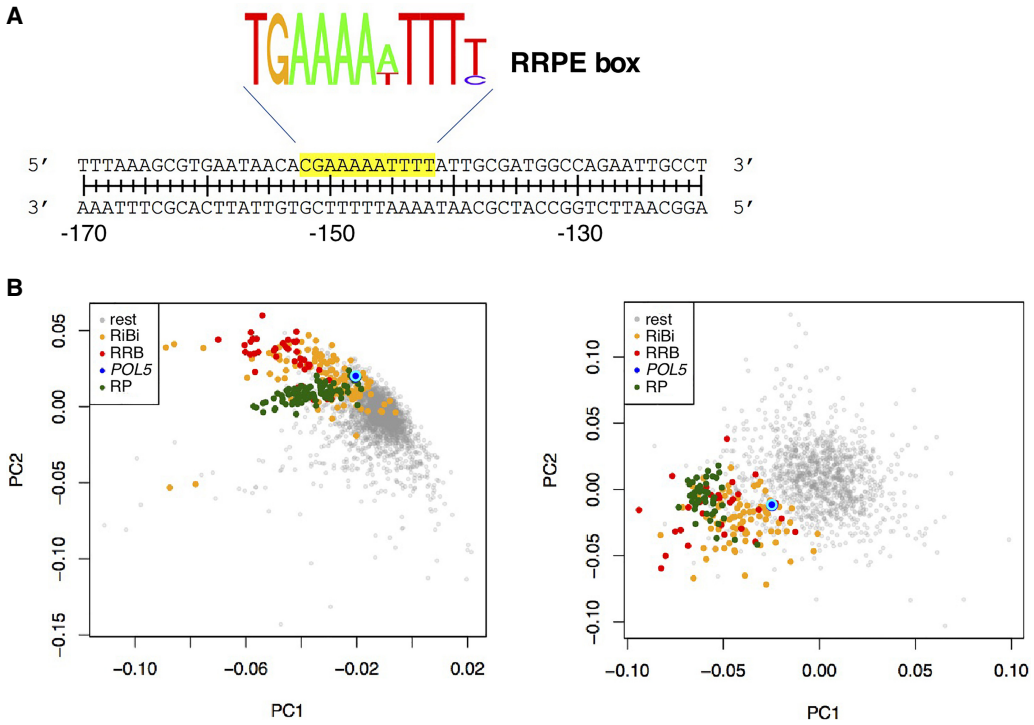


**FIGURE 1.** Pol5 is a member of the RRB/RiBi regulon. (*A*) The promoter of *POL5* contains a degenerate RRPE motif, which deviates from the consensus sequence only at the first position. As shown, the element is positioned between base pairs −152 and −142 upstream with respect to the translation start codon. (*B*) *POL5* expression is coregulated with that of RRB/RiBi genes. PCA plots at *left*: the four data sets studied in Wade et al. (2006) combined as a single data set; *right*, the expression data described in Jorgensen et al. (2002). The first (*x*-axis, PC1) and second (*y*-axis, PC2) principal components are shown. Colors were assigned to RRB, RiBi, r-protein (RP) genes, *POL5*, and the rest of yeast genes.

(C instead of T) compared with the RRPE consensus sequence. However, the *POL5* promoter apparently lacks a PAC sequence element (but see Discussion). (ii) Second, we performed a principal component analysis (PCA) of the four gene expression profile data sets used in the original analyses of Wade et al. (2001, 2006), where the RRB genes were defined as transcriptionally coregulated. These data sets correspond to cell-cycle synchronized cells by α-factor arrest and release, cells entering the sporulation pathway, and cells subjected to heat shock or osmotic stress. As shown in Figure 1B, *POL5* expression is clearly grouped within that of RRB genes. We also extended this PCA to the data set used by Jorgensen et al. (2004) to define the RiBi regulon. As above, *POL5* expression mapped within that of the bulk of the RiBi genes (Fig. 1B). Together, these findings suggest that Pol5 belongs to the RRB/RiBi regulon as it contains an almost perfect RRPE box and exhibits expression profiles similar to those of the RRB/RiBi cluster members.

## Strains for the phenotypic analysis of Pol5

Pol5 is essential for cell viability (Winzeler et al. 1999; Shimizu et al. 2002); thus, to study its role in ribosome biogenesis, we made use of two different conditional systems. First, we generated a conditional *GAL*::HA-*POL5* allele at the *POL5* genomic locus by one-step insertion of a kanMX6-*GAL1/10*::HA cassette upstream of its start codon. The resulting construct expresses an amino-terminally HA-tagged Pol5 fusion protein in media containing galactose but is repressed in media containing glucose. Growth of the *GAL*::HA-*POL5* strain was identical to that of the isogenic wild-type strain on YPGal plates, indicating that the allele is fully functional; however, it did not permit growth on YPD plates (Supplemental Fig. S2A). After transfer of the *GAL*::HA-*POL5* strain from liquid YPGal to liquid YPD medium, the growth rate remained similar to that of the wild-type strain for the first 6 h but then progressively decreased to a doubling time of >10 h after 12 h in YPD medium. Concomitantly, *GAL*::HA-*POL5* cells were depleted of the HA-Pol5 protein, as revealed by western blot analysis (Supplemental Fig. S2B).

As a second conditional system for phenotypic analysis, we used the previously described *pol5-1* and *pol5-3* ts mutants (Shimizu et al. 2002; Li et al. 2011). The *pol5-1* mutant showed apparent wild-type growth on YPD plates at 25°C but a marked slow growth (sg) at 37°C. In turn, the *pol5-3* mutant showed a mild sg at 25°C but a severe ts growth at 37°C (Supplemental Fig. S3). In liquid YPD medium, the *pol5-1* mutant and the isogenic wild-type strain showed doubling times of ∼2.0 h at 25°C, while that for *pol5-3* mutant was of ∼3 h. After 12 h at 37°C, the doubling times were of ∼7 and 13 h for the *pol5-1* and the *pol5-3* mutant, respectively, compared with the ∼1.7 h for the isogenic wild-type strain. The mutations within the *pol5-1* and *pol5-3* alleles lead to specific amino acid substitutions (V404M, K491E and Q671P for Pol5-1; W292R for Pol5-3); however, the molecular significance of the amino acid changes with respect to the ts phenotype of the respective mutants has not been determined (Shimizu et al. 2002; Li et al. 2011). Moreover, these mutations are recessive since the transformation of the *pol5-1* and the *pol5-3* mutants with YCplac111-POL5, a centromeric plasmid harboring a wild-type *POL5* allele, restored wild-type growth (see below Supplemental Fig. S10). Additionally, we used the *pol5dn* mutant, which encodes a Pol5[D623N;D625N] protein variant that lacks DNA polymerase activity in vitro (Shimizu et al. 2002). As also shown in Supplemental Figure S3, this mutant did not display any growth defect on YPD plates at any of the tested temperatures. In liquid YPD medium, it exhibited wild-type growth with a doubling time of ∼2.0 h at 25°C.

Finally, we also used a strain expressing carboxy-terminally GFP-tagged Pol5 from its genomic locus as the sole source of Pol5. This construct was obtained by a one-step insertion of a PCR-derived cassette. As shown in Supplemental Figure S4A, no growth impairment was seen for this strain in YPD plates, indicating that the fusion construct is functional. Moreover, the fusion protein could be detected by western blot analysis using anti-GFP antibodies as a single band of the expected molecular mass of ∼143 kDa (Supplemental Fig. S4B).

## Pol5 associates with pre-60S ribosomal particles

To start addressing the possible role of Pol5 in ribosome biogenesis, we determined whether a GFP-tagged Pol5 protein interacts with preribosomal particles. As stated above, this fusion protein sustains wild-type growth when it is the only source of cellular Pol5. In addition, the strain expressing Pol5-GFP showed wild-type polysome profiles, including normal levels of free 40S and 60S r-subunits (Supplemental Fig. S4C). The Pol5-GFP protein was predominantly found in the nucleolus, where it colocalized with a DsRed-Nop1 reporter (Supplemental Fig. S5). This result agrees with that of Shimizu et al. (2002), who reported that a 9xMyc-tagged Pol5 construct was restricted to the nucleolus.

To test whether Pol5 associates with r-particles, we affinity-purified Pol5-GFP containing complexes with GFP-Trap beads and analyzed which pre-rRNAs species were copurifying by northern blot hybridization (see Supplemental Fig. S1 for a scheme of pre-rRNA species). As shown in Figure 2, the $27SA_2$ and the 27SB pre-rRNAs were clearly enriched in the Pol5-GFP purification compared with the control purification performed with cell extracts derived from the isogenic wild-type strain expressing untagged Pol5. Very minor amounts of the 35S and 32S pre-rRNAs appear to also copurify with Pol5-GFP. However, neither 7S or 20S pre-rRNAs nor mature rRNAs were detected

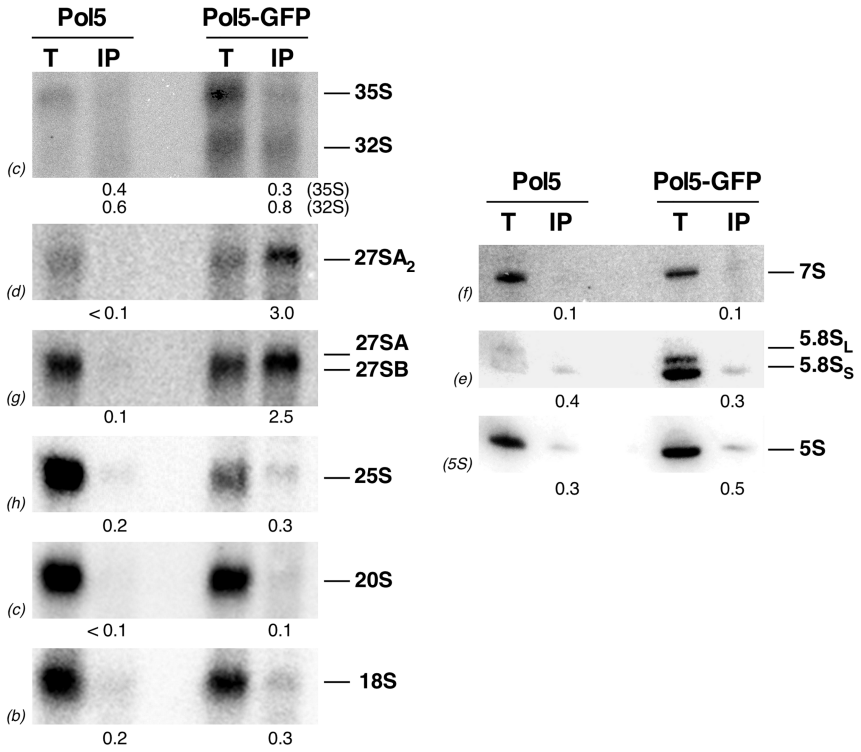


**FIGURE 2.** Pol5 is associated with pre-60S r-particles. Association of pre- and mature rRNAs with Pol5 was revealed by the GFP-Trap immunoprecipitation procedure with cell extracts of strain YMP1, which expresses the Pol5-GFP fusion protein as the sole source of cellular Pol5. Wild-type cells (untagged Pol5) were used as negative control. Cells were grown in YPD at 30°C to an $OD_{600}$ of approximately 0.8 and harvested. Total RNA was extracted from whole cell extracts (T) and immunoprecipitates (IP) and pre-rRNAs present were analyzed by northern blotting using the probes indicated between parentheses (see Supplemental Table S3 for their sequence and Supplemental Figure S1 for their location within 35S pre-rRNA). Signal intensities were measured by phosphorimager scanning; values (*below* each IP lane) refer to the percentage of each RNA species recovered after purification relative to the total extract input.

above the background levels obtained for these species in the control purification from the untagged strain (Fig. 2). Altogether, these data indicate that Pol5 is a component of early/medium nucleolar pre-60S r-particles. Pol5 apparently associates with particles at an early stage during formation of $27SA_2$ pre-rRNA and dissociates from nucle(ol)ar particles after 27SB pre-rRNA cleavage but before their nuclear export to the cytoplasm.

## Pol5 is required for 60S ribosomal subunit accumulation

The association of Pol5 with pre-60S r-particles suggests its participation in 60S r-subunit maturation. To investigate this, we studied the consequences of the loss-of-function of Pol5 on ribosome metabolism. We first examined the polysome profiles of the *GAL*::HA-*POL5* strain grown in YPGal medium or subjected to Pol5 depletion by a shift to YPD medium. In YPGal, the strain showed a normal polysome profile (Fig. 3). However, when shifted for only 3 h to YPD, there was a deficit in free 60S versus free 40S r-subunits and, accordingly, half-mer polysomes could be observed (Fig. 3). These features were even more pronounced upon a longer shift of 8 h to YPD (data not shown). The specific shortage of 60S r-subunits upon depletion of Pol5 was further confirmed by quantification of total r-subunits in low $Mg^{2+}$ sucrose gradients; thus, an $A_{254}$ 60S/40S ratio of approximately 1.8 was calculated for the *GAL*::HA-*POL5* strain grown in YPGal medium and for the isogenic wild-type counterpart grown in YPGal or YPD medium, while this ratio decreased to approximately 1.1 after transfer of the *GAL*::HA-*POL5* strain to YPD for 8 h (Supplemental Fig. S6).

Polysome analyses were also performed with cell extracts prepared from the *pol5-1* and *pol5-3* mutant and the isogenic wild-type strain grown in YPD at the permissive temperature of 25°C or 6 h after a shift to the nonpermissive temperature of 37°C. As shown in Supplemental Figure S7A, the *pol5-1* and *pol5-3* mutations also led to a significant shortage of 60S r-subunits after the temperature shift, as revealed by the deficit of free 60S versus 40S r-subunits and the appearance of half-mer polysomes. These defects were rescued by complementation of the mutants with a plasmid harboring the wild-type *POL5* gene (see later Supplemental Fig. S11 for *pol5-1*; not shown for *pol5-3*). Finally, we also analyzed polysome profiles from extracts of *pol5dn* cells grown in YPD at 30°C. In this case, the profiles were identical to those of wild-type cells (Supplemental Fig. S7B). Taken together, these results indicate that Pol5 is required for normal 60S r-subunit accumulation; however, its presumed DNA polymerase activity appears to be dispensable for its role in ribosome biogenesis.

## Pol5 is required for pre-rRNA processing

To discern whether the 60S r-subunit shortage occurring in conditional *pol5* strains is accompanied by defects in pre-rRNA processing, we analyzed the effects of Pol5 depletion on steady-state levels of pre- and mature rRNAs by northern hybridization and primer extension. As shown in Figure 4A, depletion of Pol5 resulted in a decrease of mature 25S rRNA but not of 18S rRNA. There was a clear accumulation of the 35S pre-rRNA, which was accompanied by

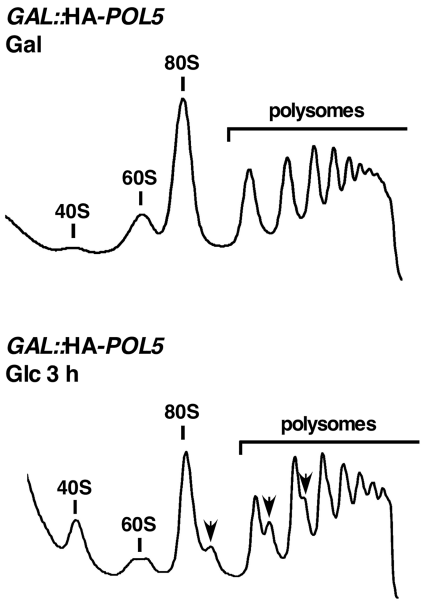


**FIGURE 3.** Depletion of Pol5 results in a 60S r-subunit shortage. YJP38 (*GAL*::HA-*POL5*) cells were grown in liquid YPGal medium (Gal) and shifted to YPD (Glc) for 3 h. Cells were harvested at an $OD_{600}$ of approximately 0.8, total cell extracts were prepared and 10 $A_{260}$ units of each extract were resolved in standard 7%–50% sucrose gradients. The $A_{254}$ was continuously measured. Sedimentation is from *left* to *right*. The peaks of free 40S and 60S r-subunits, vacant 80S ribosomes or monosomes and polysomes are indicated. Half-mers are labeled by arrows.

the appearance of an aberrant 23S species. Consistently, there was some alteration in the levels of $27SA_2$ and 20S pre-rRNAs. Importantly, a clear increase in the levels of 27SB pre-rRNAs was observed upon depletion of Pol5. Regarding low-molecular-mass rRNAs, depletion of Pol5 resulted in a very minor alteration in the accumulation of mature 5.8S rRNAs and no apparent change in the levels of the 5S rRNA or the ratios between $5.8S_L$ and $5.8S_S$ rRNAs (Fig. 4B). Nevertheless, it has been previously shown that these small mature rRNAs are more stable than 25S and 18S rRNA and depleted more slowly (e.g., Kressler et al. 1998; Thomson and Tollervey 2005). Similarly, the levels of 7S pre-rRNAs were neither clearly decreased upon Pol5 depletion, although they did not achieve the levels detected in the wild-type strain.

Primer extension analyses were performed using a probe hybridizing within the region $C_1$–$C_2$ of ITS2 (probe g; see Supplemental Table S3; Supplemental Fig. S1). Consistent with the northern blot results, minor differences in the primer extension stop at site $A_2$ but a significant increase in those at sites $B_{1L}$ and $B_{1S}$ were observed upon depletion of Pol5 (Fig. 4C). Levels of the $27SA_3$ pre-rRNA, which cannot be readily detected in northern blots, modestly increased upon depletion of Pol5 as shown by the stop at site $A_3$ (Fig. 4C). Finally, a slight decrease in the levels of the 25.5S pre-rRNA was detected following Pol5 depletion.

Together, these data demonstrate that Pol5 is required for proper ITS2 processing. Thus, depletion of Pol5 impairs processing of 27S pre-rRNAs, which is the likely cause of the reduced production of mature 60S r-subunits. As occurring for many mutants affecting 60S r-subunit synthesis (for review, see Fernández-Pevida et al. 2015), processing at the early $A_0$–$A_2$ sites is also modestly impaired.

## Depletion of Pol5 impairs nucleo-cytoplasmic transport of pre-60S r-particles

It has been well reported that diverse failures along the ribosome assembly pathway lead to the nucle(ol)ar retention of immature and/or defective preribosomal particles and hence to the impairment of their export to the cytoplasm (e.g., Fatica et al. 2003; Milkereit et al. 2003; Ferreira-Cerca et al. 2005; Pöll et al. 2009). In only a few particular cases, immature preribosomal particles gain export competence and are able to exit to the cytoplasm, where they may even be able to engage in translation (e.g.,Kressler et al. 2008; Ferreira-Cerca et al. 2014; Rodríguez-Galán et al. 2015; Sarkar et al. 2017). Thus, to determine whether Pol5 depletion impairs nuclear export of pre-60S r-particles, we assessed the intracellular localization of the reporter uL23-eGFP (formerly L25-eGFP) in the *GAL*::HA-*POL5* strain either grown in selective SGal medium or after a shift of 9 h to selective SD medium. As expected for an r-protein, uL23-eGFP was found in the cytoplasm of practically all cells when grown in liquid SGal medium (Supplemental Fig. S8A). However, following the shift to selective SD medium, uL23-eGFP accumulated in the nucle(ol)us as revealed by the colocalization with the nucleolar marker DsRed-Nop1 (Supplemental Fig. S8A). No nuclear accumulation of the uL23-eGFP reporter was observed when an isogenic *POL5* wild-type strain was analyzed in identical growth conditions (Supplemental Fig. S9A). Moreover, the 40S r-subunit reporter uS3-eGFP did not show nuclear retention in neither the *GAL*::HA-*POL5* nor the *POL5* strains when grown in liquid SGal medium or shifted up to 9 h to SD medium (Supplemental Figs. S8A, S9A). Pol5 functionally interacts with uL23 (see below). Thus, to exclude the possibility that the nucle(ol)ar accumulation of uL23-eGFP observed upon Pol5 depletion could be due to a specific failure of its assembly rather than to an impairment of nucleo-cytoplasmic export of defective pre-60S ribosomal particles, we also monitored the intracellular localization of two additional 60S r-subunit reporters, eL22A-eGFP and uL5-eGFP. The results with these reporters were identical to those with uL23-eGFP (Supplemental Figs. S8B, S9B). Taken together, these experiments indicate that both the intra-nuclear and nucleo-cytoplasmic transport of pre-60S r-particles is impaired upon Pol5 depletion.

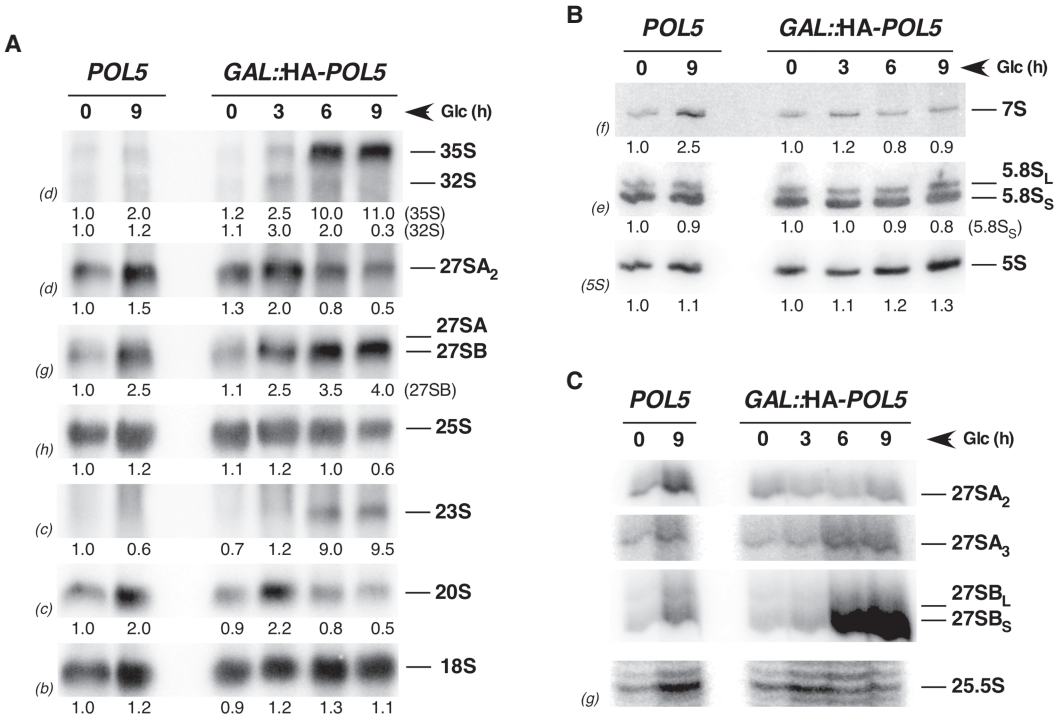


**FIGURE 4.** Depletion of Pol5 impairs pre-rRNA processing. Strains W303-1B (*POL5*) and YJP38 (*GAL::*HA-*POL5*) were grown at 30°C in liquid YPGal medium and shifted to YPD. Cells were maintained in exponential growth throughout the time course of the experiment by culture dilution with the prewarmed medium. Total RNA was extracted from the cultures at the indicated times after the shift, and equal amounts (5 µg) were subjected to northern hybridization or primer extension analyses. (*A*) Northern blot analysis of high-molecular-mass and (*B*) low-molecular-mass pre- and mature rRNAs. Probes, between parentheses, are described in Supplemental Table S3 and Supplemental Figure S1. Signal intensities were measured by phosphorimager scanning; values (*below* each lane) were normalized to those obtained for the wild-type control grown in YPGal, arbitrarily set at 1.0. (*C*) Primer extension analysis using an oligonucleotide that hybridizes within the $C_1$–$C_2$ region of ITS2 (probe g), thereby allowing detection of the 25.5S, $27SA_2$, and $27SA_3$ pre-rRNAs as well as both 27SB pre-rRNA species.

## Overexpression of uL23 and eL27A r-proteins suppresses the thermosensitive phenotype of the *pol5-1* mutant

To better understand the function of Pol5, we carried out a screen for multicopy suppressors of the ts phenotype of the *pol5-1* mutant. This mutant was transformed with a YEplac181-based multicopy library (de la Cruz et al. 1997), and the transformants (~35,000) were incubated for 5 d at the nonpermissive temperature (37°C). Plasmid DNA was isolated from the thermo-tolerant candidates, rescued in *Escherichia coli*, and back-transformed into the original *pol5-1* mutant. Four plasmids were able to reproducibly suppress the growth defect of the mutant at 37°C. One contained the wild-type *POL5* gene, two others harbored the *RPL27A* gene and the last one the *RPL25* gene. Further cloning of the PCR-amplified *RPL27A* and *RPL25* genes into YEplac181 or YEplac195, and subsequent transformation of these plasmids into the *pol5-1* mutant confirmed that these two 60S r-protein genes indeed confer the multicopy suppression (Fig. 5; Supplemental Fig. S10). To further test the allele specificity of the suppressor effect, we also transformed the *POL5* shuffle and the *pol5-3* strains with the *RPL25*- and *RPL27A*-containing multicopy plasmids. While increased gene dosage of neither *RPL25* nor *RPL27A* could overcome the essential function of Pol5 at 30°C (data not shown), overexpression of eL27A (formerly L27A) also suppressed the growth defect of *pol5-3* mutant cells to practically the wild-type extent at 37°C (Fig. 5; Supplemental Fig. S10). In addition, we also cotransformed the *pol5* ts mutants with multicopy plasmids harboring *RPL25* and *RPL27A*. Interestingly, simultaneous overexpression of uL23 (formerly L25) and eL27A resulted in better suppression of the growth defect of the *pol5-1* mutant at 37°C than the individual overexpression of each r-protein alone (Fig. 5). These results strongly suggest that the function of Pol5 during 60S r-subunit biogenesis could be related to that of 60S r-proteins uL23 and eL27A. To test whether overexpression of these r-proteins restored 60S r-subunit synthesis in the conditional *pol5* mutants, we performed polysome profile analysis. However, increased dosage of neither *RPL25* nor of *RPL27A* alone resulted in a significant improvement of the 60S r-subunit shortage detected in the *pol5* mutants and only co-overexpression of uL23 and eL27A minorly improves polysome formation in the suppressed *pol5-1* strain

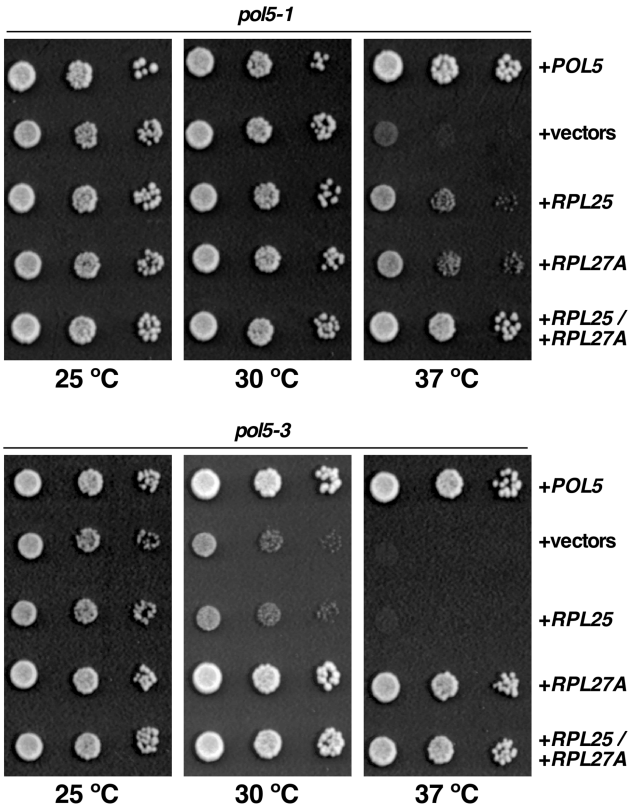


**FIGURE 5.** Suppression of the growth defects of selected *pol5* mutants by high-dosage of uL23 and eL27A. Strains Y10143 (*pol5-1*) and Y10144 (*pol5-3*) were transformed with the following combination of plasmids: YCplac111-POL5 and YCplac33 (+*POL5*), multicopy plasmids YEplac181 and YEplac195 (+vectors), YEplac181-RPL25 and YEplac195 (+*RPL25*), YEplac181 and YEplac195-RPL27A (+*RPL27A*), and YEplac181-RPL25 and YEplac195-RPL27A (+*RPL25*/+*RPL27A*). Transformants were grown in liquid SD-Leu-Ura medium and diluted to an $OD_{600}$ of 0.05. Serial 10-fold dilutions were spotted onto SD-Leu-Ura plates, which were incubated at 25°C, 30°C, and 37°C for 3 d. Note that co-overexpression of uL23 and eL27A suppresses the ts phenotype of the *pol5-1* mutant better than single overexpression of uL23 or eL27A.

grown at 37°C (Supplemental Fig. S11 for *pol5-1*; data not shown for *pol5-3*). Consistently, quantification of total r-subunits in low $Mg^{2+}$ sucrose gradients confirmed a comparable reduction of approximately 30% in the overall amount of 60S r-subunits in the *pol5-1* mutant transformed either with an empty vector or the multicopy plasmids harboring *RPL25* or *RPL27A*, grown in SD-Leu at 30°C and then transferred for 9 h to 37°C ($A_{254}$ 60S/40S ratio of 1.3), relative to the *pol5-1* strain complemented with a plasmid harboring a wild-type *POL5* gene or an isogenic wild-type strain ($A_{254}$ 60S/40S ratio of 1.8) (Supplemental Figs. S6, S12). This reduction was clearly lower (~15%) in the *pol5-1* mutant cotransformed with multicopy plasmids harboring *RPL25* and *RPL27A* ($A_{254}$ 60S/40S ratio of 1.5) (Supplemental Fig. S12). Altogether, these results suggest that higher levels of uL23 and/or eL27A in *pol5* mutants might qualitatively ameliorate ribosome production or function as we did not observe any marked increase in the absolute levels of mature r-subunits in the suppressed strains compared with the original *pol5* mutants.

### Expression of an HA-MYBBP1A construct exerts a negative effect on growth and 60S r-subunit production

Yeast Pol5 and mammalian MYBBP1A are predicted to be orthologs (Yang et al. 2003). Moreover, similarly to Pol5, MYBBP1A may have a role in ribosome biogenesis (Hochstatter et al. 2012; Tafforeau et al. 2013). Hence, we wondered whether these proteins are also functionally equivalent and can complement each other. To test this possibility, we studied the ability of the major isoform of human *MYBBP1A* cDNA to rescue the lethal phenotype of the *pol5* null mutant. This isoform, called p160 since it corresponds to the full-length 160 kDa nucleolar variant of MYBBP1A (Tavner et al. 1998), was amino terminally HA-tagged and the construct expressed from the strong yeast *GAL1-10* promoter in the *POL5* shuffle strain. As shown in Supplemental Figure S13A, expression of HA-MYBBP1A exerted a severe negative effect on the growth of this strain. Moreover, when the plasmid harboring the wild-type *POL5* allele was shuffled on 5-FOA plates containing galactose as carbon source, the strain did not support growth; thus, indicating that in addition to conferring a dominant negative phenotype, expression of the human *HA-MYBBP1A* allele was unable to complement the lethal phenotype linked to the *POL5* deletion (Supplemental Fig. S13A). Western blot analysis of the HA-tagged protein indicated that the lack of complementation was not due to deficient protein expression (Supplemental Fig. S13B). We also tested whether human HA-MYBBP1A was able to rescue the ts phenotype of the conditional *pol5-1* and *pol5-3* alleles at the nonpermissive temperature (37°C), but, as above, no complementation could be observed. Additionally, a negative effect on growth associated with expression of HA-MYBBP1A was again revealed (data not shown). Importantly, the expression of HA-MYBBP1A interferes with the correct biogenesis of 60S r-subunits as shown by polysome profile analysis (Supplemental Fig. S14). Consequently, despite the sequence homology and the fact that both proteins could have a role in 60S r-subunit biogenesis, our data are not conclusive enough to reveal that human MYBBP1A and yeast Pol5 exert the same molecular function.

## DISCUSSION

In this work, we show that Pol5 is a ribosome biogenesis factor that can be grouped into the RRB/RiBi gene class as its expression is coregulated with that of the bulk of factors belonging to these regulons. As most RRB/RiBi genes, *POL5* is a TATA-less gene (data not shown). Promoter

sequence analysis revealed that the genes within the RRB regulon are enriched in the RRPE [consensus sequence 5′ TGAAAA(A/T)TTT(C/T) 3′] and PAC [consensus sequence 5′ GCGATGAG(A/C)T 3′] motifs (Wade et al. 2006; Bosio et al. 2011). The consensus sequences of these elements are absent in the *POL5* promoter, however, an 11-base RRPE-like element 5′ CGAAAAATTTT 3′ and a PAC-like element 5′ GCGATGGCCA 3′ containing one and three mismatches (underlined bases) to the consensus sequences were found at positions −151 and −137 with respect to the first base of the start codon, respectively. Remarkably, both the positions and the relative order of these elements also coincide with that of a typical RRB gene promoter as they are found in the *POL5* promoter in the first 150 bp upstream of the start codon, with the PAC-like element being more proximal to the start codon and spaced by less than 50 bp relative to the RRPE-like element. Whether these sequence elements in the *POL5* promoter could efficiently act as recognition sites for the transcription regulators that bind the canonical elements of *bona fide* RRB/RiBi gene promoters needs to be determined (for further details, see Bosio et al. 2017), although this would be expected given the common coregulation.

Our data establish that Pol5 is a component of early/medium nucle(ol)ar pre-60S r-particles containing 27S pre-rRNAs. Previous work has identified Pol5 as a component of the t-Utp/UtpA subcomplex of 90S preribosomal particles (Gallagher et al. 2004; Krogan et al. 2004). However, more recently, it has been reported that Pol5 is rather a t-UTP/UtpA accessory factor, which dissociates from the subcomplex before the incorporation of this latter into nascent 90S preribosomal particles (Gallagher 2019). The association of Pol5 with pre-60S r-particles was not previously unveiled despite the abundant information available on the composition of different affinity-purified pre-60S complexes (e.g., Fromont-Racine et al. 2003). This fact is likely due to the weak and/or transient association of Pol5 with preribosomal particles. Indeed, the enrichment of 27S pre-rRNAs we obtained by the affinity purification of GFP-tagged Pol5 was clearly poorer than what we have previously observed for purifications of other GFP-tagged ribosome biogenesis factors or r-proteins (e.g., Babiano and de la Cruz 2010; Espinar-Marchena et al. 2018).

In this paper, we also describe the functional analysis of Pol5 in ribosome biogenesis. In vivo depletion of Pol5 leads to a deficit in 60S r-subunits as revealed by polysome analyses, which showed the appearance of half-mers, and by quantification of total r-subunits. Detailed analysis of pre-rRNA processing by northern hybridization and primer extension suggested that this deficit is the result of impaired maturation of medium pre-60S r-particles containing 27SB pre-rRNAs; hence, both $27SB_S$ and $27SB_L$ species clearly accumulated upon Pol5 depletion. Some accumulation of the upstream $27SA_3$ pre-rRNA was also detected; however, no drastic depletion of the downstream 7S and 25.5S precursors could be observed, suggesting that the pre-60S intermediates undergoing ITS2 processing are also being stabilized during the time course of Pol5 depletion. Moreover, we also observed an accumulation of the 35S pre-rRNA, the appearance of the aberrant 23S species and the concomitant mild reduction of $27SA_2$ and 20S pre-rRNAs. This is consistent with a delayed processing at the early $A_0$, $A_1$, and $A_2$ cleavage sites upon Pol5 depletion that, however, affects only minorly the overall synthesis of 18S rRNA and 40S r-subunit production. Delayed processing of 35S pre-rRNA, which is common for practically all loss-of-function mutations in genes involved in 60S r-subunit biogenesis including 60S r-proteins, has previously been well discussed (e.g., Axt et al. 2014; Espinar-Marchena et al. 2016; Talkish et al. 2016) and arises as the consequence of a functional limitation of early *trans*-acting factors that are sequestered on aberrant pre-60S intermediates and/or the specific switch of pre-rRNA processing to the so-called $A_3$ pathway (Kos-Braun et al. 2017). Recently, Gallagher has reported comparable results after assessing the steady-state levels of pre-rRNAs from an equivalent *GAL::POL5* strain shifted to glucose medium for 24 h (Gallagher 2019). It is unlikely that Pol5 plays a direct role in 27SB pre-rRNA processing. Instead, Pol5 may fulfill a more general function as a pre-60S ribosomal assembly factor. It has been reported that Pol5 has DNA polymerase activity in vitro (Shimizu et al. 2002); however, the in vivo relevance of this activity is unclear as the *pol5dn* mutant, which expresses a Pol5 [D623N;D625N] protein displaying no in vitro DNA polymerase activity, exhibits similar growth and global translation capacity, as indicated by polysome profile analysis, as the wild-type strain (Supplemental Figs. S3, S7; Shimizu et al. 2002). The pre-rRNA processing defects revealed by the depletion of Pol5 are accompanied by a retention of uL23-eGFP containing pre-60S r-particles in the nucle(ol)us. This phenotype is also very common for mutations affecting different nucle(ol)ar steps of 60S r-subunit maturation and appears as the result of a possible quality control mechanism that normally disables defective pre-60S r-particles to acquire nucleo-cytoplasmic export competence (e.g., see Thomson and Tollervey 2005; Babiano and de la Cruz 2010 and for a discussion Bassler and Hurt 2019).

The pre-rRNA processing and nuclear export defects shown by the Pol5-depleted strain closely resemble those described for loss-of-function mutations in or depletion of distinct 60S r-subunit biogenesis factors and 60S r-proteins. About 16 *trans*-acting factors, the so-called "B-factors," are required for cleavage of 27SB pre-rRNAs at site $C_2$ (Talkish et al. 2012; Woolford and Baserga 2013). Association of the B-factors with preribosomal particles takes place in two stages: most B-factors are recruited to early pre-60S r-particles containing the $27SA_2$ pre-rRNA,

while others, such as Nsa2 and Nog2, seem to associate later, just prior to cleavage at site $C_2$ (see Woolford and Baserga 2013 and references therein). Recent cryo-EM analyses of early pre-60S r-particles indicate that by the time most B-factors appear to be stably bound, the six 25S/5.8S rRNAs domains have been folded but not fully compacted (Kater et al. 2017; Sanghai et al. 2018; Bassler and Hurt 2019; Klinge and Woolford 2019). These results are consistent with those obtained by studying the composition of purified pre-60S r-particles containing progressively 3′-elongated fragments of 27S pre-rRNA (Chen et al. 2017; Chaker-Margot and Klinge 2019) and those of high-throughput RNA structure probing analyses on purified nucleolar pre-60S r-particles (Burlacu et al. 2017). Regarding r-proteins, several 60S r-subunit proteins, including uL6, uL22, eL19, uL14, uL23, uL24, eL27, eL34, uL29, and eL37 (formerly L9, L17, L19, L23, L25, L26, L27, L34, L35, and L37, respectively), have been reported to be more or less important for efficient conversion of 27SB into 7S and 25.5S pre-rRNAs (see de la Cruz et al. 2015 and references therein). All of these r-proteins assemble into pre-60S r-particles before cleavage at site $C_2$; strikingly, assembly of at least uL6, uL14, uL22, uL23, uL29, eL27, eL37 and, to a lesser extent, uL24 is necessary to properly recruit the B-factors Nsa2 and Nog2 to pre-60S r-particles, and other factors such as Noc3 and Nop53, with the latter, then acting as the recruitment factor for Mtr4 and the exosome (Babiano et al. 2012; Gamalinda et al. 2013, 2014; Ohmayer et al. 2013; Thoms et al. 2015).

To further analyze the function of Pol5 during 60S r-subunit biogenesis, we performed a multicopy suppressor screen with the *pol5-1* ts mutant and isolated the genes encoding the 60S r-proteins uL23 and eL27A, which were unable to suppress the lethality of a *pol5Δ* allele. Although high-dosage of each of the two genes, alone or together, efficiently suppressed the growth defect of the *pol5-1* mutant at 37°C, only when both are simultaneously overexpressed the 60S r-subunit shortage is minorly alleviated. This result suggests an improvement of the functional competence of the produced ribosomes in the suppressed strains rather than a restoration of the 60S r-subunit deficiency as an explanation for the suppression at the molecular level. Few other examples, where multicopy suppressors rescue the growth defect of ribosome biogenesis factor mutants without concomitantly increasing the levels of the corresponding r-subunits, have been reported. These include the suppression of certain *ebp2* mutants by eL36A/B (Wan et al. 2013), of a distinct *rrp5* allele by snR10 (Torchet and Hermann-Le Denmat 2002), of a *tsr2* mutant by uS11 (Peña et al. 2016) or of the *rrp2-1* mutant by Pop3 (Dichtl and Tollervey 1997). Very recently, it has been found by UV cross-linking and analysis of cDNAs (CRAC) that Pol5 directly contacts the 25S rRNA at several sites within domain III (J. Pérez-Fernández, pers. comm.), where, strikingly, both uL23 and eL27 also bind to intermediate pre-60S r-particles and mature 60S r-subunits (Ben-Shem et al. 2011; Wu et al. 2016; Kater et al. 2017; Sanghai et al. 2018). This suggests that Pol5 could be involved in achieving the proper structural conformation of 25S rRNA domain III by promoting the stable incorporation of distinct r-proteins, such as uL23 and eL27. The fact that the co-overexpression of uL23 and eL27 increases the suppression efficiency of the *pol5-1* mutant is consistent with this scenario. Also consistent with this hypothesis, affinity purified pre-60S r-particles depleted of Pol5 are severely deprived of uL23 and eL27 (J. Pérez-Fernández, pers. comm.).

Finally, we explored whether human MYBBP1A and yeast Pol5 are functional orthologs. However, expression of a human HA-tagged MYBBP1A construct did not complement the lethal phenotype of a *pol5Δ* null strain. Despite this negative result, some relevant coincidences make us suspect that they could perform similar functions during ribosome biogenesis: (i) Both MYBBP1A and Pol5 are nucleolar proteins (Tavner et al. 1998) and (ii) importantly, knockdown of MYBBP1A in different cultured human cell types leads to impaired pre-rRNA processing, more specifically, accumulation of 47S and 32S pre-rRNAs, which are the human equivalents to 35S and 27SB pre-rRNAs (Tafforeau et al. 2013). Strikingly, the expression of MYBBP1A exerted a strong negative effect on yeast growth and led to a deficit in 60S r-subunits. As a simple explanation for this result, MYBBP1A could be sequestering an essential factor required for 60S r-subunit biogenesis; alternatively, it could be making an unproductive binding on pre-60S r-particles. Further experiments are required to reveal whether this factor is specifically related to 60S r-subunit biogenesis in mammalian cells as Pol5 is in yeast. It has been shown that MYBBP1A connects ribosome biogenesis and growth; thus, upon inhibition of rRNA synthesis, MYBBP1A translocates to the nucleoplasm and increases the acetylation levels of p53 (Kuroda et al. 2011). A challenge for future studies will be to elucidate the molecular mechanism underlying these connections, which are crucial to understand cancer development.

## MATERIALS AND METHODS

### Strains and microbiological methods

Yeast strains used in this study are listed in Supplemental Table S1. Some are derived from the W303 strain (Thomas and Rothstein 1989): YJP38 is a conditional strain that genomically expresses an HA-tagged version of Pol5 under the control of the *GAL1* promoter. This strain (referred to in the text as *GAL::*HA-*POL5*) was generated by one-step insertion of a kanMX6-PGAL1-3xHA cassette upstream of the ATG of the *POL5* gene (Longtine et al. 1998). The YMP1 strain expresses a fully functional

carboxy-terminally GFP-tagged Pol5 under the control of its cognate promoter; the GFP(S65T) tag allele was fused by in frame one-step integration of a PCR cassette in the corresponding wild-type genomic locus of the W303-1A strain (Longtine et al. 1998). Y10143 and Y10144 strains, kindly provided by C. Boone, are genomic *pol5-1* and *pol5-3* mutant strains, respectively, in the BY4742 yeast background (Li et al. 2011); the ts *pol5-1* allele encodes the variant Pol5[V404M;K491E;Q671P] protein, while the ts *pol5-3* allele encodes the variant Pol5[W292R] protein (Shimizu et al. 2002). DGY115 is a *pol5* null haploid segregant of yeast Y20296 (BY4743 yeast background; Euroscarf), which expresses Pol5 from its cognate promoter from plasmid YCplac33.

Yeast genetic techniques and growth media have been previously described (Burke et al. 2000). Strains were grown at selected temperatures either in rich YP medium (1% yeast extract, 2% peptone) supplemented with 0.2% adenine and containing either 2% glucose (YPD) or 2% galactose (YPGal) as carbon source. We also used synthetic minimal medium (0.15% yeast nitrogen base, 0.5% ammonium sulfate) supplemented with the appropriate amino acids and bases as nutritional requirements, and containing either 2% glucose (SD) or 2% galactose (SGal) as carbon source. For in vivo depletion of Pol5, the *GAL*::HA-*POL5* strain was grown in liquid YPGal medium at 30°C until mid-exponential phase ($OD_{600}$ of 0.8), and then harvested, washed and transferred to liquid YPD broth. Cell growth was monitored over a period of 12 h, during which the cultures were regularly diluted into fresh YPD medium. Yeast transformation was done by the lithium acetate method (Gietz et al. 1992). After sporulation, tetrads were dissected using a Singer MSM micromanipulator. Standard molecular biology techniques were as previously described (Sambrook et al. 1989). *Escherichia coli* DH5α was used for cloning and propagation of plasmids (Sambrook et al. 1989).

## Multicopy suppression analysis

To isolate multicopy suppressor of *pol5* mutants, the Y10143 strain (*pol5-1* mutant) was transformed with a YEplac181-based yeast genomic library (de la Cruz et al. 1997). Transformants were spread on SD-Leu plates and incubated for 3–5 d at the non-permissive temperature of 37°C. Plasmid DNA was isolated from the thermo-tolerant candidates, rescued in *E. coli*, and back-transformed into Y10143. Four clones out of approximately 35,000 transformants were found to reproducibly suppress the ts phenotype of the *pol5-1* mutant. The inserts of the rescued plasmids were sequenced revealing that one contained the *POL5* gene, another one contained DNA corresponding to the *RPL25-TRM13* region of chromosome XV and the last two ones contained DNA fragments from chromosome VIII where only the *RPL27A* and *DIA4* genes were shared. Further cloning into YEplac181 or YEplac195 of PCR-amplified fragments containing either the *RPL27A* or *RPL25* gene defined these genes as the multicopy suppressors of the *pol5-1* mutant.

## Plasmids

All plasmids used in this study are listed in Supplemental Table S2. To construct YCplac33-, and YCplac111-POL5, a PCR was performed using yeast genomic DNA as a template and oligonucleotides placed ±1 kbp upstream and downstream from the start–stop codon of the *POL5* ORF, respectively; after the appropriate restriction digestion, the PCR product was cloned into the particular vectors. The *pol5dn* allele, which codes for a catalytically inactive Pol5[D623N;D625N] protein (Shimizu et al. 2002), was generated with the Q5 site-directed mutagenesis kit (NEB) with YCplac111-POL5 as a template. The full-length cDNA of human MYBBP1A was PCR-amplified from pACT-Flag-MYBBP1A, a gift from A. Carnero (Hochstatter et al. 2012), and cloned into the pAS24 plasmid under the transcriptional control of the *GAL1* promoter. Further details on the construction of the plasmids are available upon request. The plasmids pRS314-DsRed-NOP1, pRS316-RPS3-eGFP-NOP1-mRFP, and pRS316-RPL25-eGFP-NOP1-mRFP, a gift from J. Bassler and E. Hurt, have been previously reported (Ulbrich et al. 2009).

## Sucrose gradient centrifugation

Polyribosome and r-subunit preparations and analyses were done as described previously (Kressler et al. 1998). Gradient analysis was performed with an ISCO UA-6 system, with continuous monitoring at $A_{254}$. In all cases, extracts were performed from cell cultures harvested at an $OD_{600}$ of approximately 0.8 and about 10 $A_{260}$ units of each extract were loaded in gradients. To quantify the 60S/40S ratios, each r-subunit profile was enlarged to occupy the maximum space in an A4 size paper and printed three times; then, the area under each r-subunit peak was cut and weighed. The mean of the three independent measurements was calculated. Three independent biological replicates were performed for each experiment and the means recalculated in each case.

## Protein extractions and western blotting analyses

Total yeast protein extracts were prepared and analyzed by western blotting according to standard procedures (Sambrook et al. 1989; Ausubel et al. 1994). High-affinity mouse monoclonal anti-HA (Roche), anti-GFP (Roche), and anti-Pgk1 (Invitrogen) were used as primary antibodies. Secondary goat anti-mouse horseradish peroxidase-conjugated antibodies (Bio-Rad) were used. Proteins were detected using a chemiluminescence detection kit (Super-signal West Pico, Pierce) and a ChemiDoc MP system (Bio-Rad).

## RNA extractions and steady-state analysis of pre-rRNAs and mature rRNAs

Total RNA was extracted by the hot acidic phenol-chloroform procedure (Ausubel et al. 1994). Normally, RNA was extracted from samples corresponding to 10 $OD_{600}$ units of cells grown to mid-log phase. Northern hybridization and primer extension analyses were carried out as previously described (Kressler et al. 1998; Venema et al. 1998). Equal amounts of total RNA were loaded on gels or used for primer extension reactions. Specific oligonucleotides (see Supplemental Table S3 for their sequences) were 5′-end labeled with [γ-32P] ATP and used as probes. Hybridization signals were detected using a Typhoon FLA9400 imaging system (GE Healthcare).

### Affinity purification of Pol5-GFP

Complexes containing GFP-tagged Pol5 protein were precipitated following the one-step GFP-Trap A procedure (Chromotek) with GFP-Trap A beads, as exactly described in Babiano and de la Cruz (2010). The proteins from the purified complexes were extracted by boiling the beads with Laemmli buffer and analyzed by western blotting (Espinar-Marchena et al. 2016). Pre- and mature rRNAs were recovered from the beads by phenol–chloroform extraction and assayed by northern hybridization as indicated above.

### Fluorescence microscopy

To address the subcellular localization of Pol5, strain YMP1 was transformed with pRS314-DsRed-NOP1 and transformants grown in selective SD medium. To test preribosomal particle nuclear export, the *GAL*::HA-*POL5* strain was transformed with plasmids expressing either GFP-tagged uL23 or uS3 and Nop1-mRFP (see above); transformants were grown in selective SGal medium and shifted to selective SD medium to deplete Pol5. Cells were washed, resuspended in PBS buffer (140 mM NaCl, 8 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, 2.75 mM KCl, pH 7.3), and examined with a Leica DMR fluorescence microscope equipped with a DC350F digital camera and its software. Adobe Photoshop CC (Adobe Systems, Inc.) was used to process the images.

### Bioinformatic analysis

The promoter region of *POL5* was searched for the presence of the RRB-associated RRPE and PAC motifs by a bioinformatic analysis. A degenerated RRPE sequence was found searching the promoter region of *POL5* for the consensus sequence 5′ TGAAAA (A/T)TTT(C/T) 3′ (Bosio et al. 2011), using the function matchPattern of the Biostrings package of R, with the following settings (max.mismatch = 1, fixed = FALSE), starting at position −151 from the first base of the start codon. The PAC sequence was searched looking for the consensus sequence 5′ GCGATGAG(A/C)T 3′ (Bosio et al. 2011), using also the function matchPattern of the Biostrings package of R software (www.r-project.org). To find any relevant match, the settings had to include at least three mismatches (max.mismatch = 3, fixed = FALSE); thus, the sequence 5′ GCGATGGCCA 3′ was found starting at position −137 and the sequence 5′ CCCATCAGCT 3′ was found starting at position −403 relative to the start codon.

A list of 65 RRB genes has been published in Wade et al. (2001). A list of 236 RiBi genes is described in Jorgensen et al. (2004). The four data sets employed in this study were described in Wade et al. (2006), corresponding to expression profiles of cells subjected to release of an α-factor arrest (Spellman et al. 1998), progressing through the sporulation pathway (Chu et al. 1998), or subjected to heat shock (Gasch et al. 2000) or osmotic stress (Gasch et al. 2000). All data of these four expression data sets were consolidated in a single data table for the combined analysis. The PCA was performed using the *prcomp* function of the R base package with default settings. The values of cDNA expression were extracted as normalized, background corrected Cy3:Cy5 ratios from the different original publications and used as input for the analysis. Genes with missing values were excluded from the analysis. The PCA with the data contained in Jorgensen et al. (2004) was performed with the whole expression data set except for one of the time points that was excluded from the analysis due to missing the value for *POL5*.

## SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

## ACKNOWLEDGMENTS

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# Pol5 is an essential ribosome biogenesis factor required for 60S ribosomal subunit maturation in *Saccharomyces cerevisiae*

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