The ATPase and helicase activities of Prp43p are stimulated by the G-patch protein Pfa1p during yeast ribosome biogenesis

Simon Lebaron^{1,3,4}, Christophe Papin^{1,3,4},
Régine Capeyrou^{1,3}, Yan-Ling Chen^{1,3},
Carine Froment^{2,3}, Bernard Monsarrat^{2,3},
Michèle Caizergues-Ferrer^{1,3},
Mikhail Grigoriev^{1,3,*} and Yves Henry^{1,3,*}

¹Centre National de la Recherche Scientifique, Laboratoire de Biologie Moléculaire Eucaryote, Toulouse, France, ²Centre National de la Recherche Scientifique, Institut de Pharmacologie et de Biologie Structurale, Toulouse, France and ³Université de Toulouse, UPS, Toulouse, France

Prp43p is a RNA helicase required for pre-mRNA splicing and for the synthesis of large and small ribosomal subunits. The molecular functions and modes of regulation of Prp43p during ribosome biogenesis remain unknown. We demonstrate that the G-patch protein Pfa1p, a component of pre-40S pre-ribosomal particles, directly interacts with Prp43p. We also show that lack of Gno1p, another G-patch protein associated with Prp43p, specifically reduces Pfa1p accumulation, whereas it increases the levels of the pre-40S pre-ribosomal particle component Ltv1p. Moreover, cells lacking Pfa1p and depleted for Ltv1p show strong 20S pre-rRNA accumulation in the cytoplasm and reduced levels of 18S rRNA. Finally, we demonstrate that Pfa1p stimulates the ATPase and helicase activities of Prp43p. Truncated Pfa1p variants unable to fully stimulate the activity of Prp43p fail to complement the 20S pre-rRNA processing defect of $\Delta pfa1$ cells depleted for Ltv1p. Our results strongly suggest that stimulation of ATPase/helicase activities of Prp43p by Pfa1p is required for efficient 20S pre-rRNA-to-18S rRNA conversion.

The EMBO Journal (2009) 28, 3808–3819. doi:10.1038/emboj.2009.335; Published online 19 November 2009

Subject Categories: proteins

Keywords: Gno1p; Pfa1p; Prp43p; ribosome biogenesis; RNA helicase

Introduction

Ribosome biogenesis is an intricate process involving the synthesis of ribosomal protein pre-mRNAs by RNA polymerase II, 5S rRNA precursor by RNA polymerase III, and

a common polycistronic precursor to 18S, 5.8S and 25/28S rRNAs by RNA polymerase I (Henras *et al.*, 2008). In the course of its transcription by RNA PolI in the nucleolus, the polycistronic pre-rRNA begins to associate with small nucleolar ribonucleoprotein particles (RNPs), and with a sub-set of ribosomal proteins and non-ribosomal proteins, so-called because they are absent from mature cytoplasmic ribosomes (Fromont-Racine *et al.*, 2003). Such associations generate pre-ribosomal particles, within which the pre-rRNA will be chemically modified and will undergo a series of endo- and exonucleolytic cleavage steps, leading to the removal of spacer sequences and the release of mature 18S, 5.8S and 25/28S rRNAs (Henras *et al.*, 2008). The pre-rRNA can be fully transcribed before being cleaved. In that case, initial so-called 90S pre-ribosomal particles are generated. Cleavage at site A2, situated within the internal transcribed spacer 1 separating the 18S and 5.8S sequences, then splits maturing 90S particles into pre-40S and pre-60S pre-ribosomal particles that will be independently matured into functional 40S and 60S ribosomal subunits, respectively (Henras *et al.*, 2008). By genetic and more recently by large-scale tandem affinity purification approaches, close to 200 non-ribosomal protein components of pre-ribosomal particles have been characterized (Fromont-Racine *et al.*, 2003; Henras *et al.*, 2008). About 20 of these are members of the DEXD/H family of RNA helicases, the largest group of such enzymes involved in a given cellular process (Bleichert and Baserga, 2007). The vast majority of DEXD/H proteins involved in ribosome biogenesis are essential for viability, underscoring the fact that they have non-redundant essential roles. Most are either involved in the synthesis of the large or small ribosomal subunit. Only two such proteins, Has1p and Prp43p, intervene in both pathways (Emery *et al.*, 2004; Lebaron *et al.*, 2005; Rocak *et al.*, 2005; Combs *et al.*, 2006; Leeds *et al.*, 2006). Although the ribosome biogenesis steps affected by lack of a given DEXD/H protein have been characterized, their precise molecular functions remain elusive. The DEXD/H RNA helicases have been demonstrated or are believed to use ATP hydrolysis to unwind RNA duplexes or to disrupt RNA–protein complexes (Jankowsky *et al.*, 2001; Tanner and Linder, 2001; Fairman *et al.*, 2004; Bleichert and Baserga, 2007; Jankowsky and Fairman, 2007). They could also act as clamp holders, being released from their RNA substrate by ATP hydrolysis. In the context of ribosome biogenesis, DEXD/H RNA helicases could, for instance, promote conformational rearrangements of pre-rRNA, remove non-ribosomal proteins bound to pre-rRNA or dissociate small nucleolar RNAs from pre-rRNA. Indeed, it has recently been shown that lack of Dbp4p, Has1p or Rok1p leads to the retention of specific snoRNAs on pre-rRNAs, suggesting that these enzymes remove these snoRNAs from pre-rRNAs (Kos and Tollervey, 2005; Liang and Fournier, 2006; Bohnsack *et al.*, 2008). However, a demonstration that Dbp4p, Has1p or Rok1p directly unwind

*Corresponding authors. M Grigoriev or Y Henry, Centre National de la Recherche Scientifique, Laboratoire de Biologie Moléculaire Eucaryote, 118 route de Narbonne, Toulouse 31062, France.

Tel.: + 33 561 335 919; Fax: + 33 561 335 886;

E-mail: grigor@ibcg.biotoul.fr or

Tel.: + 33 561 335 953; Fax: + 33 561 335 886;

E-mail: henry@ibcg.biotoul.fr

⁴These authors contributed equally to this work

Received: 5 June 2009; accepted: 15 October 2009; published online: 19 November 2009

pre-rRNA/snoRNA complexes has not yet been provided. The Dob1p/Mtr4p RNA helicase is required for 7S pre-rRNA maturation (de la Cruz *et al*, 1998) and could facilitate 7S processing catalysed by the exosome by unwinding secondary structures within the internal transcribed spacer 2. Apart from these examples, the RNA and/or protein targets of the other DEXD/H RNA helicases involved in ribosome biogenesis remain totally unknown.

Among DEXD/H RNA helicases required for ribosome synthesis, Prp43p is particularly intriguing. It acts not only in ribosome synthesis but also in pre-mRNA splicing, being required for the release of the spliced-out lariat intron from the U2/U5/U6 post-spliceosome and for the disassembly of this post-spliceosome (Arenas and Abelson, 1997; Martin *et al*, 2002; Tsai *et al*, 2005). Moreover, yeast Prp43p seems to be associated with pre-ribosomal particles from the initial transcriptional steps of the pre-rRNA to the last maturation steps of the pre-ribosomal particles in the cytoplasm (Lebaron *et al*, 2005), suggesting it acts at several steps of the ribosome biogenesis pathways on several different substrates. The nature of these substrates and the way the activities of Prp43p are controlled during ribosome biogenesis remain unknown. It has previously been shown, however, that Prp43p directly interacts with Ntr1p (Tsai *et al*, 2005; Tanaka *et al*, 2007), a protein required for splicing that contains a G-patch, a glycine-rich domain proposed to mediate protein-protein or protein-RNA interactions (Aravind and Koonin, 1999). Ntr1p is required for Prp43p interaction with the spliceosome and stimulates its helicase activity. In this study, we demonstrate that Pfa1p, a G-patch-containing protein involved in ribosome biogenesis, directly binds to Prp43p. We show that binding of Pfa1p to Prp43p stimulates its ATPase and helicase activities and we present evidence suggesting that activation of the ATPase/helicase activities of Prp43p by Pfa1p is important for 20S pre-rRNA processing.

Results

G-patch protein Pfa1p/Sqs1p can directly bind to Prp43p through two distinct structural domains

How Prp43p is targeted to pre-ribosomal particles and how its activities are controlled within these particles remain open questions. To start addressing these, we searched for factors involved in ribosome biogenesis that either directly interact with Prp43p or are closely connected to Prp43p. Our attention was drawn to Gno1p and Pfa1p (also known as Sqs1p) for the following reasons: (1) Gno1p interacts with Prp43p in a double-hybrid assay and is found among the proteins co-purifying with Prp43p after tandem affinity purification (Lebaron *et al*, 2005). (2) Pfa1p is the most abundant polypeptide co-purified with Prp43p after tandem affinity purification (Lebaron *et al*, 2005). (3) Both Gno1p and Pfa1p contain a G-patch domain thought to mediate protein-protein or protein-RNA interactions (Aravind and Koonin, 1999). (4) Finally, both Gno1p and Pfa1p are linked to ribosome biogenesis. Yeast cells lacking Gno1p are strongly impaired in growth and display early pre-rRNA processing defects (Guglielmi and Werner, 2002), whereas we have previously shown that Pfa1p is a component of pre-40S pre-ribosomal particles (Lebaron *et al*, 2005).

To test whether Gno1p and Pfa1p directly interact with Prp43p, pull-down assays were performed using recombinant

proteins purified in *Escherichia coli*. His-tagged Prp43p, Pfa1p and Nob1p were purified using affinity chromatography on nickel columns followed by size exclusion chromatography. Nob1p is a specific component of pre-40S pre-ribosomal particles (Fatica *et al*, 2003, 2004), which was used as a negative control in pull-down experiments. Full-length His-tagged Gno1p could not be sufficiently expressed in *E. coli* to allow its purification, although we could obtain sufficient full-length GST-tagged Gno1p expression to purify it by glutathione-Sepharose chromatography followed by size exclusion chromatography. The integrity of the purified proteins and the purity of the preparations were checked by Coomassie blue staining (Figure 1A and data not shown) and western blotting (data not shown). Purified Pfa1p-His or Nob1p-His proteins were mixed with purified Prp43p-His and incubated on Sepharose beads coated with polyclonal anti-Prp43p antibodies. Hence, only proteins specifically interacting with Prp43p were retained on the beads after stringent washing. Pfa1p-His was detected in the bead fraction and depleted from the supernatant (Figure 1B, lanes 5 and 6). In contrast, Nob1p-His was fully recovered in the supernatant fraction (Figure 1B, lanes 7 and 8). These data indicate that Pfa1p specifically interacts with Prp43p *in vitro*. Interactions between GST-Gno1p and Prp43p-His were assessed by GST pull-down experiments using glutathione-Sepharose beads. No reproducible *in vitro* interaction between GST-Gno1p and Prp43p-His could be obtained. Hence it remains unclear at present whether Gno1p can directly bind to Prp43p.

Hydrophobic cluster analysis of Pfa1p (Callebaut *et al*, 1997) reveals that this protein is composed of three structural domains (Figure 1A). The G-patch is located in the C-terminal structural domain of Pfa1p. Truncated versions of His-tagged Pfa1p were purified from *E. coli* (Figure 1A) and tested for their ability to interact with Prp43p-His using the procedures outlined above. This analysis revealed that the N-terminal domain (amino acids 1–202) and C-terminal G-patch-containing domain (amino acids 574–767) of Pfa1p can independently interact with Prp43p-His (Figure 1C, lanes 13 and 15). We conclude that Pfa1p contains two independent Prp43p interaction domains. However, much greater proportions of Pfa1p(1–202) and Pfa1p(574–767) are recovered in the supernatant fraction (Figure 1C, lanes 14 and 16) compared, respectively, with Pfa1p(1–565) (Figure 1C, lane 12) and Pfa1p(201–767) (Figure 1C, lane 18), demonstrating that the middle domain of Pfa1p stabilizes the interactions of the N- and C-terminal domains with Prp43p.

Gno1p is likely present in 90S pre-ribosomal particles, whereas Pfa1p is present in 90S, pre-40S and pre-60S pre-ribosomal particles

As a first step to analyse the functions of Gno1p and Pfa1p, we attempted to characterize the pre-ribosomal particles with which they associate. We consistently failed to precipitate HA-tagged or TAP-tagged Gno1p from yeast extracts. Hence, we analysed the sedimentation profile of TAP-tagged Gno1p on a sucrose gradient instead (Supplementary Figure S1). In parallel experiments, we also analysed the sedimentation profile of TAP-tagged Pfa1p (Supplementary Figure S1), which we previously found almost exclusively associated with 20S pre-rRNA by precipitation analyses (Lebaron *et al*, 2005). Gno1p-TAP co-sedimented with 35S pre-rRNA, sug-

Figure 1 Pfa1p interacts directly with Prp43p *in vitro*. (A) Pfa1p domain structure (top) and Coomassie blue staining (bottom) of purified Prp43p-His, Pfa1p-His, Nob1p-His and truncated versions of Pfa1p-His. (B, C) Pull-down experiments. Purified proteins were mixed in the indicated combinations and incubated with IgG-Sepharose beads coated with polyclonal anti-Prp43p antibodies. Proteins remaining in the supernatant fractions (Sup) were precipitated using TCA. After washing the beads, pulled-down proteins (IP) were re-suspended in SDS-PAGE loading buffer. Proteins were detected by the western blotting procedure using anti-histidine-HRP antibodies.

gesting that it is a component of 90S pre-ribosomal particles. This is fully consistent with the nucleolar localization of Gno1p and with the inhibition of early pre-rRNA processing steps detected in cells lacking Gno1p (Guglielmi and Werner, 2002). Prp43p, detected using anti-Prp43p antibodies, was found in most fractions of the gradient, consistent with it being a component of 90S, pre-40S and pre-60S pre-ribosomal particles, as well as of post-splicing complexes. Pfa1p-TAP was detected in fractions containing 40S riboso-

mal subunits and 20S pre-rRNA, as would be expected for a component of pre-40S pre-ribosomal particles. However, to our surprise, it was also present in fractions containing 27SA2 or 35S pre-rRNAs, suggesting that it is also a component of 90S and pre-60S particles. To confirm this finding, the protein partners of Pfa1p-TAP were identified by tandem affinity purification of this protein followed by mass-spectrometry identification of the co-purifying proteins. Among the protein partners of Pfa1p-TAP, we observed not only components

of pre-40S particles, but also numerous protein factors associated with 90S and pre-60S pre-ribosomal particles (Supplementary Figure S2 and Supplementary Table I). This finding is corroborated by a recent large scale genetic interaction screen that found that lack of the *PFA1* gene induces a growth defect when combined with the deletion of the *ALB1*, *ARX1* or *FPR4* genes encoding components of pre-60S particles (Decourty *et al*, 2008). From these studies, we conclude that Pfa1p is present in some 90S, pre-60S and pre-40S pre-ribosomal particles.

Pfa1p, Gno1p and Ltv1p are linked

As Pfa1p and Gno1p are closely associated with Prp43p *in vivo* and as Pfa1p can directly bind to Prp43p *in vitro*, we next checked whether Pfa1p and Gno1p are required for normal accumulation of Prp43p. Western blot analyses using anti-Prp43p antibodies and total protein extracts from $\Delta gno1$ or $\Delta pfa1$ cells showed that total Prp43p levels are unaffected by

lack of Gno1p or Pfa1p (data not shown). However, we made the surprising observation that the steady-state levels of Pfa1p are strongly decreased in $\Delta gno1$ cells and, moreover, that the Pfa1p protein migrates faster on denaturing SDS-polyacrylamide gels, consistent with it being truncated (Figure 2A, lanes 1 and 2). On the contrary, the absence of Pfa1p has no effect on the steady-state levels of Gno1p (data not shown). As Pfa1p is detected through its TAP tag fused at the C-terminus, the truncated version of Pfa1p detected in $\Delta gno1$ cells lacks some of the N-terminus. Such an observation could be explained by postulating that Gno1p and Pfa1p interact directly and that Pfa1p becomes susceptible to protease cleavage when not bound to Gno1p. However, we have so far failed to obtain convincing evidence for a direct interaction between Gno1p and Pfa1p *in vitro*. Another possibility is that lack of Gno1p, which results in reduced steady-state levels of 20S pre-rRNA (Guglielmi and Werner, 2002), could lead to a general destabilization of components

Figure 2 The pre-40S pre-ribosomal particle composition in $\Delta gno1$ cells. (A) Western blot analysis of the steady-state accumulation of the indicated TAP-tagged non-ribosomal pre-40S pre-ribosomal particle components in $\Delta gno1$ (ΔG) and isogenic wild-type cells (WT). ' α PAP' refers to the PAP rabbit antibodies (DAKO) used to detect the TAP tag fused to the proteins of interest. (B) Western blot analysis of the interaction between Pfa1p-TAP and Prp43p in yeast extracts. Extracts from $\Delta gno1$ cells expressing Pfa1p-TAP (lanes 5 and 6) or from isogenic wild-type cells expressing Pfa1p-TAP (lanes 3 and 4) or wild-type Pfa1p (lanes 1 and 2) were incubated with IgG-Sepharose beads. Total proteins were extracted from the bead pellets (IP) or from 1/30th of the initial input extracts used for immunoprecipitations (Tot) and analysed by western blotting. (C) Interactions between 20S pre-rRNA and non-ribosomal pre-40S pre-ribosomal particle components in wild-type (WT) and $\Delta gno1$ (ΔG) cells. Extracts from $\Delta gno1$ or isogenic wild-type cells expressing the indicated TAP-tagged proteins were incubated with IgG-Sepharose beads. Total RNAs were extracted from the bead pellets (IP) or from 1/30th of the initial input extracts used for immunoprecipitations (Tot). 20S pre-rRNA was detected by northern blotting. Percentages of input 20S pre-rRNA precipitated are indicated below the IP lanes.

of pre-40S pre-ribosomal particles. To investigate this possibility, we generated $\Delta gno1$ and isogenic wild-type strains expressing TAP-tagged versions of eight different non-ribosomal pre-40S pre-ribosomal particle components (Rio2p, Dim2p, Slx9p, Enp1p, Tsr1p, Fap7p, Nob1p and Ltv1p) and assessed their steady-state accumulation by western blot analysis. Strikingly, none of these proteins displayed significantly altered steady-state accumulation in $\Delta gno1$ cells, except Ltv1p, the steady-state levels of which were strongly elevated (Figure 2A, compare lanes 17 and 18). Thus, there seems to be a specific connection between Gno1p, Pfa1p and Ltv1p. Moreover, we observed that the 'truncated' version of Pfa1p detected in $\Delta gno1$ cells is unable to interact with Prp43p and 20S pre-rRNA (Figure 2B, lanes 5 and 6 and Figure 2C, lanes 3–6), possibly, in part, because this truncated Pfa1p variant lacks some of its N-terminal domain shown to bind to Prp43p (Figure 1C). In contrast, lack of Pfa1p does not affect the ability of Gno1p to interact with Prp43p, nor does it increase the proportion of Prp43p associated with Gno1p (data not shown). Interestingly, a greater proportion of 20S pre-rRNA is precipitated by Ltv1p-TAP from $\Delta gno1$ compared with isogenic wild-type cell extracts (Figure 2C, lanes 27–30). Thus, in $\Delta gno1$ cells, pre-40S pre-ribosomal particles seem devoid of Pfa1p and a greater

proportion of these contain Ltv1p. This latter effect may result from partial stalling of Ltv1p within pre-40S pre-ribosomal particles due to lack of Pfa1p and/or other pre-40S particle defect resulting from the absence of Gno1p.

Pfa1p is implicated in 35S and 20S pre-rRNA processing

To strengthen the case for a specific connection between Gno1p, Pfa1p and Ltv1p, we analysed the phenotypes of $GAL::ltv1/\Delta gno1$ or $GAL::ltv1/\Delta pfa1$ cells depleted for Ltv1p by shifting these cells from galactose- to glucose-containing medium. The $GAL::ltv1/\Delta gno1$ cells did not display stronger growth impairment after shifting them from galactose to glucose-containing medium at any of the temperatures tested (25, 30 or 37°C), although we checked that Ltv1p depletion effectively took place (data not shown). This suggests that *GNO1* is epistatic to *LTV1*. In contrast, we observed that shifting $GAL::ltv1/\Delta pfa1$ cells from galactose- to glucose-containing medium led to a growth defect at 30°C that was exacerbated by lowering the temperature to 25°C (data not shown; see also Figure 4A). To assess whether $GAL::ltv1/\Delta pfa1$ cells depleted for Ltv1p displayed a pre-rRNA processing defect, we analysed, using the northern blotting procedure, the accumulation of pre-rRNAs extracted from $GAL::ltv1/\Delta pfa1$, $\Delta pfa1$ or $\Delta ltv1$ cells grown at 25°C in galactose- or glucose-containing medium

Figure 3 The 20S pre-rRNA strongly accumulates in $GAL::ltv1/\Delta pfa1$ cells depleted for Ltv1p. (A) Structure of the primary RNA Pol I transcript. Cleavage sites are indicated as well as the hybridization position of probes used in the northern blot analysis. (B) Northern blot analysis of pre-rRNAs extracted from $GAL::ltv1/\Delta pfa1$, $\Delta pfa1$ or $\Delta ltv1$ cells grown at 25°C in galactose- (Gal) or glucose-containing (Glu) medium, as indicated, is shown. The probes used are indicated above each hybridization panel.

(Figure 3; see Supplementary Figure S3 for a cartoon of the pre-rRNA processing steps in *Saccharomyces cerevisiae*). This analysis revealed a massive accumulation of 20S pre-rRNA in *GAL::ltv1/Δpfa1* cells depleted for Ltv1p (Figure 3, lane 20), compared to un-depleted *GAL::ltv1/Δpfa1* cells (Figure 3, lane 19), *Δpfa1* (lanes 21 and 22) or *Δltv1* (lanes 23 and 24) cells. Phosphorimager quantification shows that there is approximately 1.8-fold less 18S rRNA relative to 25S rRNA in *Δltv1* cells compared with *Δpfa1* cells grown on galactose, whereas, as expected, no difference is observed in relative 18S rRNA levels between *GAL::ltv1/Δpfa1* and *Δpfa1* cells grown on galactose. In contrast, when glucose is present in the growth medium, there is an approximately four-fold reduction in 18S rRNA levels relative to 25S rRNA in *GAL::ltv1/Δpfa1* cells compared with *Δpfa1* cells, whereas a 1.6-fold reduction in relative 18S rRNA levels is observed in *Δltv1* cells compared with *Δpfa1* cells, similar to what was observed in galactose medium. Thus, although lack of Ltv1p already leads to a reduction in 18S rRNA levels, the effect is more dramatic when Pfa1p is also absent. Interestingly, an aberrant RNA migrating faster than 18S rRNA could be detected with a probe ('18S mid') hybridizing in the middle of 18S rRNA in samples from *GAL::ltv1/Δpfa1* cells depleted for Ltv1p (Figure 3, lane 8). This RNA probably corresponds to the '17S' rRNA that is often detected in cells displaying 20S pre-rRNA processing defects (Jakovljevic *et al*, 2004; Granneman *et al*, 2005) and lacks the 3' end of 18S rRNA, as it is not detected by a probe ('18S 3') that hybridizes to the 3' end of 18S rRNA (Figure 3, lane 14). Production of the 22S pre-rRNA, which extends from site A0 to site A3, seems to result from the absence of Ltv1p, as it is detected at similar levels in both *Δltv1*

and *GAL::ltv1/Δpfa1* cells grown on glucose (Figure 3, lanes 2 and 6). The above-described aberrant pre-rRNA accumulation profiles are not the result of a destabilization of Gno1p, levels of which are unaffected in *Δltv1* cells or *GAL::ltv1/Δpfa1* cells depleted for Ltv1p (data not shown). Altogether, these data suggest that Pfa1p has a role, direct or indirect, in 20S pre-rRNA transport to the cytoplasm and/or in 20S pre-rRNA to 18S rRNA processing. To distinguish between these two possibilities, we checked whether 20S pre-rRNA that accumulates in *GAL::ltv1/Δpfa1* cells depleted for Ltv1p is present in the cytoplasm. This was done by two approaches. First, we assessed by primer extension (Supplementary Figure S4A) whether this 20S pre-rRNA contains two methylated adenines ($m^6A_{1779}m^6A_{1780}$) at the 3' end of the 18S rRNA sequence. This modification is introduced by the Dim1p dimethylase (Lafontaine *et al*, 1995) and is believed to be a late, maybe cytoplasmic, maturation event of 20S pre-rRNA (Brand *et al*, 1977). Second, we more directly assessed the sub-cellular distribution of 20S pre-rRNA by performing a cell fractionation experiment and assessing by northern blot analysis the presence of various pre-rRNAs in the nuclear and cytoplasmic fractions (Supplementary Figure S4B). Our data unambiguously show that the bulk of 20S pre-rRNAs produced in *GAL::ltv1/Δpfa1* cells depleted for Ltv1p are cytoplasmic, as they contain the two methylated adenines and are retrieved in the cytoplasmic fraction.

To identify the domains of Pfa1p required for its function, we transformed *GAL::ltv1/Δpfa1* cells with centromeric plasmids directing the expression of ZZ-tagged truncated versions of Pfa1p, and analysed the growth of the resulting transformants on glucose-containing medium at 25°C (Figure 4A). None of the truncated versions of Pfa1p restored the growth

Figure 4 Phenotypes of *GAL::ltv1/Δpfa1* cells expressing truncated variants of Pfa1p. *GAL::ltv1/Δpfa1* cells were transformed with centromeric plasmids directing expression of wild-type ZZ-tagged Pfa1p (Pfa1p-ZZ), the ZZ tag alone, or truncated variants of Pfa1p, Pfa1p(1-565)-ZZ, Pfa1p(574-767)-ZZ, Pfa1p(1-202)-ZZ, Pfa1p(201-565)-ZZ and Pfa1p(201-767)-ZZ. (A) Growth of the transformed cells was assessed by performing serial dilutions of the cultures, aliquots of which were spotted on glucose-containing minimal medium plates incubated at 25°C for 4 days. (B) Western blot analysis of the expression of ZZ-tagged Pfa1p or truncated derivatives in *GAL::ltv1/Δpfa1* cells grown in galactose- or glucose-containing medium. (C) Northern blot analysis of pre-rRNA and rRNA steady-state levels in *GAL::ltv1/Δpfa1* cells expressing ZZ-tagged Pfa1p or truncated derivatives and grown in galactose- or glucose-containing medium at 25°C.

of *GAL::ltv1/Δpfa1* cells depleted for Ltv1p at 25°C, except Pfa1p(201–767)-ZZ that fully complemented the growth defect (Figure 4A). A lack of growth restoration was not caused by reduced expression of the truncated proteins that displayed essentially similar steady-state levels (Figure 4B). Northern blot analysis of pre-rRNA and rRNA levels revealed that *GAL::ltv1/Δpfa1* cells depleted for Ltv1p at 25°C and expressing Pfa1p(1–565)-ZZ, Pfa1p(574–767)-ZZ, Pfa1p(1–202)-ZZ or Pfa1p(201–565)-ZZ display essentially the same pre-rRNA/rRNA accumulation pattern, namely increased steady-state levels of 20S pre-rRNA, decreased steady-state levels of 18S rRNA relative to 25S rRNA (approximately 1.3–1.5-fold reduction as assessed by phosphorimager quantification, a lesser effect as the one detected in rich medium), and appearance of the aberrant '17S' species (Figure 4C, lanes 9–12). Unexpectedly, expression of Pfa1p(201–767)-ZZ in *GAL::ltv1/Δpfa1* cells depleted for Ltv1p at 25°C led to an earlier pre-rRNA processing defect: 35S and 23S pre-rRNA steady-state levels increased, whereas 20S pre-rRNA levels decreased and 32S and 27SA2 pre-rRNAs were totally undetectable (Figure 4C, lane 13). The same processing defects were observed in *GAL::ltv1/Δpfa1* cells expressing Pfa1p(201–767)-ZZ grown on galactose-containing medium, that is, expressing Ltv1p, except that steady-state levels of 35S pre-rRNA were decreased (Figure 4C, lane 6). The *GAL::ltv1/Δpfa1* cells expressing Pfa1p(574–767)-ZZ grown on galactose-containing medium at 25°C displayed an intermediate phenotype, namely decreased 32S and 27SA2 pre-rRNA steady-state levels (Figure 4C, lane 3).

Altogether, these data suggest that the central and the G-patch-containing structural domains of Pfa1p are essential for its function in 20S pre-rRNA processing. They also suggest that Pfa1p is involved in 35S pre-rRNA processing at the A1 and A2 sites. The involvement of Pfa1p in 35S pre-rRNA processing is probably direct, as we observed that numerous protein components of 90S pre-ribosomal particles co-purify with TAP-tagged Pfa1p after tandem affinity purification (Supplementary Figure S2 and Supplementary Table I). Our results suggest that the N-terminal Prp43p interaction domain of Pfa1p is required for its function in 35S pre-rRNA processing. Strikingly, it seems that the presence of the other C-terminal Prp43p interaction domain (and presumably, therefore, interaction with Prp43p) is necessary for Pfa1p-truncated variants lacking domain 1–202 to exert their toxic effect on 32S and 27SA2 pre-rRNA accumulation.

Gno1p and Pfa1p are not essential for integration or retention of Prp43p within pre-ribosomal particles

Gno1p and Pfa1p may function in ribosome biogenesis by promoting integration or retention of Prp43p within pre-ribosomal particles. This hypothesis was first assessed by determining the sub-cellular localization of Prp43p in *Δgno1* or *Δpfa1* cells, and in the corresponding wild-type parental strains. Prp43p was visualized by immunostaining using specific polyclonal anti-Prp43p antibodies. The nucleolus was stained using anti-Nop1p antibodies. The merged Prp43p and Nop1p localization images (Supplementary Figure S5) reveal that both in *Δgno1* and *Δpfa1* cells, Prp43p is mostly concentrated in the nucleolus, as in wild-type cells. We next assessed the efficiency of co-precipitation of different pre-rRNAs with tagged Prp43p from *Δgno1*, *Δpfa1* or control parental cell extracts. We found that lack of Gno1p

has little effect on the efficiency of co-precipitation of pre-rRNAs with tagged Prp43p (Supplementary Figure S6A). Similarly, the efficiency of co-precipitation of 27SB pre-rRNA was unaffected by lack of Pfa1p (Supplementary Figure S6B). However, we noted that co-precipitation of 20S pre-rRNA was reduced approximately two-fold by the lack of Pfa1p. We conclude that Gno1p and Pfa1p are not essential for integration or retention of Prp43p within pre-ribosomal particles, although in the absence of Pfa1p, the association of Prp43p with pre-40S pre-ribosomal particles is destabilized.

Pfa1p stimulates the ATPase activity of Prp43p in vitro

As Pfa1p has no essential role in the association of Prp43p with pre-ribosomal particles and directly interacts with Prp43p, we tested next whether Pfa1p can modulate the enzymatic activities of Prp43p. First, the rates of ATP hydrolysis by Prp43p–His were measured as a function of ATP concentration in the absence of RNA and in the absence or presence of Pfa1p–His (Figure 5A). To derive these rates, individual ATP hydrolysis time courses were performed at 30°C by incubating Prp43p–His (100 nM) with ATP (up to 2.5 mM) using [α -³²P]-ATP as a tracer and quantified after thin layer chromatography. The maximal rate of ATP hydrolysis by Prp43p–His was 1 ± 0.02 per min (mole ATP/mole protein/min; Figure 5A). When Pfa1p–His was added to the reaction mixture to a final concentration of 500 nM, ATP hydrolysis increased approximately 30-fold (36 ± 3 per min). In contrast, addition of purified Nob1p–His, an RNA-binding protein, or GST did not stimulate ATP hydrolysis (data not shown). Pfa1p–His itself did not show any detectable ATPase activity in the absence of Prp43p–His or in the presence of RNA (data not shown). To rule out the possibility that the observed activation of ATP hydrolysis by Pfa1p–His could be caused by RNA contaminants in our Pfa1p–His preparation, we treated this protein with RNase A before its addition to the reaction mixture. This treatment had no effect on ATP hydrolysis by Prp43p–His (data not shown). We conclude from these results that direct binding of Pfa1p–His to Prp43p–His increases its ability to hydrolyse ATP in the absence of RNA.

In the presence of saturating concentrations of RNA (150 μ M as nucleotides), the rate of ATP hydrolysis by Prp43p–His was stimulated approximately 180-fold compared with that measured in the absence of RNA (180 ± 6 versus 1 ± 0.02 per min; Supplementary Figure S7). The addition of Pfa1p–His (500 nM) further increased the rate of ATP hydrolysis to 394 ± 14 per min (Figure 5B). We conclude that Pfa1p–His and RNA can synergistically stimulate ATP hydrolysis by Prp43p–His.

Pfa1p could stimulate the rate of ATP hydrolysis by Prp43p by increasing its affinity for ATP or, alternatively, by increasing the rate of hydrolysis itself. To test the first hypothesis, we performed UV cross-linking of Prp43p–His (1 μ M) with [γ -³²P]-ATP followed by SDS-gel electrophoresis and autoradiography (Figure 5C). Prp43p–His, detected as a 95-kDa band, was efficiently cross-linked to ATP in the absence or in the presence of RNA (Figure 5C, compare top and bottom panels, lane 2). It should be noted that, in the presence of increasing concentrations of Pfa1p–His (Figure 5C, lanes 3–5), the amount of cross-linked species did not significantly vary compared with that obtained in the absence of this protein (Figure 5C, lane 2). This result indicates that binding of ATP

Figure 5 Stimulation of the ATPase activity of Prp43p by Pfa1p. (A) Rate of ATP hydrolysis by Prp43p-His (100 nM) as a function of ATP concentration in the absence of RNA and in the absence (solid circles) or in the presence (open circles) of Pfa1p-His at 500 nM (two independent experiments are shown). Experimental points were fitted with the Michaelis-Menten equation. Deviations are from the fit. (B) Same as in (A) except that total yeast RNA (150 μ M) was added. (C) Pfa1p-His does not increase the affinity of Prp43p-His for ATP. UV cross-linking of Prp43p-His (1 μ M) with [γ -³²P]-ATP was performed in the absence (top panel) or presence (bottom panel) of total yeast RNA at 150 μ M (as nucleotides). Pfa1p-His was added to the reactions loaded in lanes 3–5 at the following concentrations: lane 3: 0.5 μ M; lane 4: 0.75 μ M; lane 5: 1 μ M. After irradiation, cross-linked Prp43p-His was subjected to SDS-polyacrylamide gel electrophoresis and detected by autoradiography. (D) Stimulation of ATP hydrolysis requires the G-patch-containing domain of Pfa1p. Where indicated, ATP hydrolysis was performed with Prp43p-His (100 nM) and either full-length Pfa1p-His or various truncated Pfa1p-His variants (500 nM). ATP and ADP were separated by TLC and the percentage of ADP produced quantified by phosphorimager.

to Prp43p is not affected by Pfa1p. We conclude from these results that the observed induction of Prp43p-His ATPase activity by Pfa1p-His most probably reflects an increase in the catalytic efficiency of Prp43p-His.

To determine which structural domains of Pfa1p are required for the stimulation of Prp43p ATPase activity, ATPase assays were performed with Prp43p-His and truncated variants of Pfa1p (Figures 1A and 5D). As expected, Pfa1p(201–565)-His, which is unable to interact with Prp43p, failed to stimulate ATP hydrolysis. Truncated variants Pfa1p(1–565)-His and Pfa1p(1–202)-His that can interact with Prp43p-His through the N-terminal structural domain, but lack the C-terminal Prp43p interaction domain also failed to stimulate ATP hydrolysis. In contrast, the C-terminal G-patch-containing domain (Pfa1p(574–767)-His) that can independently bind to Prp43p was able to stimulate ATP hydrolysis, although the magnitude of the stimulation was halved in comparison with full-length Pfa1p-His. Interestingly, the Pfa1p(201–767)-His-truncated variant stimulated ATP hydrolysis to a greater extent than wild-type Pfa1p-His. We conclude that the N-terminal Prp43p interaction domain of Pfa1p is not required for the stimulation of ATP hydrolysis and could even have an inhibitory effect. The G-patch-containing Prp43p interaction domain of Pfa1p is necessary for the stimulation but is not sufficient for full stimulation, which requires the addition of the central structural domain.

Pfa1p stimulates the helicase activity of Prp43p

Prp43p has been shown to function as a RNA helicase *in vitro* (Tanaka and Schwer, 2006; Tanaka *et al*, 2007). We therefore investigated whether the stimulation of Prp43p ATPase activity by Pfa1p might also be accompanied by a stimulation of Prp43p helicase activity. We constructed two RNA–DNA hybrid helicase substrates, containing either 3′- or 5′-single-stranded tails, to allow detection of 3′–5′ or 5′–3′ unwinding by Prp43 (Figure 6A). We first tested the ability of Prp43p-His and Pfa1p-His to interact with these RNA–DNA substrates through electrophoretic mobility shift assays (EMSAs) in the absence of ATP (Supplementary Figure S8). Prp43p-His bound both substrates with a similar affinity (mid-points at 7 ± 0.6 nM for the 3′–5′ substrate and 10 ± 0.2 nM for the 5′–3′ substrate). Pfa1p-His showed a modest preference for binding to the 5′–3′, compared with the 3′–5′ substrate (mid-points at 16 ± 0.6 and 33 ± 2 nM, respectively). The addition of Pfa1p-His to Prp43p-His led to the formation of a stable ternary complex in a concentration-dependent manner (see supershift in Figure 6B). Binding occurred with similar affinities for both helicase substrates (compare left and right panels). The formation of this ternary complex may result from binding of Pfa1p-His to RNA-bound Prp43p-His, or binding of Prp43p-His and Pfa1p-His to the same RNA molecule, or a combination of both.

Having shown that Prp43p-His together with Pfa1p-His can form a stable complex with both helicase substrates, we

Figure 6 Pfa1p-His stimulates the helicase activity of Prp43p-His. (A) Schematics of the RNA-DNA hybrid substrates used for the electrophoretic mobility shift assays (EMSAs) and helicase assays. The 113 nucleotide-long RNA probe corresponds to the 5' stem loop and H box of snR5 snoRNA. It was annealed to either one of two 21 nucleotide-long radiolabelled DNA oligonucleotides. Annealing produced either a 3' single-stranded overhang (3'-5' helicase substrate) or a 5' single-stranded overhang (5'-3' substrate). (B) EMSAs performed with the 3'-5' (left panel) or the 5'-3' (right panel) substrates, Prp43p-His at a concentration of 10 nM and increasing concentrations of Pfa1p-His (from 0.5 to 100 nM). Positions of the free probe, the Prp43p-His-bound probe and the Prp43p-His/Pfa1p-His/probe ternary complex ('supershift') are indicated. (C) Helicase assays performed with the 3'-5' substrate (left panel) or the 5'-3' substrate (right panel). Positions of the RNA/labelled DNA substrate and of the unwound oligonucleotide are indicated. Where indicated, Prp43p-His and/or Pfa1p-His were added to the unwinding reactions at 100 nM and 500 nM, respectively. (D) Duplex unwinding is ATP dependent. The unwinding reaction was performed with Prp43p-His at 100 nM, Pfa1p-His at 500 nM, and different nucleotides at 1 mM. (E) Pfa1p domains required for the stimulation of Prp43p-His helicase activity. Helicase assays were performed with the 3'-5' substrate as in (C), Prp43p-His (100 nM) and either full-length Pfa1p-His or various truncated Pfa1p-His variants (500 nM), as indicated.

then performed helicase assays by mixing the 3'-5' or 5'-3' substrates (1 nM) with Prp43p-His (100 nM) with or without Pfa1p-His (500 nM) in the presence of ATP (1 mM) and analysing unwinding of substrates by polyacrylamide gel electrophoresis (Figure 6C). In the absence of Pfa1p-His, no Prp43p-mediated unwinding could be detected on either substrate under these experimental conditions (Figure 6C, lanes 4 and 9). Only when the concentration of Prp43p-His was increased to 1 μ M could unwinding of the 3'-5' substrate be detected (data not shown). However, when Pfa1p-His was included in the reaction containing Prp43p-His at 100 nM, the 3'-5' and 5'-3' substrates were efficiently unwound (Figure 6C, lanes 5 and 10). This helicase activity was ATP dependent, as little or no unwinding was observed when AMP or ADP were added to the reactions (Figure 6D, lanes 4

and 5). Furthermore, unwinding was dependent on ATP hydrolysis, as poorly or non-hydrolysable ATP analogues, ATP γ S or AMP-PNP, failed to fuel unwinding reactions (Figure 6D, lanes 7 and 8).

Finally, we performed helicase assays with Prp43p-His and truncated variants of Pfa1p-His to identify the structural domains of Pfa1p involved in the stimulation of the helicase activity of Prp43p (Figure 6E). All Pfa1p variants that failed to stimulate Prp43p ATPase activity also failed to stimulate its helicase activity. Conversely, all truncated variants of Pfa1p that stimulated the ATPase activity of Prp43p also stimulated its helicase activity (compare Figures 5D and 6E). This strongly suggests that the stimulation by Pfa1p of Prp43p helicase activity chiefly results from the stimulation of Prp43p ATPase activity.

Discussion

Several examples of RNA helicase co-factors that are required for the recruitment of the helicase to its substrate and/or enhance the ATPase activity of the RNA helicase *in vitro* have been described previously (Silverman *et al*, 2003; Bleichert and Baserga, 2007). All the published examples deal with helicase co-factors involved in splicing, translation initiation, RNA turnover, viral replication, RNA export or RNAi, with one exception, the Esf2p protein, which is involved in ribosome biogenesis (Granneman *et al*, 2006). We have now identified one additional helicase co-factor involved in ribosome biogenesis, the G-patch protein Pfa1p, which is a direct binding partner of Prp43p and stimulates its activity. Moreover, we demonstrate that Gno1p, another G-patch protein necessary for ribosome biogenesis and associated with Prp43p *in vivo*, is specifically required for normal accumulation of Pfa1p and for its ability to interact with Prp43p and 20S pre-rRNA. The reverse is not true, that is, Pfa1p is neither necessary for the accumulation of Gno1p, nor for its ability to interact with Prp43p. Our data suggest that Pfa1p and Gno1p are both associated, directly or indirectly, with Prp43p within some 90S pre-ribosomal particles, whereas only Pfa1p remains in pre-40S and pre-60S pre-ribosomal particles. Pfa1p and Gno1p are both involved in early pre-rRNA cleavages at sites A1 and A2. Pfa1p is also involved in cleavage at site D that converts 20S pre-rRNA into 18S rRNA. Finally, our data also strongly suggest that Pfa1p exerts its effect in 20S pre-rRNA maturation by directly binding to Prp43p and stimulating its enzymatic activities. Interestingly, recent data show that overexpression of Pfa1p can inhibit both pre-mRNA splicing and ribosome biogenesis (Pandit *et al*, 2009). The effect on splicing is probably indirect, resulting from a reduction in the Prp43p pool available for splicing, as we failed to find physical interactions between Pfa1p and splicing factors (Supplementary Table I).

G-patch proteins, regulators of DEAH proteins

Both Gno1p and Pfa1p contain a G-patch domain, characterized by the presence of six conserved glycines and one invariant leucine precisely positioned within or in the immediate vicinity of two putative α -helical structures (Aravind and Koonin, 1999). Strikingly, the G-patch protein Ntr1p also interacts with Prp43p (Tsai *et al*, 2005; Tanaka *et al*, 2007), whereas the G-patch protein Spp2p interacts with another DEAH helicase required for pre-mRNA splicing, Prp2p (Roy *et al*, 1995; Silverman *et al*, 2004). In all instances, a domain containing the G-patch has been shown to mediate interactions of these G-patch proteins with their DEAH box protein partner. In the case of Spp2p, one amino-acid substitution in the G-patch can abolish interactions between Spp2p and Prp2p *in vivo* (Silverman *et al*, 2004), whereas the first 122 N-terminal amino acids encompassing the G-patch of Ntr1p are necessary and sufficient for its interaction with Prp43p *in vivo* (Tsai *et al*, 2005) and *in vitro* (Tanaka *et al*, 2007). Furthermore, amino-acid substitutions within Ntr1p G-patch abolish interactions of the N-terminal 1–120 Ntr1p domain with Prp43p *in vitro* (Tanaka *et al*, 2007). We demonstrate here that the G-patch-containing C-terminal domain of Pfa1p, encompassing residues 574–767, can independently bind to Prp43p *in vitro*. However, binding affinity is increased by addition of the central domain (amino acids 201–573).

Two properties have been assigned to G-patch-containing proteins, namely targeting of DEAH box helicase proteins to the spliceosome (Silverman *et al*, 2004; Tsai *et al*, 2005) and activation of DEAH proteins (Tanaka *et al*, 2007). Spp2p and Ntr1p are required for the association of Prp2p to the spliceosome and of Prp43p to the post-spliceosome, respectively. We show that Gno1p and Pfa1p are not strictly required for the association of Prp43p with pre-ribosomal particles, although lack of Pfa1p reduces the efficiency of 20S pre-rRNA co-precipitation with Prp43p. Similar to Pfa1p, Ntr1p is able to activate the 5'–3' and 3'–5' duplex-unwinding activities of Prp43p *in vitro* (Tanaka *et al*, 2007). However, Tanaka *et al* (2007) have reported a failure of Ntr1p to stimulate the ATPase activity of Prp43p, whereas we demonstrate here that Pfa1p can have this effect. Our results suggest that Pfa1p binding to Prp43p increases the catalysis of ATP hydrolysis as such, as ATP binding seems unaffected. Moreover, we show that the G-patch-containing C-terminal domain of Pfa1p plays a key role in the stimulation because it alone can increase the ATPase and helicase activities of Prp43p, and because its presence is necessary for the stimulation. Similarly, the 1–120 N-terminal domain of Ntr1p containing the G-patch is capable of stimulating the helicase activity of Prp43p. Altogether, our results suggest that Pfa1p increases the helicase activity of Prp43p mainly by stimulating the catalysis of ATP hydrolysis through direct binding of the C-terminal G-patch-containing domain to Prp43p. Molecular details of the mechanism of stimulation will require crystal structures of Prp43p in complex with Pfa1p, ATP and RNA coupled with detailed mutational analyses.

In addition to the G-patch-containing domains, additional regions of Ntr1p and Pfa1p have important roles *in vivo*. For example, the central domain of Ntr1p is required for the interaction with Ntr2p, another essential factor required for lariat intron release from the post-spliceosome (Tsai *et al*, 2005). The central domain of Pfa1p plays an important role in 20S pre-rRNA maturation, maybe, in part, because it increases the affinity of the G-patch-containing C-terminal domain of Pfa1p for Prp43p.

Roles of Gno1p and Pfa1p within 90S pre-ribosomal particles

Both Gno1p and Pfa1p are present in 90S pre-ribosomal particles and involved in early cleavages at sites A1 and A2. The role of Gno1p is more important, as its absence leads to a strong reduction in growth and inhibition of A1 and A2 cleavages (Guglielmi and Werner, 2002). Total lack of Pfa1p has no detectable effect on growth and pre-rRNA processing under standard laboratory growth conditions. However, expression in Δ pfa1 cells of a truncated version of Pfa1p (Pfa1p(201–767)) lacking the N-terminal Prp43p interaction domain totally inhibits 32S and 27SA2 pre-rRNA accumulation and leads to a reduction in 20S pre-rRNA steady-state levels. This effect requires the absence of the C-terminal G-patch-containing domain, as expression of the central (amino acids 201–565) domain of Pfa1p has no effect on 32S and 27SA2 pre-rRNA steady-state levels. The expression of the C-terminal G-patch-containing domain alone (amino acids 574–767) in Δ pfa1 cells has also an inhibitory effect on 32S and 27SA2 pre-rRNA accumulation, although less drastic. Strikingly, Pfa1p(201–767)-His binds with greater

affinity to Prp43p and stimulates Prp43p ATPase activity to a greater degree than Pfa1p(574–767)–His does. Thus, it seems that stimulation in 90S pre-ribosomal particles of Prp43p ATPase activity by a Pfa1p variant lacking its N-terminal Prp43p-binding domain inhibits A1 and A2 cleavages.

Role of Pfa1p in pre-40S particle maturation

Pfa1p, which associates with 20S pre-rRNA (Lebaron *et al*, 2005), likely plays a role in 20S pre-rRNA-to-18S rRNA maturation. This role can be uncovered when Ltv1p is depleted in cells lacking Pfa1p. Under such conditions, 18S rRNA levels are reduced and 20S pre-rRNA levels are strongly elevated. Although lack of Ltv1p already leads to an increase in 20S pre-rRNA levels and to a reduction in 18S rRNA levels, the effect is much more dramatic when Pfa1p is absent in addition, demonstrating that Pfa1p intervenes in 20S maturation. Pfa1p is most probably involved in the cytoplasmic conversion of 20S pre-rRNA to 18S rRNA, as the bulk of 20S pre-rRNAs accumulating in Δ pfa1 cells depleted for Ltv1p are cytoplasmic. The role of Pfa1p in 20S pre-rRNA processing is probably linked to its ability to stimulate Prp43p helicase activity. Indeed, truncated versions of Pfa1p (Pfa1p(1–202), Pfa1p(201–565) and Pfa1p(1–565)) unable to stimulate Prp43p helicase activity *in vitro* are not capable of restoring normal 20S pre-rRNA and 18S rRNA levels in Δ pfa1 cells depleted for Ltv1p at 25°C. In contrast, Pfa1p(201–767) that is capable of full stimulation of Prp43p ATPase and helicase activities can restore normal 18S rRNA accumulation in these cells. The target RNA structure of the Prp43p–Pfa1p complex on the 20S pre-rRNA remains unknown. In the published secondary structure of the internal transcribed spacer 1, the 3' end of 18S rRNA is buried within a double-stranded stem (van Nues *et al*, 1994). The Prp43p–Pfa1p complex may be required to open up this structure and/or to remove any protein bound to it to allow access of the D-site endonuclease, which is thought to be Nob1p (Fatica *et al*, 2003, 2004).

Why do strains lacking Pfa1p fail to display an obvious growth defect?

Unlike strains lacking the two other known G-patch partners of Prp43p, Ntr1p and Gno1p, strains lacking Pfa1p fail to display noticeable growth defects under standard laboratory conditions. This can have several explanations. The role of the stimulation of Prp43p activity by Pfa1p may be to optimize pre-rRNA processing and be dispensable under growth conditions not requiring optimal ribosome biogenesis. Alternatively, the stimulation of Prp43p activity within 90S, pre-40S and/or pre-60S particles may be important for growth, but Prp43p stimulation within these particles may be carried out by another G-patch protein partner when Pfa1p is absent. The two most likely candidates for taking over the function of Pfa1p are Ntr1p and Gno1p. Ntr1p has not been shown to be involved in ribosome biogenesis, but has been reported to be present in the nucleolus (Herrmann *et al*, 2007). Arguing against a role for Ntr1p in ribosome biogenesis is the fact that we failed to detect co-purification of pre-ribosomal particle components (non-ribosomal proteins or pre-rRNAs) with TAP-tagged Ntr1p (Supplementary Table II and data not shown). Gno1p presumably does not activate Prp43p within pre-40S pre-ribosomal particles because it seems to be absent from these particles under normal conditions. Moreover, lack of Pfa1p does not increase the proportion of Prp43p

associated with Gno1p (data not shown). However, we cannot rule out the possibility that Ntr1p associates with pre-ribosomal particles or that Gno1p remains bound to pre-40S particles when Pfa1p is absent. Finally, one cannot exclude the possibility that the G-patch protein of unknown function encoded by the YLR271W open-reading frame can take over the function of Pfa1p. Clearly, the possibility for Pfa1p to be functionally replaced by another G patch protein partner remains open.

Materials and methods

Pull-down assays

A total of 2 µg of each His-tagged protein (alone or in combination) was incubated with 50 ml of stacked protein A/protein G Sepharose beads (GE Healthcare) in 600 µl of Ipp500 buffer (25 mM Tris–HCl (pH 8.0), 10% glycerol, 0.1% NP-40, 5 mM MgCl₂, 500 mM KCl, 1 mM DTT supplemented with complete EDTA-free protease inhibitor cocktail (Roche)) with gentle shaking for 1 h at 4°C. The supernatant was collected and added to 50 µl of stacked protein A/protein G Sepharose beads, previously incubated with 20 µl of purified anti-Prp43p antibodies and 750 µl of Ipp500 buffer with gentle shaking at room temperature for 1 h and then washed thrice with 1 ml Ipp500 buffer. Incubation between protein A/protein G Sepharose beads coupled to anti-Prp43p antibodies and pre-cleared His-tagged recombinant proteins was performed for 1 h at 4°C with gentle shaking. The supernatant was collected and proteins precipitated with TCA. Beads were washed four times with 1 ml Ipp500 buffer and proteins retained on the beads were eluted with 70 µl SDS-PAGE loading buffer (100 mM Tris–HCl (pH 8.0), 4% SDS, 20% glycerol, 0.04% bromophenol blue, 200 mM DTT). Proteins were analysed by western blotting using six HIS mAb HRP conjugate (Clontech).

ATPase assays

The ATPase activity of the proteins (100 nM of Prp43p–His and/or 500 nM of Pfa1p–His as indicated) was measured in a 5-µl reaction volume containing 25 mM HEPES (pH 8.0), 2.5 mM Mg(CH₃COO)₂, 100 mM KCl, 0.2 mM DTT, 100 µg/ml BSA (Sigma) and 0.6 µCi/µl [α -³²P]-ATP and unlabelled ATP up to 2.5 mM. The reaction mixtures were incubated at 30°C, stopped on ice and analysed by thin layer chromatography on PEI-Cellulose plates (Merck) using 0.75 M KH₂PO₄ as a migration buffer. Plates were dried and quantified on a Fuji BAS 3000 phosphorimager.

UV cross-linking

Prp43p–His (1 µM) alone or mixed with Pfa1p–His (at 0.5, 0.75 or 1 µM) were incubated in a 20-µl reaction volume in a buffer containing 25 mM HEPES–KOH (pH 8.0), 2.5 mM Mg(CH₃COO)₂, 0.2 mM DTT, 100 µg/ml BSA (Sigma), 0.2 µCi/ml [α -³²P]-ATP, with or without total RNA (50 µM) followed by UV cross-linking at 254 nm. Samples were separated by electrophoresis on 4–20% SDS–Tris–glycine gels.

Electrophoretic mobility shift assays

Recombinant proteins were incubated in a 10-µl reaction volume containing 25 mM HEPES–KOH (pH 8.0), 2.5 mM Mg(CH₃COO)₂, 100 mM KCl, 0.2 mM DTT, 100 µg/ml BSA (Sigma). Helicase substrates (1 nM) were incubated for 45 min at 4°C and electrophoresed on native 6% polyacrylamide gels (19:1) using 1 × TBE as a running buffer. Gels were dried and quantified on a Fuji BAS 3000 phosphorimager.

Helicase assays

The 5'–3' or 3'–5' helicase substrate (1 nM) was pre-incubated with 100 nM Prp43p–His and/or 500 nM Pfa1p–His, as indicated, for 15 min on ice. The reaction mixtures were complemented or not with 1 mM nucleotide co-factor, as indicated, and incubated at 30°C. The reactions were stopped by addition of 2 µl of a solution containing 1 mg/ml proteinase K, 1.25% SDS, 10 mM Tris–HCl, 0.06% bromophenol blue, 0.06% xylene cyanol and 30% glycerol in the presence of 100-fold excess of the trap oligonucleotide. The samples were analysed by native electrophoresis on 8% polyacrylamide gels using 1 × TBE as running buffer. Gels were dried and quantified on a Fuji BAS 3000 phosphorimager.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank B Guglielmi and M Werner for the gift of the *Δgno1* and isogenic wild-type strains. We also thank Emmanuel Käs and members of the Ferrer-Henry and Grigoriev laboratories for helpful discussions.

References

- Aravind L, Koonin EV (1999) G-patch: a new conserved domain in eukaryotic RNA-processing proteins and type D retroviral polyproteins. *Trends Biochem Sci* **24**: 342–344
- Arenas JE, Abelson JN (1997) Prp43: an RNA helicase-like factor involved in spliceosome disassembly. *Proc Natl Acad Sci USA* **94**: 11798–11802
- Bleichert F, Baserga SJ (2007) The long unwinding road of RNA helicases. *Mol Cell* **27**: 339–352
- Bohnsack MT, Kos M, Tollervey D (2008) Quantitative analysis of snoRNA association with pre-ribosomes and release of snR30 by Rok1 helicase. *EMBO Rep* **9**: 1230–1236
- Brand RC, Klootwijk J, Van Steenberg TJ, De Kok AJ, Planta RJ (1977) Secondary methylation of yeast ribosomal precursor RNA. *Eur J Biochem* **75**: 311–318
- Callebaut I, Labesse G, Durand P, Poupon A, Canard L, Chomilier J, Henrissat B, Mornon JP (1997) Deciphering protein sequence information through hydrophobic cluster analysis (HCA): current status and perspectives. *Cell Mol Life Sci* **53**: 621–645
- Combs DJ, Nagel RJ, Ares Jr M, Stevens SW (2006) Prp43p is a DEAH-box spliceosome disassembly factor essential for ribosome biogenesis. *Mol Cell Biol* **26**: 523–534
- de la Cruz J, Kressler D, Tollervey D, Linder P (1998) Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3' end formation of 5.8S rRNA in *Saccharomyces cerevisiae*. *EMBO J* **17**: 1128–1140
- Decourty L, Saveanu C, Zemam K, Hantraye F, Frachon E, Rousselle JC, Fromont-Racine M, Jacquier A (2008) Linking functionally related genes by sensitive and quantitative characterization of genetic interaction profiles. *Proc Natl Acad Sci USA* **105**: 5821–5826
- Emery B, de la Cruz J, Rocak S, Deloche O, Linder P (2004) Has1p, a member of the DEAD-box family, is required for 40S ribosomal subunit biogenesis in *Saccharomyces cerevisiae*. *Mol Microbiol* **52**: 141–158
- Fairman ME, Maroney PA, Wang W, Bowers HA, Gollnick P, Nilsen TW, Jankowsky E (2004) Protein displacement by DEXH/D 'RNA helicases' without duplex unwinding. *Science* **304**: 730–734
- Fatica A, Oeffinger M, Dlakic M, Tollervey D (2003) Nob1p is required for cleavage of the 3' end of 18S rRNA. *Mol Cell Biol* **23**: 1798–1807
- Fatica A, Tollervey D, Dlakic M (2004) PIN domain of Nob1p is required for D-site cleavage in 20S pre-rRNA. *RNA* **10**: 1698–1701
- Fromont-Racine M, Senger B, Saveanu C, Fasiolo F (2003) Ribosome assembly in eukaryotes. *Gene* **313**: 17–42
- Granneman S, Lin C, Champion EA, Nandineni MR, Zorca C, Baserga SJ (2006) The nucleolar protein Esf2 interacts directly with the DEXH/H box RNA helicase, Dbp8, to stimulate ATP hydrolysis. *Nucleic Acids Res* **34**: 3189–3199
- Granneman S, Nandineni MR, Baserga SJ (2005) The putative NTPase Fap7 mediates cytoplasmic 20S pre-rRNA processing through a direct interaction with Rps14. *Mol Cell Biol* **25**: 10352–10364
- Guglielmi B, Werner M (2002) The yeast homolog of human PinX1 is involved in rRNA and small nucleolar RNA maturation, not in telomere elongation inhibition. *J Biol Chem* **277**: 35712–35719
- Henras AK, Soudet J, Grus M, Lebaron S, Caizergues-Ferrer M, Mougn A, Henry Y (2008) The post-transcriptional steps of eukaryotic ribosome biogenesis. *Cell Mol Life Sci* **65**: 2334–2359
- Herrmann G, Kais S, Hoffbauer J, Shah-Hosseini K, Bruggenolte N, Schober H, Fasi M, Schar P (2007) Conserved interactions of the splicing factor Ntr1/Spp382 with proteins involved in DNA double-strand break repair and telomere metabolism. *Nucleic Acids Res* **35**: 2321–2332

This study was supported by the CNRS, Universit Paul Sabatier and grants from the Agence Nationale de la Recherche, the Ligue Contre le Cancer, the Fondation pour la Recherche Mdicale ('Programme Grands Equipements') and the Gnpole Toulouse Midi-Pyrnes.

Conflict of interest

The authors declare that they have no conflict of interest.

- Jakovljevic J, de Mayolo PA, Miles TD, Nguyen TM, Lger-Silvestre I, Gas N, Woolford Jr JL (2004) The carboxy-terminal extension of yeast ribosomal protein S14 is necessary for maturation of 43S preribosomes. *Mol Cell* **14**: 331–342
- Jankowsky E, Fairman ME (2007) RNA helicases—one fold for many functions. *Curr Opin Struct Biol* **17**: 316–324
- Jankowsky E, Gross CH, Shuman S, Pyle AM (2001) Active disruption of an RNA–protein interaction by a DEXH/D RNA helicase. *Science* **291**: 121–125
- Kos M, Tollervey D (2005) The putative RNA helicase Dbp4p is required for release of the U14 snoRNA from preribosomes in *Saccharomyces cerevisiae*. *Mol Cell* **20**: 53–64
- Lafontaine D, Vandenhaute J, Tollervey D (1995) The 18S rRNA dimethylase Dim1p is required for pre-ribosomal RNA processing in yeast. *Genes Dev* **9**: 2470–2481
- Lebaron S, Froment C, Fromont-Racine M, Rain JC, Monsarrat B, Caizergues-Ferrer M, Henry Y (2005) The splicing ATPase prp43p is a component of multiple preribosomal particles. *Mol Cell Biol* **25**: 9269–9282
- Leeds NB, Small EC, Hiley SL, Hughes TR, Staley JP (2006) The splicing factor Prp43p, a DEAH box ATPase, functions in ribosome biogenesis. *Mol Cell Biol* **26**: 513–522
- Liang XH, Fournier MJ (2006) The helicase Has1p is required for snoRNA release from pre-rRNA. *Mol Cell Biol* **26**: 7437–7450
- Martin A, Schneider S, Schwer B (2002) Prp43 is an essential RNA-dependent ATPase required for release of lariat-intron from the spliceosome. *J Biol Chem* **277**: 17743–17750
- Pandit S, Paul S, Zhang L, Chen M, Durbin N, Harrison SM, Rymond BC (2009) Spp382p interacts with multiple yeast splicing factors including possible regulators of Prp43 DEXH/H-box protein function. *Genetics* **183**: 195–206
- Rocak S, Emery B, Tanner NK, Linder P (2005) Characterization of the ATPase and unwinding activities of the yeast DEAD-box protein Has1p and the analysis of the roles of the conserved motifs. *Nucleic Acids Res* **33**: 999–1009
- Roy J, Kim K, Maddock JR, Anthony JG, Woolford Jr JL (1995) The final stages of spliceosome maturation require Spp2p that can interact with the DEAH box protein Prp2p and promote step 1 of splicing. *RNA* **1**: 375–390
- Silverman E, Edwalds-Gilbert G, Lin RJ (2003) DEXH/H-box proteins and their partners: helping RNA helicases unwind. *Gene* **312**: 1–16
- Silverman EJ, Maeda A, Wei J, Smith P, Beggs JD, Lin RJ (2004) Interaction between a G-patch protein and a spliceosomal DEXH/H-box ATPase that is critical for splicing. *Mol Cell Biol* **24**: 10101–10110
- Tanaka N, Aronova A, Schwer B (2007) Ntr1 activates the Prp43 helicase to trigger release of lariat-intron from the spliceosome. *Genes Dev* **21**: 2312–2325
- Tanaka N, Schwer B (2006) Mutations in PRP43 that uncouple RNA-dependent NTPase activity and pre-mRNA splicing function. *Biochemistry* **45**: 6510–6521
- Tanner NK, Linder P (2001) DEXH/H box RNA helicases: from generic motors to specific dissociation functions. *Mol Cell* **8**: 251–262
- Tsai RT, Fu RH, Yeh FL, Tseng CK, Lin YC, Huang YH, Cheng SC (2005) Spliceosome disassembly catalyzed by Prp43 and its associated components Ntr1 and Ntr2. *Genes Dev* **19**: 2991–3003
- van Nues RW, Rientjes JM, van der Sande CA, Zerp SF, Sluiter C, Venema J, Planta RJ, Rau HA (1994) Separate structural elements within internal transcribed spacer 1 of *Saccharomyces cerevisiae* precursor ribosomal RNA direct the formation of 17S and 26S rRNA. *Nucleic Acids Res* **22**: 912–919