Sdo1p, the yeast orthologue of Shwachman-Bodian-Diamond syndrome protein, binds RNA and interacts with nuclear rRNA-processing factors. Yeast

ARTICLE *in* YEAST · MAY 1990

Impact Factor: 1.63 · DOI: 10.1002/yea.1668 · Source: PubMed

5 AUTHORS, INCLUDING:

Luz Juliana S.
São Paulo State University
16 PUBLICATIONS **247** CITATIONS
SEE PROFILE

Carloskhamus Henrique Gomes
University of São Paulo
1 PUBLICATION **15** CITATIONS
SEE PROFILE

Glaucia Machado-Santelli
University of São Paulo
84 PUBLICATIONS **674** CITATIONS
SEE PROFILE

Carla C Oliveira
University of São Paulo
38 PUBLICATIONS **825** CITATIONS
SEE PROFILE

Yeast
Yeast 2009; **26**: 287–298.
Published online 6 April 2009 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/yea.1668

Research Article

Sdo1p, the yeast orthologue of Shwachman–Bodian–Diamond syndrome protein, binds RNA and interacts with nuclear rRNA-processing factors

Juliana S. Luz[1#], Raphaela C. Georg[1#], Carlos H. Gomes[1], Gláucia M. Machado-Santelli[2] and Carla C. Oliveira[1*]

[1] *Department of Biochemistry, Institute of Chemistry, University of São Paulo, São Paulo, SP 05508-000, Brazil*
[2] *Department of Cellular and Development Biology, Institute of Biomedical Sciences, University of São Paulo, SP, 05508-000, Brazil*

**Correspondence to:*
Carla C. Oliveira, Departamento de Bioquímica, Instituto de Química, USP, Av. Prof. Lineu Prestes, 748, São Paulo, SP, 05508-000, Brazil.
E-mail: ccoliv@iq.usp.br

[#] *These authors contributed equally to this work.*

Received: 27 November 2008
Accepted: 8 February 2009

Abstract

The Shwachman–Bodian–Diamond syndrome protein (SBDS) is a member of a highly conserved protein family of not well understood function, with putative orthologues found in different organisms ranging from Archaea, yeast and plants to vertebrate animals. The yeast orthologue of SBDS, Sdo1p, has been previously identified in association with the 60S ribosomal subunit and is proposed to participate in ribosomal recycling. Here we show that Sdo1p interacts with nucleolar rRNA processing factors and ribosomal proteins, indicating that it might bind the pre-60S complex and remain associated with it during processing and transport to the cytoplasm. Corroborating the protein interaction data, Sdo1p localizes to the nucleus and cytoplasm and co-immunoprecipitates precursors of 60S and 40S subunits, as well as the mature rRNAs. Sdo1p binds RNA directly, suggesting that it may associate with the ribosomal subunits also through RNA interaction. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: SBDS orthologue; rRNA processing; RNA binding; Nip7p; ribosome maturation

Introduction

Eukaryotic ribosomal biogenesis is a complex process, starting with the transcription of rDNA repeats by RNA polymerase I in the nucleolus, which generates a large polycistronic precursor rRNA (*35S* pre-rRNA) that contains the sequences for the mature ribosomal RNAs (*18S*, *5.8S*, *25S* rRNA), and two external (ETS) and two internal (ITS) transcribed spacers. This primary transcript is subsequently chemically modified at numerous sites and subjected to many endo- and exonucleolytic processing steps to produce the mature rRNAs. *5S* rRNA is independently transcribed as a precursor by RNA polymerase III (Kressler *et al*., 1999; Venema and Tollervey, 1999). During the maturation of ribosomal rRNAs and assembly into ribosomal subunits, in addition to the ribosomal proteins, a large number (~150) of non-ribosomal proteins associate with the pre-rRNAs (Woolford, 1991; Warner, 1989, 1999; Tschochner and Hurt, 2003). The first ribosome precursor that can be isolated is the 90S pre-ribosome, which is composed of the *35S* pre-rRNA, ribosomal proteins and *trans*-acting factors. The separation of the 90S pre-ribosome into the pre-40S and pre-60S particles occurs with the cleavage reactions in ITS1, after which the two subunit precursors have largely independent pathways (Venema and Tollervey, 1999).

Several *trans*-acting factors bind to the pre-60S ribosome promoting its maturation and correct export to cytoplasm. These non-ribosomal factors interact with pre-60S ribosomes in an ordered manner and can localize to the nucleolus, nucleus or cytoplasm. Some non-ribosomal proteins accompany the pre-60S particles from the nucleus to the cytoplasm (Nissan *et al*., 2002). Of the 50 non-ribosomal proteins that associate with the earliest nucleolar pre-60S ribosomes, five remain bound to the most mature pre-60S subunits after export to the cytoplasm (Nissan *et al*., 2002). These findings emphasize the enormous re-engineering of 60S pre-ribosomes on their path from the nucleolus to the cytoplasm.

Ribosome dysfunction has been implicated in several syndromes, including some that cause human bone marrow failure and cancer predisposition. Diamond–Blackfan anaemia is associated with mutations in the *RPS19* (Draptchinskaia *et al*., 1999) and *RPS24* (Gazda *et al*., 2006) genes that encode structural components of the 40S ribosomal subunit. X-linked dyskeratosis congenita is caused by mutations in the *DKC1* gene encoding dyskerin (the orthologue of yeast Cbf5p), a pseudouridine synthase that post-transcriptionally modifies rRNA by converting uridine to pseudouridine (Heiss *et al*.,1998; Lafontaine *et al*., 1998). Recently, the Shwachman–Diamond syndrome has also been associated with ribosome disfunction (Boocock *et al*., 2003).

Shwachman–Diamond syndrome (SDS; OMIM 260 400) is a rare autosomal recessive disorder with clinical features that include haematological dysfunction, pancreatic exocrine insufficiency and skeletal abnormalities, as well as a significant predisposition to the development of myelodysplasia and leukemia (Shwachman *et al*., 1964; Bodian *et al*., 1964; Ginzberg *et al*., 1999). The Shwachman–Diamond gene (*SBDS*) is a member of a highly conserved protein family of not well understood function with putative orthologues found in different organisms ranging from Archaea, yeast and plants to vertebrate animals (Boocock *et al*., 2003). Archaeal *SBDS* orthologue genes are located within highly conserved operons that include homologues of RNA-processing genes, suggesting that SDS may be caused by a deficiency in an aspect of RNA metabolism.

Consistent with this suggestion, the *Saccharomyces cerevisiae SBDS* orthologue, *Sdo1p*, has been clustered with genes encoding RNA-processing enzymes on the basis of microarray expression profile analysis (Wu *et al*., 2002; Peng *et al*., 2003), and high-throughput affinity capture mass spectrometry experiments have identified potential interactions between *Sdo1p* and ribosome biogenesis factors (Krogan *et al*., 2006). Recently, it was demonstrated that Sdo1p is critical for the release and recycling of Tif6p from pre-60S ribosomes. Tif6p is a nucleolar shuttling factor and its release from pre-60S is a key step in the maturation and translational activation of ribosomes (Menne *et al*., 2007). Furthermore, that work suggests that Sdo1p is involved in a pathway in conjunction with elongation factor-like 1 (Efl1p) to facilitate the release and recycling of Tif6p from late cytoplasmic pre-60S (Menne *et al*., 2007).

The crystal structure of the *Archaeoglobus fulgidus* SBDS protein orthologue was solved and shown to be composed of three domains. The N-terminal (FYSH) domain, which is the most frequent target for disease mutations and contains a novel mixed α/β-fold identical to the single domain yeast protein encoded by *YHR087W*, which is implicated in RNA metabolism. The central domain consists of a three-helix bundle, whereas the C-terminal domain has a ferredoxin-like fold. In addition, the C-terminal domain shares structural homology with known RNA-binding domains (Shammas *et al*., 2005; Savchenko *et al*., 2005).

In the present study, we identified through two-hybrid assays the interaction of Sdo1p with the rRNA processing factors Nip7p, Prp43p and Spp382p, and with Rpl3p and Asc1p, which are components of the large and small ribosomal subunits, respectively, and the translation elongation factor Eft2p. To assess the involvement of Sdo1p in rRNA metabolism, we tested its ability to bind RNA through co-immunoprecipitation and *in vitro* RNA-binding assays. The results show that Sdo1p co-precipitates the pre-rRNAs *27S* and *23S* and the mature rRNAs *25S*, *5.8S* and *18S*. Interestingly, Sdo1p binds RNA in a non-sequence-specific but length-dependent manner. This interaction might be mediated by the putative C-terminal RNA recognition motif (RRM) of Sdo1p and stabilized by protein interactions. The results shown here shed new light on the role of Sdo1p in rRNA processing, by indicating that Sdo1p binds pre-ribosomal subunits in the nucleus, and remains bound to them through their transport to the cytoplasm.

DOI: 10.1002/yea

Materials and methods

DNA analyses and plasmid construction

DNA cloning and analyses were performed as described (Sherman *et al*., 1986). DNA was sequenced by the Big Dye method (Perkin-Elmer). Cloning strategies are briefly described below. *LexA::SDO1* fusion used in the two-hybrid assay was constructed by inserting a 753 bp *Eco*RI/*Xho*I DNA fragment containing the PCR-amplified *SDO1* ORF into pBTM-116, previously digested with *Eco*RI/*Xho*I, generating the plasmid pBTM–SDO1. pACT–SDO1 codes for the hybrid protein GAL4p activation domain, Sdo1p. YCpGAL1-A–SDO1 was constructed by inserting the *SDO1* ORF into pBlueScript and subcloning the *Spe*I/*Xho*I *SDO1*-containing insert into YCp33GAL1–A plasmid previously digested with *Xba*I/*Sal*I. pACT–PRP43 was constructed by inserting *PRP43 Sma*I/*Pvu*II DNA fragment into pGAD–C1, digested with *Sma*I. For the construction of pET–SDO1 and pGFP–SDO1, the PCR-amplified *SDO1* (*Eco*RI/*Xho*I) ORF was inserted, respectively, into the pET–28a and pUG34 vectors digested with *Eco*RI and *Xho*I.

Yeast two-hybrid screen for proteins that interact with Sdo1p

The host strain for the two-hybrid screen, L40 (Vojtek and Hollenberg, 1995), contains both yeast *HIS3* and *Escherichia coli lacZ* reporter genes integrated into the genome. Transformation of L40 was carried out using the lithium acetate method (Gonzales *et al*., 2005). Yeast strains were maintained in yeast extract–peptone medium (YP) or synthetic medium (YNB), as described previously (Gonzales *et al*., 2005). Glucose or galactose was added as carbon source to a final concentration of 2%. For the two-hybrid screen, L40/pBTM–SDO1 was either transformed with a yeast cDNA library fused to the *GAL4* activation domain (pACT–cDNA; ATCC 87 002) or with pACT or pGAD vectors containing the exosome proteins genes, the box C/D and box H/ACA core snoRNPs protein genes. L40/pBTM–SDO1 transformed with the pACT–cDNA library was plated directly onto selective plates (−His). Of 620 colonies, 64 had the His^+ phenotype confirmed on second plates, out of which 50 were also $lacZ^+$. Sequence analysis of some of these clones showed that two contained *ASC1* cDNA, and one clone of each of *NTR1, RPL3* and *EFT2*. Prp3p and Nip7p interactions with Sdo1p were tested separately, after cloning the respective genes into two-hybrid system vectors. For the study of Sdo1p–Nip7p interactions, L40 was transformed with pBTM–NIP7, pBTM–Nip7-1, pBTM–PUA, pBTM–N–NIP7 (Granato *et al*., 2005) and pACT–SDO1. Transformants were plated onto YNB medium lacking histidine for selection of Sdo1p-interacting proteins. His^+ clones were tested for *lacZ* expression by transferring cells to nitrocellulose filters and analysing β-galactosidase (β-Gal) activity (Vojtek and Hollenberg, 1995). L40 strains containing either pBTM–NIP7 + pACT–RRP43 or pBTM–NIP7 + pACT–NOP8 were used as positive controls, and L40 strain transformed with pBTM–SDO1 + pGAD–C1 was used as the negative control for two-hybrid interaction (Granato *et al*., 2005).

Co-immunoprecipitation of RNAs

Total cellular extracts were prepared from W303 strains expressing either ProtA (YCpGAL1-A) or ProtA–Sdo1p (YCpGAL1-A–SDO1) and added to IgG–Sepharose beads (GE Healthcare) as described previously (Peltz *et al*., 1999), with some modifications. Briefly, total cellular extracts were incubated with IgG–Sepharose beads in buffer A (20 mM Tris–Cl, pH 8.0, 5 mM magnesium acetate, 0.1% Triton X-100, and protease inhibitors) containing different concentrations of potassium acetate (25, 75 or 100 mM). Immunoprecipitation was performed at 4 °C for 2 h. IgG–Sepharose beads were washed with buffer A and the respective concentrations of potassium acetate used during immunoprecipitation. RNA was isolated from bound fractions by adding phenol directly to the beads. After precipitation, the recovered RNA was denatured and separated by electrophoresis on 1.5% agarose gels and transferred to nylon membranes. For comparison, 1% of RNA recovered from the total extract was loaded onto the gel. Hybridization was performed as described above, using probes specific to RNAs, and visualized on a phosphorimager.

RNA binding assay

The sequence of pre-rRNA *5′ETS* (up to A_0), *ITS2*, *5.8S* and *25S* were PCR-amplified using *S. cerevisiae* genomic DNA and cloned into vector pGEM-T (Promega), generating *pGEM-5′ETS,*

DOI: 10.1002/yea

pGEM–ITS2, pGEM–5.8S and *pGEM-25S*, respectively. The PCR-amplified inserts were cleaved with specific enzymes and used in *in vitro* RNA transcription reactions in the presence of SP6 or T7 RNA polymerase and 50 μCi α-^{32}P-UTP. Approximately 1 pM radiolabelled RNA was incubated in buffer B (20 mM Tris–HCl, pH 8.0, 150 mM potassium acetate, 5 mM magnesium acetate, 0.2% Triton, 10 mM DTT and 0.8 U RNasin) with different amounts of purified protein for 15 min at 37 °C, followed by UV crosslinking on ice for 15 min. The samples were resolved on a 6% denaturing polyacrylamide gel and visualized on a phosphorimager. The oligoribonucleotide-binding assays were carried out with 1 pM ^{32}P 5′-labelled 10-, 12- and 14-mer poly(rA), poly(rU), 13-mer poly(rC), 21-mer poly(rAU) 21-mer poly(rGC) and 25-mer poly(rG). The assays were performed under the same conditions as described above. Different amounts of protein were incubated with the substrate RNA at 37 °C for 30 min. The samples were resolved on 8% native polyacrylamide gels and visualized on a phosphorimager.

Protein expression and purification

The pET–SDO1 expression vector was transformed into the *E. coli* BL21-CodonPlus (DE3) RIL strain. To obtain a soluble His–Sdo1p recombinant protein, transformed bacteria were grown in 2YT medium at 30 °C, and the protein expression was induced by adding 10 mM lactose to the medium for 16 h. After this period, cells were collected and lysed in a French press in the presence of 50 mM Tris–HCl, pH 7.0, 500 mM NaCl, 1 mM PMSF and 5 mM imidazole. Total extract was subjected to streptomycin sulphate precipitation (2% w/v) for 1 h at 4 °C with agitation. The supernatant fraction was incubated with a nickel column (Ni-NTA, Qiagen). His–Sdo1p protein was then eluted through an imidazole gradient.

Subcellular localization of Sdo1p

The subcellular localization of Sdo1p was analysed by monitoring the fluorescence signal produced by a GFP fusion to Sdo1p. The subcellular localization of Cwc24p was analysed by monitoring the DsRed fluorescence, which was fused to the N-terminus of Cwc24p. GFP–Sdo1p and DsRed–Cwc24p proteins were expressed in strain W303, transformed with plasmid vector pGFP–SDO1 bearing the *SDO1* gene, and pDsRed–CWC24. Cells were immobilized on polylysine-coated slides and observed in a laser scanning confocal microscope (LSM510, Zeiss). The fluorescent images were obtained by confocal laser scanning with lasers of argon (458, 488 and 514 nm), helium–neon 1 (543 nm), and helium–neon 2 (633 nm) connected to an inverted fluorescence microscope (Zeiss Axiovert 100 M). The profile module of LSM510 software was used to analyse the green and red fluorescence co-localization.

Pull-down and Western blot

The pGEX-Nip7 expression vector was transformed into the *E. coli* BL21-CodonPlus (DE3) RIL strain. To obtain a soluble GST–Nip7p recombinant protein, transformed bacteria were grown in 2YT medium at room temperature, and the protein expression was induced by adding 10 mM lactose to the medium for 16 h. After this period, cells were collected and add to His-Sdo1 cells, which were lysed in a French press in the presence of 50 mM Tris–HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.2% Triton, 0.2 mM DTT and 1 mM PMSF. Total extract was subjected to streptomycin sulphate precipitation (2% w/v) for 1 h at 4 °C with agitation. The supernatant fraction was incubated with a glutathione column (GE Healthcare). The proteins were then eluted in the same buffer plus 20 mM glutathione reduced. The eluted proteins were resolved on SDS–PAGE and transferred to PVDF membranes, which were incubated with anti-poly-His monoclonal antibody (GE Healthcare), with anti-GST antiserum (Sigma) or anti-Nip7 polyclonal antibody. The immunoblots were developed using the Super Signal West Pico Chemiluminescent substrate (Thermo Scientific).

The pulled down proteins were subject to gel filtration assay in the Superdex 75 column. A flow rate of 0.5 ml/min in the same buffer was used, and fractions were stored at −20 °C. Apparent molecular weight was assessed based on the retention time of molecular weight markers (GE Healthcare: aldolase, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 25kDa).

DOI: 10.1002/yea

Results

Sdo1p interacts with RNA-processing proteins

Yeast Sdo1p has been reported to be present in complexes with many RNA-processing factors; among those were Prp43p (Peng *et al*., 2003), a helicase involved in both splicing and pre-rRNA processing (Peng *et al*., 2003; Lebaron *et al*., 2005; Leeds *et al*., 2006; Combs *et al*., 2006), and the nucleolar shuttling factor Tif6p (Menne *et al*., 2007). In addition, human SBDS has been shown to interact with the pre-60S processing factor hNip7p (Hesling *et al*., 2007). In order to identify other proteins interacting with Sdo1p, we used the two-hybrid system, screening a cDNA library and also testing the Sdo1p interaction with some proteins known to participate in different steps of RNA processing. We identified the interactions of Sdo1p with the 60S ribosomal protein Rpl3p (Peltz *et al*., 1999), with the translation elongation factor Eft2p (Perentesis *et al*., 1992) and with the helicase Prp43p (Arenas and Abelson, 1997) (Figure 1A). In addition, we found the interaction between Sdo1p and Spp382p/Ntr1p (Figure 1A), a protein also found in complex with Prp43p (Pandit *et al*., 2006; Tsai *et al*., 2007). Furthermore, we also identified the interaction between Sdo1p and Asc1p (Figure 1A), a G-protein β-subunit that binds the 40S ribosomal subunit and is involved in translational repression (Zeller *et al*., 2007). Interestingly, Asc1p was also identified in the same complex with Prp43p (Gavin *et al*., 2002).

These results identify new Sdo1p partners and confirm its involvement in the rRNA-processing pathway. Furthermore, the new nuclear Sdo1p interactions identified here strengthen the data on the involvement of Sdo1p in ribosome biogenesis and control. We also identified the interaction between Sdo1p and Nip7p, a pre-60S rRNA processing protein (Figure 1B). In order to map the region of Nip7p involved in the interaction with Sdo1p, mutants of Nip7p were used in the two-hybrid system. Interestingly, Sdo1p interacts with the full-length wild-type Nip7p, with the temperature-sensitive mutant nip7-1p (Zanchin *et al*., 1997) and with the Nip7p C-terminal PUA domain, but not with the truncated mutant containing only the N-terminal region of Nip7p (Figure 1B). Sdo1p interaction with Nip7p, Prp43p,

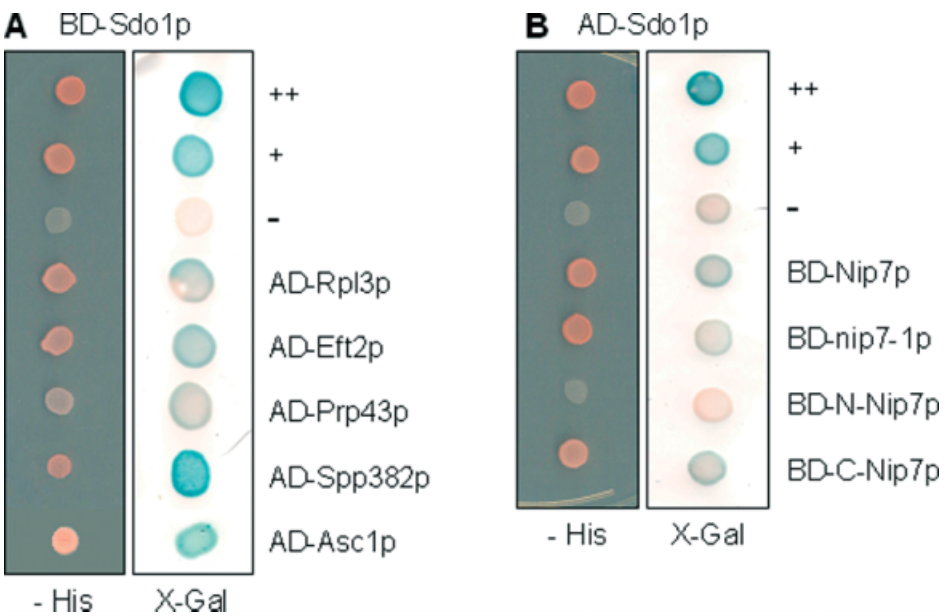

Figure 1. Interaction between Sdo1p and RNA processing proteins. Protein interactions were analysed through the two-hybrid system, testing for expression of the reporter genes *HIS3* and *lacZ*. (A) Test of interaction between BD–Sdo1p and proteins fused to the Gal4p activation domain, from the cDNA library (AD-cDNA). (B) Test of interaction between wild-type or mutant Nip7p fused to the lexA DNA binding domain and AD–Sdo1p, in the presence of 1 mM 3AT. ++, positive control, L40/pBTM–Nip7/pACT–NOP8; +, positive control, L40/pBTM–Nip7/pACT–Rrp43; –, negative control, L40/pBTM–Sdo1/pACT

DOI: 10.1002/yea

Rpl3p and Eft2p corroborates the function proposed for Sdo1p in pre-60S processing. In addition, Sdo1p interaction with Prp43p, Spp382p/Ntr1p and Asc1p suggest that it is also involved in pre-40S maturation. Furthermore, Sdo1p–Nip7p interaction shows the evolutionary conservation of function of these proteins.

In order to confirm the direct interaction between Sdo1p and Nip7p, the recombinant proteins GST–Nip7p and His–Sdo1p were used in protein pull-down assays. In these experiments, GST or GST–Nip7p was bound to a glutathione–Sepharose column and the co-purification of His–Sdo1p was analysed. The results show that GST–Nip7p (but not GST) co-purifies His–Sdo1p (Figure 2). Although we cannot exclude the possibility that bacterial RNA can mediate or stabilize the interaction between GST–Nip7p and His–Sdo1p, these experiments confirm that no other yeast protein factor is necessary for this interaction. In addition, protein elution fractions from the pull-down assay were subjected to size-exclusion chromatography. The results further confirm the interaction between Nip7p and Sdo1p, since both proteins are recovered as a complex, and not as monomers (Figure 2B). The elution profile suggests that two copies of both proteins (GST–Nip7 and His–Sdo1) were eluted together. The formation of this tetramer, however, could be the result of GST dimerization. Co-immunoprecipitation assays were also performed in the presence or absence of RNase to analyze whether the interaction between these two proteins could be mediated by RNA. The results show that ProtA-Sdo1p co-immunoprecipitates Nip7p in both conditions, although less Nip7p is recovered in the presence of RNase (Figure S1). These results indicate that Nip7p-Sdo1p interaction is direct, but may be stabilized in the presence of RNA.

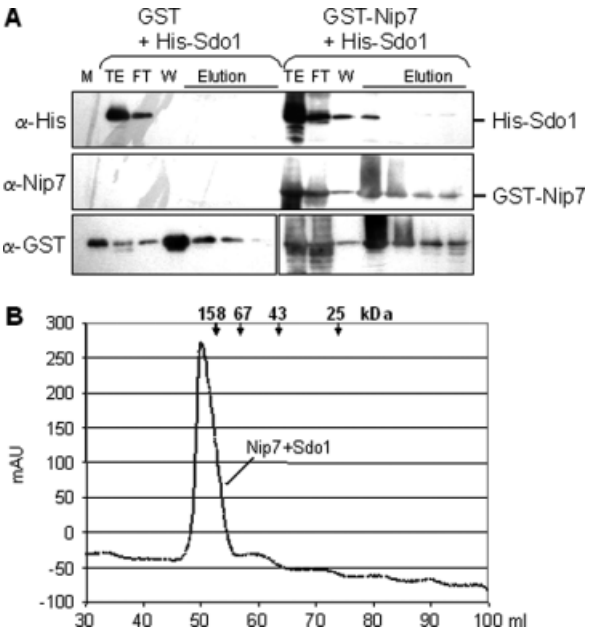

Figure 2. Pull-down of recombinant proteins GST–Nip7p and His–Sdo1p. (A) GST or GST–Nip7p was bound to glutathione–Sepharose beads, followed by incubation with His–Sdo1p. After washing the beads, proteins were eluted, showing the co-purification of His–Sdo1p with GST–Nip7p and not with GST. Proteins were detected with anti-His monoclonal antibody, anti-Nip7 or anti-GST antiserum. M, molecular weight marker; TE, total extract; W, wash; Elution, different fractions of elution with increasing concentration of reduced glutathione. (B) Size exclusion chromatography of proteins eluted from the pull-down assay. GST–Nip7p and His–Sdo1p co-eluted as a large protein complex. Molecular mass standards used in this experiments were: aldolase, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; chymotrypsinogen, 25 kDa

Sdo1p co-immunoprecipitates rRNAs

Based on the information that Sdo1p interacts with Tif6p (Menne *et al.*, 2007) and Nip7p (Figure 1), two proteins that are present in the pre-60S complex, and with Prp43p, which is involved in pre-rRNA processing (Lebaron *et al.*, 2005; Leeds *et al.*, 2006; Combs *et al.*, 2006), we wanted to investigate whether Sdo1p also associates with pre-ribosomal complexes. Therefore, RNA co-immunoprecipitation assays were performed with either Protein A or ProtA–Sdo1p immobilized on IgG–Sepharose columns in different salt concentrations. The results show that ProtA–Sdo1p co-immunoprecipitates the pre-rRNA *27S*, as well as the mature *25S* and *5.8S* rRNAs, even in the highest salt concentration used (Figure 3A). These results corroborate the protein interaction data and prediction of the presence of Sdo1p in the pre-60S complex. Interestingly, Sdo1p also co-precipitated the *18S* rRNA, which is a component of the 40S ribosome subunit (Figure 3A). The co-immunoprecipitation of mature *25S* and *5.8S* rRNAs with ProtA–Sdo1p indicates that Sdo1p remains bound to mature *60S* in the cytoplasm, and possibly also during translation, since *18S* was also

DOI: 10.1002/yea

co-precipitated. Accordingly, Sdo1p has been identified in association mainly with the 60S ribosomal subunit, but also with polysomes (Menne *et al*., 2007) and, as shown here, Sdo1p also interacts with the cytoplasmic 40S-associated factors Eft2p and Asc1p. Further investigation of the rRNAs co-precipitated with ProtA–Sdo1p was performed in the presence of an intermediate salt concentration (50 mM potassium acetate). The results show that, in addition to the mature rRNAs *25S*, *5.8S* and *18S*, ProtA–Sdo1p also co-precipitates *23S* pre-rRNA (Figure 3B). These observations indicate that Sdo1p interacts with both pre-rRNP complexes, pre-60S and pre-40S, in the nucleus and accompanies them during transport to the cytoplasm. These data corroborate the Sdo1p–Prp43p interaction results, since Prp43p has been demonstrated to be involved in maturation of both ribosomal subunits (Lebaron *et al*., 2005; Leeds *et al*., 2006; Combs *et al*., 2006).

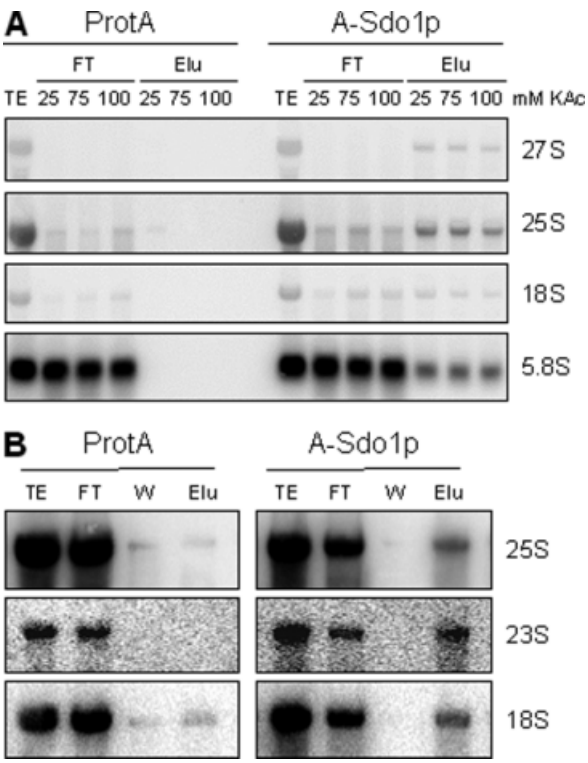

Figure 3. Co-immunoprecipitation of rRNAs with ProtA–Sdo1p. (A) Total cell extracts from W303/ProtA and W303/ProtA–Sdo1 strains were mixed with IgG–Sepharose beads in the presence of different salt concentrations (25, 75 or 100 mM potassium acetate) for co-immunoprecipitation of rRNAs with ProtA–Sdo1p. RNA extracted from different fractions was separated on an agarose gel. Bound RNA was detected by hybridization against probes specific for rRNAs, as indicated. TE, total extract; FT, flow through corresponding to the samples incubated with 25, 75 or 100 mM potassium acetate; Elu, bound fraction (beads) corresponding to the samples incubated with the same potassium acetate concentrations. (B) As (A) but in the presence of 50 mM KOAc. RNA extracted from the different fractions was hybridized against probes for *25S*, *23S* and *18S* rRNAs

Sdo1p binds RNA *in vitro*

As Sdo1p co-immunoprecipitates rRNAs, we evaluated whether this interaction is direct by means of *in vitro* RNA-binding assays. We performed these experiments by using recombinant histidine-tagged Sdo1p and *in vitro* transcribed RNAs corresponding to fragments of four distinct regions of the pre-rRNA: the *5.8S* and *25S* rRNAs and the 5′ETS and ITS2 spacer sequences. The interaction experiments showed that Sdo1p binds RNA directly in a concentration-dependent, but not sequence-specific, manner (Figure 4). Although the *in vitro* transcribed RNAs had been purified after electrophoresis on denaturing gels, multiple bands are visualized on the gels of the RNA-binding assays, which can be explained by the presence of secondary structures.

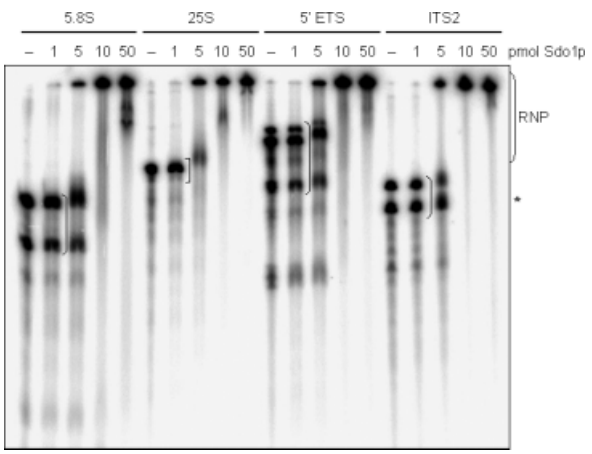

Figure 4. Analysis of yeast Sdo1p interaction with RNA *in vitro*. 1–50 pM Sdo1p was incubated with ~1 pM *in vitro*-transcribed [^{32}P]-labelled fragments of *5.8S*, *25S*, *5′-ETS* or *ITS2* RNAs. RNA–protein complexes were cross-linked, subjected to denaturing polyacrylamide gel electrophoresis and analysed by phosphorimaging. *Brackets indicate free RNAs

DOI: 10.1002/yea

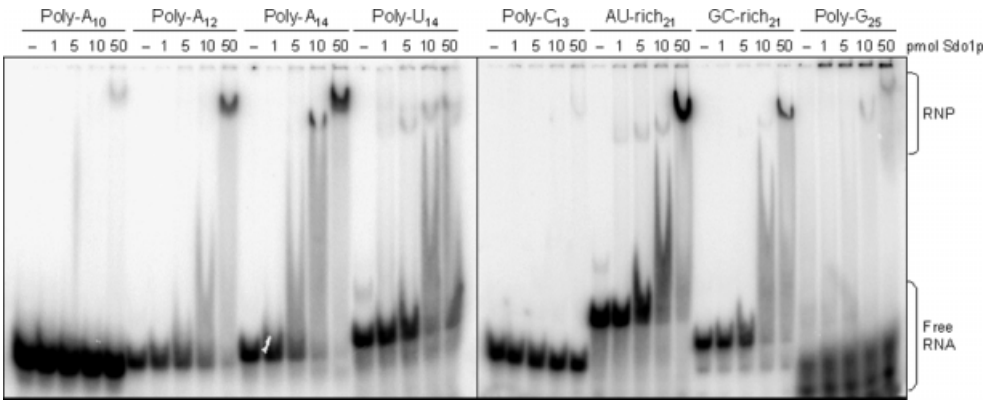

Figure 5. Analysis of Sdo1p interaction with oligoribonucleotides. The [^{32}P]-labelled oligoribonucleotides indicated (poly-A_{10}, poly-A_{12}, poly-A_{14}, poly-U_{14}, poly-C_{13}, AU-rich$_{21}$, GC-rich$_{21}$ and poly-G_{25}) were incubated with 1–50 pM Sdo1p. RNA–protein complexes were subjected to native polyacrylamide gel electrophoresis and analysed by phosphorimaging

In order to determine whether Sdo1p has higher affinity for any RNA sequence, RNA binding experiments using oligoribonucleotides revealed that Sdo1p binds *poly-A* and *poly-U* RNAs with higher efficiency than *poly-C*, and binds longer *poly-A* RNA oligos with higher affinity than shorter oligos (Figure 5). Sdo1p also bound *poly-G* and *poly-GC* RNAs, predicted to form secondary structures, and displayed an increased binding affinity for an *AU*-rich RNA, predicted to form a hairpin structure and double-molecule base pairs. These data suggest that Sdo1p has a binding preference for long *A*- and *AU*-rich and structured RNAs.

Sdo1p localizes to the nucleus and cytoplasm

Although initial global yeast protein localization experiments had indicated that Sdo1p was a cytoplasmic protein, its interaction with nuclear proteins shown here and the co-purification of pre-rRNAs with the fusion ProtA–Sdo1p indicated to us that Sdo1p might also be present in the nucleus. In order to determine the Sdo1p subcellular localization, it was fused to GFP tag (GFP–Sdo1p). We also used the fusion GFP–Rps2p as a control of a cytoplasmic protein. Confocal images of split fluorescence channels showed that GFP–Sdo1p is present in both the nucleus and the cytoplasm (Figure 6). When the nuclear protein Cwc24p (Goldfeder and Oliveira, 2008) was used as a nuclear control, DsRed–Cwc24p and GFP–Sdo1p co-localized in the nucleus (Figure 6). The colocalization of GFP–Sdo1p and DsRed–Cwc24p was confirmed through the fluorescence profiles in several cell images (Figure 6). Accordingly, the human Sdo1p orthologue SBDS has been shown to localize to the nucleolus (Andersen *et al*., 2005). These results further confirm Sdo1p interaction with pre-ribosome particles in the nucleus.

Discussion

In the last 5 years, human SBDS and its yeast homologue, Sdo1p, have been the object of several studies aiming at the elucidation of the molecular mechanisms involved in the Shwachman–Diamond syndrome (Boocock *et al*., 2003; Menne *et al*., 2007; Savchenko *et al*., 2005; Austin *et al*., 2005; Ganapathi *et al*., 2007; Calado *et al*., 2007). First reports indicated that these proteins were involved in 60S ribosome subunit biogenesis. Recently, Menne and collaborators (2007) demonstrated that Sdo1p is responsible for the release and recycling of the nucleolar shuttling factor Tif6p from pre-60S ribosomes, a key step in the biogenesis of 60S ribosomal subunit.

To assess whether Sdo1p interacts with other ribosomal or non-ribosomal proteins, we performed two-hybrid assays. Consistent with the proposed association of Sdo1p with polysomes (Menne *et al*., 2007), we detected its interaction with the 60S ribosomal protein Rpl3p and with the translation elongation factor Eft2p. We also detected the interaction of Sdo1p with Nip7p, a nucleolar yeast protein that is required for 60S biogenesis (Zanchin *et al*., 1997) and that interacts with

DOI: 10.1002/yea

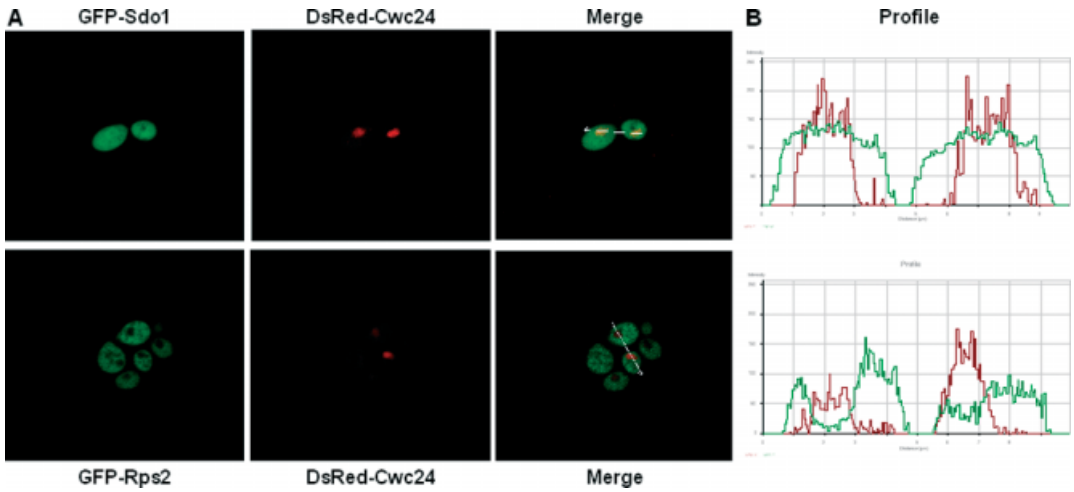

Figure 6. Subcellular localization of Sdo1p. Yeast strain *W303* expressing GFP–Sdo1p and DsRed–Cwc24p was analysed by laser scanning confocal microscopy. (A) Each channel labelling is shown separately and merged in the right-hand panel. As a control, cytoplasmic protein GFP–Rps2p is shown in the lower panels not to co-localize with DsRed–Cwc24p. (B) A representative profile of green and red fluorescence is presented, indicative of the DsRed–Cwc24p (red line) and GFP–Sdo1p (green line) or GFP–Rps2p (green line) localization

the other rRNA-processing proteins Nop8p, the exosome subunit Rrp43p (Zanchin and Goldfarb, 1999) and the nucleolar protein Nop53p (Granato *et al*., 2005). Confirming our two-hybrid analysis, we observed that Sdo1p co-immunoprecipitates Nip7p. Interestingly, the Sdo1p interaction with Nip7p is conserved throughout evolution, since a recent study demonstrated that SBDS, the human orthologue of Sdo1p, interacts with human Nip7p (Hesling *et al*., 2007).

We also observed a positive interaction between Sdo1p and Prp43p, a DExD/H-box helicase involved in the late steps of splicing (Martin *et al*., 2002; Tsai *et al*., 2005). Corroborating these data, the interaction Sdo1p–Prp43p had been previously observed through TAP-tag experiments (Peng *et al*., 2003). Interestingly, Prp43p has been recently shown to localize to the nucleolus and to interact with pre-ribosomal particles, thereby playing an important role in ribosome biogenesis of both ribosomal subunits (Lebaron *et al*., 2005; Leeds *et al*., 2006; Combs *et al*., 2006). Therefore, it is possible that Sdo1p and Prp43p might be acting together in pre-60S ribosome processing. Furthermore, DExD/H-box RNA helicases show very little substrate specificity for unwinding RNA duplexes *in vitro*. It is speculated that these helicases might require extrinsic factors for targeting to specific substrates to execute their normal functions (Tsai *et al*., 2005). In addition, Sdo1p also interacted with Spp382p/Ntr1p, a protein that acts together with Prp43p in the late steps of the splicing process (Tsai *et al*., 2007; Pandit *et al*., 2006). Interestingly, recent data indicate that Spp382p/Ntr1p is also involved in pre-rRNA processing and ribosome biogenesis (T. Auchynnikava and J. D. Beggs, personal communication). The Sdo1p interaction with both Spp382p and Prp43p further indicate Sdo1p participation in nuclear ribosome maturation.

As shown here, Sdo1p also interacts with Asc1p, the yeast orthologue of human RACK1, which is involved in translational control and in the release of the human homologue of Tif6p, eIF6, from pre-60S ribosomes (Gerbasi *et al*., 2004; Ceci *et al*., 2003). All the protein factors interacting with Sdo1p identified in this work are involved in different steps of ribosomal maturation, either in the nucleus or in the cytoplasm, or else are shuttling factors. These are strong evidences that Sdo1p binds pre-ribosome complexes in the nucleus and remain associated with them through transport to the cytoplasm.

In order to confirm Sdo1p association with the pre-ribosomal complexes, we carried out RNA co-immunoprecipitation assays. Interestingly, we identified the interaction of Sdo1p with the *27S* pre-rRNA and the mature *25S* and *5.8S* rRNAs,

DOI: 10.1002/yea

indicating that Sdo1p binds the pre-60S subunit. Furthermore, the fact that Sdo1p binds the 27S pre-rRNA and interacts with Nip7p suggests that Sdo1p associates with the pre-60S ribosomal particle in the nucleus, and remains bound to it during the late steps of maturation and transport to the cytoplasm. This hypothesis is coherent with the nuclear and cytoplasmatic localization of Sdo1p (Figure 6) (Huh *et al*., 2003). In the cytoplasm, Sdo1p and Efl1p are responsible for the release of Tif6p from 60S ribosomes (Menne *et al*., 2007). The majority of pre-60S factors assemble during or after the cleavage of pre-90S into pre-40S and pre-60S. During the transport from the nucleolus towards the nuclear pore, many of the pre-60S factors are released from the nascent subunit in subsequent steps, whereas a few processing proteins remain associated to the pre-60S until its export to the cytoplasm (Tschochner and Hurt, 2003). Sdo1p seems to be one of these few proteins. In addition, Sdo1p co-immunoprecipitated *23S* pre-rRNA and mature *18S* rRNA, indicating that it also interacts with pre-40S complexes, and might participate in different processing steps. Interestingly, Prp43p, which interacts with Sdo1p, functions in both small and large ribosomal subunit biogenesis (Lebaron *et al*., 2005; Leeds *et al*., 2006; Combs *et al*., 2006). It is interesting to note that both Prp43p and Efl1p, two Sdo1p-interacting proteins, hydrolyse ATP and GTP, respectively. It has been suggested that Sdo1p may be required to recruit Efl1p to the GTPase centre of cytoplasmic 60S ribosomal subunits, where GTP hydrolysis by Efl1p may then drive a structural rearrangement of the Tif6p-bound preribosome, thereby facilitating Tif6p release (Menne *et al*., 2007). The 60S and 40S ribosomal subunits can only start protein translation after the release of Tif6p (Basu *et al*., 2001), which makes Sdo1p an important factor in ribosome biogenesis process.

We also tested the ability of Sdo1p to interact directly with rRNAs *in vitro*. We verified that Sdo1p binds rRNAs efficiently in a concentration-dependent manner. Although Sdo1p does not show clear sequence specificity, RNA binding experiments using oligoribonucleotides revealed that Sdo1p shows higher affinity for at least 12-nucleotide long A-rich and AU-rich structured RNAs. These data indicate that Sdo1p binds RNA in a non-sequence-specific, but length-dependent, manner. Mtr4p is another example of a yeast protein involved in RNA processing and degradation, which shows higher affinity for poly-A RNA longer than 20 nucleotides (Bernstein *et al*., 2008). Since Sdo1p did not show sequence specificity for binding rRNAs, it might be directed to pre-ribosomal complexes through the interactions with proteins present in those complexes, such as Nip7p and Prp43p.

In summary, we identified new Sdo1p interacting partners and, among them, Nip7p, a nucleolar rRNA-processing factor. Sdo1p also interacts with the pre-rRNAs *27S* and *23S*, indicating that this protein binds pre-ribosomal subunits in the nucleus and is probably transported to the cytoplasm with these complexes. Sdo1p also interacts with the large ribosomal subunit protein Rpl3p and with Eft2p, a translation elongation factor, and co-precipitates the mature rRNAs *5.8S*, *25S* and *18S*. These results corroborate the data on the association of Sdo1p with mature ribosomes, and its role in the release of Tif6p and activation of 60S for translation. The association of Sdo1p with the ribosomal subunits may occur through direct RNA interaction via a conserved C-terminal RRM, and may be directed or stabilized through protein–protein interactions.

Acknowledgements

We would like to thank Nilson I.T. Zanchin for anti-Nip7p antiserum, and Tereza C. Lima Silva and Zildene G. Correa for DNA sequencing. J.S.L. and R.C.G. were recipients of Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) and C.H.G was recipient of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) fellowships. This work was supported by FAPESP (Grant No. 05/56493-9 to C.C.O).

Supporting Information

Supporting information may be found in the online version of this article.

References

Andersen JE, Lam YW, Leung AKL, *et al*. 2005. Nucleolar proteome dynamics. *Nature* **433**: 77–83.

Arenas JE, Abelson JN. 1997. Prp43: an RNA helicase-like factor involved in spliceosome disassembly. *Proc Natl Acad Sci USA* **28**: 11798–11802.

Austin KM, Leary RJ, Shimamura A. 2005. The Shwachman–Diamond SBDS protein localizes to the nucleolus. *Blood* **15**: 1253–1258.

Basu U, Si K, Warner JR, Maitra U. 2001. The *Saccharomyces cerevisiae TIF6* gene encoding translation initiation factor 6 is required for 60S ribosomal subunit biogenesis. *Mol Cell Biol* **21**: 1453–1462.

Bernstein J, Patterson DN, Wilson GM, Toth EA. 2008. Characterization of the essential activities of *Saccharomyces cerevisiae* Mtr4p, a 3′–5′ helicase partner of the nuclear exosome. *J Biol Chem* **283**: 4930–4942.

Bodian M, Sheldon W, Lightwood R. 1964. Congenital hypoplasia of the exocrine pancreas. *Acta Pediatr* **53**: 282–293.

Boocock GR, Morrison JA, Popovic M, *et al*. 2003. Mutations in *SBDS* are associated with Shwachman–Diamond syndrome. *Nat Genet* **33**: 97–101.

Calado RT, Graf SA, Wilkerson KL, *et al*. 2007. Mutations in the *SBDS* gene in acquired aplastic anemia. *Blood* **110**: 1141–1146.

Ceci M, Gaviraghi C, Gorrini C, *et al*. 2003. Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. *Nature* **426**: 579–584.

Combs DJ, Nagel RJ, Ares M, Stevens SW. 2006. Prp43p is a DEAH-box spliceosome disassembly factor essential for ribosome biogenesis. *Mol Cell Biol* **26**: 523–534.

Draptchinskaia N, Gustavsson P, Andersson B, *et al*. 1999. The gene encoding ribosomal protein S19 is mutated in Diamond–Blackfan anaemia. *Nat Gene* **21**: 169–175.

Ganapathi KA, Austin KM, Lee CS, *et al*. 2007. The human Shwachman–Diamond syndrome protein, SBDS, associates with ribosomal RNA. *Blood* **110**: 1458–1465.

Gavin AC, Bösche R, Krause M, *et al*. 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* **415**: 123–124.

Gazda HT, Grabowska A, Merida-Long LB, *et al*. 2006. Ribosomal protein S24 gene is mutated in Diamond–Blackfan anemia. *Am J Hum Genet* **79**: 1110–1118.

Gerbasi VR, Weaver CM, Hill S, *et al*. 2004. Yeast Asc1p and mammalian RACK1 are functionally orthologous core 40S ribosomal proteins that repress gene expression. *Mol Cell Biol* **24**: 8276–8287.

Ginzberg H, Shin J, Ellis L, *et al*. 1999. Shwachman syndrome: phenotypic manifestations of sibling sets and isolated cases in a large patient cohort are similar. *J Pediatr* **135**: 81–88.

Goldfeder MB, Oliveira CC. 2008. Cwc24p, a novel *Saccharomyces cerevisiae* nuclear RING-finger protein, interacts with Nop17p and affects pre-snoRNA *U3* splicing. *J Biol Chem* **283**: 2644–2653.

Gonzales FA, Zanchin NIT, Luz JS, Oliveira CC. 2005. Characterization of *Saccharomyces cerevisiae* Nop17p, a novel Nop58p-interacting protein that is involved in pre-rRNA processing. *J Mol Biol* **346**: 437–455.

Granato DC, Gonzales FA, Luz JS, *et al*. 2005. Nop53p, an essential nucleolar protein that interacts with Nop17p and Nip7p, is required for pre-rRNA processing in *Saccharomyces cerevisiae*. *FEBS J* **272**: 4450–4463.

Heiss NS, Knight SW, Vulliamy TJ, *et al*. 1998. X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. *Nat Genet* **19**: 32–38.

Hesling C, Oliveira CC, Castilho BA, Zanchin NIT. 2007. The Shwachman–Bodian–Diamond syndrome associated protein interacts with HsNip7 and its down-regulation affects gene expression at the transcriptional and translational levels. *Exp Cell Res* **313**: 4180–4195.

Huh WK, Falvo JV, Gerke LC, *et al*. 2003. Global analysis of protein localization in budding yeast. *Nature* **16**: 686–691.

Kressler D, Linder P, de La Cruz PJ. 1999. Protein trans-acting factors involved in ribosome biogenesis in *Saccharomyces cerevisiae*. *Mol Cell Biol* **19**: 7897–7912.

Krogan NJ, Cagney G, Yu H, *et al*. 2006. Global landscape of protein complexes in the yeast *Saccharomyces cerevisiae*. *Nature* **30**: 637–643.

Lafontaine DL, Bousquet-Antonelli C, Henry Y, *et al*. 1998. The box H + *ACA* snoRNAs carry Cbf5p, the putative rRNA pseudouridine synthase. *Genes Dev* **12**: 527–537.

Lebaron S, Froment C, Fromont-Racine M, *et al*. 2005. The splicing ATPase Prp43p is a component of multiple preribosomal particles. *Mol Cell Biol* **25**: 9269–9282.

Leeds NB, Small EC, Hiley SL, *et al*. 2006. The splicing factor Prp43p, a DEAH box ATPase, functions in ribosome biogenesis. *Mol Cell Biol* **26**: 513–522.

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* **17**: 17743–17750.

Menne TF, Goyenechea B, Sánchez-Puig N, *et al*. 2007. The Shwachman–Bodian–Diamond syndrome protein mediates translational activation of ribosomes in yeast. *Nat Genet* **39**: 486–495.

Nissan TA, Bassler J, Petfalski E, *et al*. 2002. 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. *EMBO J* **15**: 5539–5547.

Pandit S, Lynn B, Rymond BC. 2006. Inhibition of a spliceosome turnover pathway suppresses splicing defects. *Proc Natl Acad Sci USA* **103**: 13700–12705.

Peltz SW, Hammell AB, Cui Y, *et al*. 1999. Ribosomal protein L3 mutants alter translational fidelity and promote rapid loss of the yeast killer virus. *Mol Cell Biol* **19**: 384–391.

Peng WT, Robinson MD, Mnaimneh S, *et al*. 2003. A panoramic view of yeast noncoding RNA processing. *Cell* **27**: 919–933.

Perentesis JP, Phan LD, Gleason WB, *et al*. 1992. *Saccharomyces cerevisiae* elongation factor 2. Genetic cloning, characterization of expression, and G-domain modeling. *J Biol Chem* **267**: 1190–1197.

Savchenko A, Krogan N, Cort JR, *et al*. 2005. The Shwachman–Bodian–Diamond syndrome protein family is involved in RNA metabolism. *J Biol Chem* **13**: 19213–19220.

Shammas C, Menne TF, Hilcenko C, *et al*. 2005. Structural and mutational analysis of the SBDS protein family. Insight into the leukemia-associated Shwachman–Diamond Syndrome. *J Biol Chem* **13**: 19221–19229.

Sherman F, Fink G, Hicks JB. 1986. *Laboratory Course Manual for Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Shwachman H, Diamond LK, Oski FA, Khaw KT. 1964. The syndrome of pancreatic insufficiency and bone marrow dysfunction. *J Pediatr* **65**: 645–663.

Tsai RT, Fu RH, Yeh FL, *et al*. 2005. Spliceosome disassembly catalyzed by Prp43 and its associated components Ntr1 and Ntr2. *Genes Dev* **15**: 2991–3003.

Tsai RT, Tseng CK, Lee PJ, *et al*. 2007. Dynamic interactions of Ntr1–Ntr2 with Prp43 and with U5 govern the recruitment of

Prp43 to mediate spliceosome disassembly. *Mol Cell Biol* **27**: 8027–8037.
Tschochner H, Hurt E. 2003. Pre-ribosomes on the road from the nucleolus to the cytoplasm. *Trends Cell Biol* **13**: 255–263.
Venema J, Tollervey D. 1999. Ribosome synthesis in *Saccharomyces cerevisiae*. *Annu Rev Genet* **33**: 261–311.
Vojtek AB, Hollenberg SM. 1995. Ras–Raf interaction: two-hybrid analysis. *Methods Enzymol* **255**: 331–342.
Warner JR. 1989. Synthesis of ribosomes in *Saccharomyces cerevisiae*. *Microbiol Rev* **53**: 256–271.
Warner JR. 1999. The economics of ribosome biosynthesis in yeast. *Trends Biochem Sci* **24**: 437–440.
Woolford JL. 1991. The structure and biogenesis of yeast ribosomes. *Adv Genet* **29**: 63–118.
Wu LF, Hughes TR, Davierwala AP, *et al*. 2002. Large-scale prediction of *Saccharomyces cerevisiae* gene function using overlapping transcriptional cluster. *Nat Genet* **31**: 255–265.
Zanchin NIT, Goldfarb DS. 1999. Nip7p interacts with Nop8p, an essential nucleolar protein required for 60S ribosome biogenesis, and the exosome subunit Rrp43p. *Mol Cell Biol* **19**: 1518–1525.
Zanchin NIT, Roberts P, DeSilva A, *et al*. 1997. *Saccharomyces cerevisiae* Nip7p is required for efficient 60S ribosome subunit biogenesis. *Mol Cell Biol* **17**: 5001–5015.
Zeller CE, Parnell SC, Dohlman HG. 2007. The RACK1 ortholog Asc1 functions as a G-protein β-subunit coupled to glucose responsiveness in yeast. *J Biol Chem* **24**: 25168–25176.

DOI: 10.1002/yea