Nop53p, an essential nucleolar protein that interacts with Nop17p and Nip7p, is required for pre-rRNA processing in Saccharomyces cerevisiae

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In eukaryotes, pre-rRNA processing depends on a large number of non-ribosomal trans-acting factors that form large and intriguingly organized complexes. A novel nucleolar protein, Nop53p, was isolated by using Nop17p as bait in the yeast two-hybrid system. Nop53p also interacts with a second nucleolar protein, Nip7p. A carbon source-conditional strain with the NOP53 coding sequence under the control of the GAL1 promoter did not grow in glucose-containing medium, showing the phenotype of an essential gene. Under nonpermissive conditions, the conditional mutant strain showed rRNA biosynthesis defects, leading to an accumulation of the 27S and 7S pre-rRNAs and depletion of the mature 25S and 5.8S mature rRNAs. Nop53p did not interact with any of the exosome subunits in the yeast two-hybrid system, but its depletion affects the exosome function. In pull-down assays, protein A-tagged Nop53p coprecipitated the 27S and 7S pre-rRNAs, and His–Nop53p also bound directly 5.8S rRNA in vitro, which is consistent with a role for Nop53p in pre-rRNA processing.

The factors involved in rRNA processing in eukaryotes assemble cotranscriptionally onto the nascent pre-rRNAs and include endonucleases, exonucleases, RNA helicases, GTPases, modifying enzymes and snoRNPs (small nucleolar ribonucleoproteins). The precursor of three of the four eukaryotic mature rRNAs contains the rRNA sequences flanked by two internal (ITS1 and ITS2) and two external (5′-ETS and 3′-ETS) spacer sequences that are removed during processing [1,2]. The pre-rRNA is first assembled into a 90S particle that contains U3 snoRNP and 40S subunit-processing factors [3,4]. The early pre-rRNA endonucleolytic cleavages at sites A₀, A₁ and A₂ occur within the 90S particles [3,5]. A₂ cleavage releases the first pre60S particle, which differs in composition from the known 90S particle. Pre60S particles contain 27S rRNA, ribosomal L proteins and many nonribosomal proteins [6].

As they mature, pre60S particles migrate from the nucleolus to the nucleoplasm and their content of non-ribosomal factors changes [7,8]. Nip7p was among the proteins identified in the early pre60S particle [6–8], and has been shown to participate in the processing of 27S pre-rRNA to the formation of 25S [9]. Interestingly, Nip7p also binds the exosome subunit Rrp43p [10]. The exosome complex is responsible for the degradation of the excised 5′-ETS and for the 3′–5′ exonucleolytic processing of 7S pre-rRNA to form the mature 5.8S rRNA. The exosome is also involved in the processing of snoRNAs and in mRNA degradation [11–13].

During processing, pre-rRNA undergoes covalent modifications that include isomerization of some uridines into pseudouridines and addition of methyl groups to specific nucleotides, mainly at the 2′-O posi-

Abbreviations
ETS, external transcribed spacer; β-Gal, β-galactosidase; GFP, green fluorescent protein; GST, glutathione S-transferase; ITS, internal transcribed spacer; RFP, red fluorescent protein; snoRNP, small nucleolar ribonucleoprotein.
tion of the ribose. These nucleotide modifications are directed by snoRNPs, which select the nucleotide through complementary base-pairing between the snoRNA and the rRNA substrate. The snoRNAs involved in rRNA modification can be divided into two major classes based on conserved sequence elements and on the association with evolutionarily conserved core proteins [14–16]. The box C/D class of guide snoRNAs contains the core proteins Nop1p, Nop58p, Nop56p and Snu13p, and is involved in cleavage and methylation of pre-rRNA. The box H/ACA guide snoRNAs are associated with the core proteins Cbf1p, Gar1p, Nhp2p and Nop10p and function in the conversion of uridine into pseudouridine [17–23].

In addition to the core snoRNP proteins, other proteins have been found to be associated with the snoRNPs and to participate in cleavage reactions as well as methylation and pseudouridylation of specific nucleotides of rRNA [24–28]. Among these proteins is Nop17p, which interacts with the box C/D snoRNP subunit Nop58p and with the exosome subunit Rrp43p [28]. Characterization of Nop17p function showed that it is required for proper localization of the core proteins of the box C/D snoRNP Nop1p, Nop56p, Nop58p and Snu13p [28]. In addition, cells depleted of Nop17p show pre-rRNA processing defects that include increased primer extension products at certain box C/D methylation sites, indicating that Nop17p is required for proper pre-rRNA methylation [28]. A third Nop17p-interacting partner isolated using the yeast two-hybrid system is the protein encoded by the open reading frame (ORF) YPL146C, Nop53p. Nop53p is an essential nucleolar protein, which was also recently identified as a subunit in pre60S particles [6,7].

In this study, we show that Nop53p is required for the late steps of rRNA processing. Consistent with its copurification with the pre60S particle, Nop53p depletion affects exonucleolytic cleavage of the 3'-end of the 7S pre-rRNA, a processing step that requires the function of the exosome [11]. In addition, protein A-tagged Nop53p coprecipitated the 27S and 7S pre-rRNAs and the mature 5.8S rRNA. Purified His–Nop53p also bound in vitro transcribed 5.8S rRNA, showing that it must play an important role in ribosome biogenesis, possibly related to the exosome function.

Results

Nop53p interacts with the pre-rRNA processing proteins Nop17p and Nip7p

*Saccharomyces cerevisiae* Nop53p, a previously uncharacterized essential protein (SGD), is encoded by the YPL146C ORF and was identified in the yeast nuclear pore complex [29] and as a component of the pre60S complex [6,7]. In this study, Nop53p was isolated in a two-hybrid screen as a protein interacting with Nop17p, which is involved in the early steps of prerRNA processing [28]. Nop17p and Nop53p interacted in the two-hybrid system independently of the tag, but the interaction was stronger when Nop17p was fused to the DNA binding domain (BD-Nop17p; Fig. 1). Further protein interaction studies in the two-hybrid system revealed that Nop53p also interacts with Nip7p (Fig. 1), a protein component of the pre60S complex that is involved in processing of 27S preRNA [6,7,9]. The interaction between Nop53p and Nip7p in the two-hybrid system confirms the finding of these two proteins in the pre60S complex. The two-hybrid system was also used to test the interaction between Nop53p and the exosome subunits and between Nop53p and snoRNPs of box C/D (Nop1p, Nop56p, Nop58p and Snu13p) and of box H/ACA (Cbf1p, Nop10p, Gar1p and Nhp2p), although no interaction was detected (data not shown).

The Nop53p–Nop17p interaction was confirmed by pull-down assays carried out using *Escherichia coli* expressed His–Nop53p and GST–Nop17p fusion proteins. The results obtained show that His–Nop53p was pulled-down by GST–Nop17p (Fig. 1C). A parallel negative control experiment was carried out using glutathione S-transferase (GST), which showed no precipitation of His–Nop53p (Fig. 1C).

Depletion of Nop53p correlates with loss of viability

A diploid *NOP53* deletion strain (2n, *NOP53*/Δnop53), obtained from Euroscarf (Table 2), was transformed with a plasmid containing a copy of *NOP53* fused to protein A under control of the regulated GAL1 promoter (Table 1) and induced to sporulation. Haploid Δnop53/A-NOP53 was not able to grow on glucose plates, confirming that *NOP53* is an essential gene for cell viability (Fig. 2A). A growth curve in liquid medium showed that the growth rate of Δnop53/A-NOP53 decreases 4 h after shifting cells from galactose-containing medium to glucose (Fig. 2B). The analysis of A-NOP53 expression in Δnop53/A-NOP53 cells shows that after 4 h on glucose, the A-NOP53 mRNA can no longer be detected (Fig. 2C). The two bands corresponding to A-NOP53 mRNA are due to the lack of an efficient transcription termination sequence in the plasmid YCp33Gal-A-NOP53. The fusion protein A-Nop53p can be detected by immunoblots up to 8 h after shift to glucose-containing medium,
although by this time the levels of the protein are very low (Fig. 2D). The fusion Protein A–Nop53p is functional, supporting growth of the \( \Delta \text{nop53} \) strain in galactose-containing medium. The detection of A–Nop53p after 8 h of transcriptional repression of the \( \text{GAL1} \) promoter indicates that this is a stable protein, probably because it is not free in the cell, but part of the pre60S complex.

**Table 1.** List of plasmid vectors used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBTM116</td>
<td>lexA DNA binding domain, ( \text{TRP1} ) 2 ( \mu \text{m} )</td>
<td>[41]</td>
</tr>
<tr>
<td>pBTM-NIP7</td>
<td>lexA::NIP7, ( \text{TRP1} ) 2 ( \mu \text{m} )</td>
<td>[42]</td>
</tr>
<tr>
<td>pBTM-NOP17</td>
<td>lexA::NOP17, ( \text{TRP1} ) 2 ( \mu \text{m} )</td>
<td>[28]</td>
</tr>
<tr>
<td>pBTM-NOP53</td>
<td>lexA::NOP53, ( \text{TRP1} ) 2 ( \mu \text{m} )</td>
<td>This study</td>
</tr>
<tr>
<td>pACT-NOP8</td>
<td>GAL4::NOP8, ( \text{LEU2} ) 2 ( \mu \text{m} )</td>
<td>[42]</td>
</tr>
<tr>
<td>pGADc2</td>
<td>GAL4 activation domain, ( \text{LEU2} ) 2 ( \mu \text{m} )</td>
<td>[43]</td>
</tr>
<tr>
<td>pGAD-NOP17</td>
<td>GAL4::NOP17, ( \text{LEU2} ) 2 ( \mu \text{m} )</td>
<td>[28]</td>
</tr>
<tr>
<td>pGAD-NOP53</td>
<td>GAL4::NOP53, ( \text{LEU2} ) 2 ( \mu \text{m} )</td>
<td>This study</td>
</tr>
<tr>
<td>YCp33GAL-A</td>
<td>GAL1::ProtA, ( \text{URA3, CEN4} )</td>
<td>Tavares and Oliveira, unpublished</td>
</tr>
<tr>
<td>YCp111-His-NOP53</td>
<td>GAL1::His-NOP53, ( \text{URA2} ) ( \text{CEN4} )</td>
<td>This study</td>
</tr>
<tr>
<td>pRS313</td>
<td>pBluescript, ( \text{HIS3, CEN6, ARSH4} )</td>
<td>[44]</td>
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<td>pRS-GAL-His-NOP53</td>
<td>GAL1::His-NOP53, ( \text{LEU2} ) ( \text{CEN4} )</td>
<td>This study</td>
</tr>
<tr>
<td>pGFP-N-FUS</td>
<td>( \text{MET25::GFP, URA3} )</td>
<td>[45]</td>
</tr>
<tr>
<td>pGFP-N-NOP53</td>
<td>( \text{MET25::NOP53, URA3, CEN6} )</td>
<td>This study</td>
</tr>
<tr>
<td>pRFP-NOP1</td>
<td>( \text{ADH1::RFP-NOP1, LEU2, 2} ( \mu \text{m} )</td>
<td>[28]</td>
</tr>
<tr>
<td>pGEX-NOP17</td>
<td>GST::NOP17, ( \text{Amp}^R )</td>
<td>[28]</td>
</tr>
<tr>
<td>pET-NOP53</td>
<td>His::NOP53, ( \text{Kan}^R )</td>
<td>This study</td>
</tr>
</tbody>
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GFP–Nop53p colocalizes with RFP–Nop1p

The interaction of Nop53p with Nop17p, a nucleolar protein [28], and Nip7p, a protein that localizes to the nucleus and the cytoplasm [9], raised the question of where Nop53p would localize in the cell. This was assessed by the utilization of a green fluorescent protein (GFP) fusion (GFP–Nop53p) and a red fluorescent protein (RFP)–Nop1p fusion protein as a nucleolar marker. ∆nop53 cells were cotransformed with plasmids expressing GFP–Nop53p and RFP–Nop1p and observed by confocal microscopy. GFP–Nop53p colocalizes with RFP–Nop1p (Fig. 3), showing a predominantly nucleolar localization. The colocalization was confirmed by using the profile module of LSM 510 software. The GFP–Nop53p fusion protein was functional in these cells, because it complemented the growth of ∆nop53/GAL-His–NOP53/GFP–NOP53 in the presence of glucose (data not shown).

∆nop53 shows defects in pre-rRNA processing

Because all the evidence pointed to a role for Nop53p in pre-rRNA processing, the kinetics of pre-rRNA processing was analyzed by pulse-chase labeling with both [3H]uracil and [methyl-3H]methionine. Following incubation of wild-type and ∆nop53/A-NOP53 cells for 12 h in glucose medium, pulse-chase-labeling experiments showed a severe delay in 25S and 5.8S rRNA formation, with accumulation of the 35S, 27S and 7S pre-rRNAs (Fig. 4). Pulse-chase labeling with 3H-uracil showed that although mature 5.8S rRNA could be detected in the NOP53 strain after 3 min of chase, in ∆nop53/A-NOP53 7S pre-rRNA was still visible after 60 min, showing a defect for processing 27S into 5.8S.
and 25S rRNAs (Fig. 4A,C). Pulse-chase labeling with [methyl-3H]methionine also showed the delay in 25S formation in Δnop53p compared with the much less affected formation of mature 18S rRNA (Fig. 4B).

Analysis of pre-rRNA and rRNA steady-state levels by means of northern blot was performed using specific oligonucleotide probes that hybridize in the pre-rRNA spacer sequences and in the mature rRNAs. Analyses of RNA isolated from cells subjected to growth in glucose medium for up to 12 h, which leads to Nop53p depletion, also detected pre-rRNA processing defects including accumulation of 35S, 27S and 7S pre-rRNAs and a corresponding decrease in the concentration of the mature 25S and 5.8S rRNAs, as compared with the control strain (Fig. 5). Accumulation of the 7S pre-rRNA indicates that Nop53p may be required for proper exosome function, because defective processing of the 7S pre-rRNA 3'-end is a typical phenotype of exosome mutants [10–13,30]. Although
The depletion of Nop53p does not seem to affect the formation of 18S rRNA, an accumulation of 23S and 35S pre-rRNAs results in a slight decrease in the concentration of 18S rRNA (Fig. 5).

The lower concentrations of mature 25S and 5.8S rRNAs detected by steady-state analysis are consistent with the data obtained from the pulse-chase-labeling experiments and indicate that Nop53p is involved in the late steps of rRNA processing. To further investigate the effects of Nop53p deficiency on pre-rRNA cleavages we performed primer extension experiments using primers that anneal in the regions of the mature rRNAs close to the 5’-end of those rRNAs. Extension of primer P2, that anneals to nucleotides 34–53 downstream of the 18S rRNA 5’-end, showed that depletion of Nop53p leads to shorter 18S rRNA at the 5’-end (Fig. 6A). A similar decrease in the amount of primer extension product is observed for the extension reactions using primer P4 that anneals to nucleotides 42–64 downstream of the 5.8S rRNA 5’-end (Fig. 6B). Extension of primer P7 (complementary to nucleotides 80–105 downstream of 25S rRNA 5’-end) also resulted in a decrease of concentration of the band corresponding to the 5’-end of the 25S rRNA. Processing at sites C1 and C2 separates the mature 25S rRNA from the 7S pre-rRNA. This pre-rRNA is subsequently processed exonucleolytically to generate the mature 5.8S pre-rRNA. A fraction of the 27SA2 pre-rRNA is processed at the 5’-end by a different mechanism and, following processing at the remaining sites, gives rise to the 5.8S, (5.8S long) rRNA, which is 6–8 nucleotides longer than the 5.8S pre-rRNA at the 5’-end.

Fig. 5. Northern blot analysis of pre-rRNA processing. (A) Total RNA was extracted from cells incubated in glucose medium for different time intervals and hybridized against specific oligonucleotide probes. The relative positions of the probes on the 35S pre-rRNA are indicated in (B). Bands corresponding to the major intermediates and to the mature rRNAs are indicated on the right-hand side. The lower panel shows a northern blot detecting the actin mRNA, used as an internal control. (B) Structure of the 35S pre-rRNA and major intermediates of the rRNA processing pathway in S. cerevisiae. The positions of the probes used for northern blot hybridizations are indicated below the 35S pre-rRNA. Processing of 35S pre-rRNA starts with endonucleolytic cleavages at sites A0 and A1 in the 5’-ETS, generating 32S pre-rRNA. The subsequent cleavage at site A2, in ITS1, generates the 20S and 27SA2 pre-rRNAs (dotted arrows indicate a possible pathway including the aberrant intermediate 23S). The 20S pre-rRNA is then processed at site D to the mature 18S rRNA. The major processing pathway of the 27SA2 pre-rRNA involves cleavage at site A3, producing 27SA3, which is digested quickly by exonucleases to generate the 27SBs (27S short) pre-rRNA. The subsequent processing step occurs at site B2, at the 3’-end of the mature 25S rRNA. Processing at sites C1 and C2 separates the mature 25S rRNA from the 7S pre-rRNA. This pre-rRNA is subsequently processed exonucleolytically to generate the mature 5.8S pre-rRNA. A fraction of the 27SA2 pre-rRNA is processed at the 5’-end by a different mechanism and, following processing at the remaining sites, gives rise to the 5.8S, (5.8S long) rRNA, which is 6–8 nucleotides longer than the 5.8S pre-rRNA at the 5’-end.
not functional and rRNA processing is defective, precursor and intermediate rRNAs may undergo 5′–3′ degradation. Interestingly, \( \Delta \text{nop}53/A-\text{NOP53} \) cells showed the same phenotype, indicating that Nop53p affects exosome function.

**Nop53p coprecipitates pre-rRNAs and binds 5.8S rRNA**

In order to find out whether Nop53p interacts with pre-rRNAs, \( \text{NOP53} \) strains expressing either Protein A or A–Nop53p fusion protein were constructed to test coimmunoprecipitation of pre-rRNAs on IgG-Sepharose affinity columns. The results obtained showed that A–Nop53p coprecipitates the 27S and 7S pre-rRNAs, and 5.8S mature rRNA (Fig. 7). A–Nop53p also coprecipitated snR37, a box H/ACA snoRNA involved in pseudouridylation of the 25S rRNA. A–Nop53p did not coprecipitate box C/D snoRNAs U3 and U14, involved in processing of 18S rRNA (Fig. 7; data not shown). Compared with the control Protein A, A–Nop53p coprecipitated 4.31-fold more snR37, 4.67-fold more 5.8S, and 50-fold more 7S. These results indicate that Nop53p participates in the pre60S complex, affecting the processing of the 27S and more strongly the processing of the 7S pre-rRNA. Purified His–Nop53p was also tested for binding to \( \text{in vitro} \) transcribed 5.8S rRNA and the results show that it binds directly to this RNA (Fig. 8). These results support the hypothesis that Nop53p depletion results in a defective function of the exosome.

**Nop53p has a putative human homolog**

Database searches were performed to identify possible homologs of \( S. \text{cerevisiae NOP53} \), and Nop53p was found to be a conserved protein in eukaryotes, showing a higher conservation in lower eukaryotes (Fig. 9). Despite the fact that Nop53p binds RNA, no RNA recognition motif was identified in its sequence. A putative human ortholog (glioma tumor suppressor candidate, Accession no. NP056525) shares 21% of identity with its \( S. \text{cerevisiae} \) counterpart, but 41% identity at the C-terminal region. Interestingly, hNop53p was also localized to the nucleolus [31], supporting the hypothesis of Nop53p having a conserved function throughout evolution.

**Discussion**

Protein interaction studies have established a functional link between several proteins involved in pre-rRNA processing. The exosome subunit Rrp43p interacts with Rrp46p, Nip7p and Nop17p [10,13,28]. Nop17p interacts with Nop58p and Nop53p [28] (this study). The circle is closed by the interaction of Nop53p and Nip7p, which was determined here. The exosome subunits Rrp43p and Rrp46p and Nip7p are found both in the nucleus and in the cytoplasm, whereas Nop58p, Nop17p and Nop53p are restricted to the nuclear compartment, showing a predominantly nucleolar localization [9,10,12,20,28]. The subcellular distribution and the interactions of these proteins are consistent with their function in...
pre-rRNA processing and ribosome biogenesis. Nop53p colocalizes with the nucleolar protein Nop1p [17] and its localization is consistent with the data reported in the global yeast protein localization program [32].

The interaction with Nip7p indicated that Nop53p is involved in the late steps of rRNA processing. Evidence supporting this hypothesis was obtained from the Nop53p–rRNA coprecipitation analyses. Nop53p coimmunoprecipitated the 27S and 7S pre-rRNAs and the mature 5.8S rRNAs. In vitro RNA-binding assays showed that Nop53p actually binds 5.8S rRNA. Analysis of rRNA processing showed that depletion of Nop53p leads to an accumulation of the 27S and 7S pre-rRNAs, confirming a role for Nop53p in late steps of processing. Accumulation of unprocessed 27S pre-rRNA was observed for cells depleted of Nip7p [9], which is consistent with a functional interaction with Nop53p. Accumulation of the 7S pre-rRNA, by contrast, is a defect typical of a deficient exosome [10–13].
Although Nop53p did not interact with any of the exosome subunits in the two-hybrid system (data not shown), it might be connected to the exosome via Nip7p. Similar to exosome mutants $D_{\text{nop53}}$/$A_{\text{NOP53}}$ strain showed higher levels of 7S pre-rRNA, indicating a defective 3'→5' exonucleolytic cleavage of this precursor and therefore that the exosome is not fully active in the absence of Nop53p. Interestingly, the accumulated 7S pre-rRNA in cells depleted of Nop53p contains aberrant 5'→3'-end, indicating that this pre-rRNA is being degraded by a 5'→3' exonuclease, probably Rat1p or Xrn1p [33,34]. Rapid degradation of pre-rRNAs has been reported for many strains with defects in pre-rRNA processing [35–37]. The finding that the depletion of Nop53p leads to the accumulation of 7S pre-rRNA indicates that Nop53p could mediate the signal for the processing of this pre-rRNA to the exosome. Alternatively, the interaction of Nop53p with Nip7p, that binds the exosome subunit Rrp43p [10] could activate the exosome for processing of the 7S pre-rRNA. However, since $nip7$ mutants do not show accumulation of 7S pre-rRNA [9], the former hypothesis seems more likely.

Nop53p also coprecipitated the box H$^{\text{ACA}}$ snoRNA snR37, but not box C$^{\text{D}}$ snoRNAs involved in 18S processing. This result raised the possibility that Nop53p could participate in processing or assembly of box H$^{\text{ACA}}$ snoRNPs. However, the deficiency of

Fig. 9. Multiple sequence alignment of Nop53p. The full sequence of Nop53p and its putative eukaryotic orthologs were aligned. Numbers correspond to amino acid position in each protein. Proteins access numbers: C. glabrata, CAG62427; K. lactis, XP_455604; E. gossypii, AAS51532; S. pombe, CAB52719; Homo sapiens, NP_056525; Mus musculus, AAH25810. * identity; : strong similarity; . weak similarity. CLUSTALW was used for the sequence alignment [50].
Nop53p did not affect box H/ACA snoRNAs stability (data not shown). It remains to be determined whether Nop53p binds directly box H/ACA snoRNAs, or whether snR37 coimmunoprecipitated as part of the pre60S particle.

The data on the identification of Nop53p interaction with Nop17p, a protein involved in the assembly and/or stabilization of box C/D snoRNPs [28] indicates that these interactions take place on the pre60S particle. Interestingly, the modification of nucleotides at the peptidyl transferase center has been reported to occur late in processing, accounting for the copurification of snoRNPs of box C⁄D and H/ACA with the pre60S particles [7,27,38]. The interactions reported here between Nop53p and Nop17p, and between Nop53p and Nip7p could occur in the context of the pre60S particles, which is formed by a different number of proteins associated with the 27S rRNA, depending on the phase of processing and transit from the nucleolus to the cytoplasm.

In conclusion, the results obtained with the conditional A-nop53/A-NOP53 strain showed that rRNA processing is affected in the absence of Nop53p, leading to a reduction in rRNA synthesis and accumulation of the pre-rRNAs 27S and 7S. The finding that depletion of Nop53p affects more strongly the late processing reactions responsible for the formation of the mature 5.8S rRNA, indicates that this novel protein is important for proper exosome function.

During the final preparation of this article a study was published on Nop53p [39]. In that study it is reported that Nop53p is involved in the processing of 27S pre-rRNA, consistent with the data shown here. However, contrary to our data, the authors found that the depletion of Nop53p has stronger effects on the maturation of the 25S rRNA, and not on the 5.8S. Our data show that Nop53p coprecipitates the 27S and 7S preRNAs and the mature 5.8S rRNA, binding directly to the 5.8S rRNA region. These discrepancies may be the result of the different strain background, because Sydorsky et al. [39] used their own deletion strain, in which NOP53 was not essential, whereas the strain we used was purchased from the yeast deletion collection at Euroscarf.

**Experimental procedures**

**DNA analyses and plasmid construction**

DNA cloning and analyses were performed as described elsewhere [40]. DNA was sequenced by using the Big Dye method (Perkin-Elmer, USA). Plasmids used in this study are summarized in Table 1, and cloning strategies are briefly described below. The lexA::NOP53 fusion used in the two-hybrid assay was constructed by inserting a 1.3 kb BamHI/SalI DNA fragment containing the PCR-amplified NOP53 ORF into pBTM-116, which was previously digested with BamHI/SalI restriction enzymes, generating the plasmid pBTM-NOP53. Plasmid pACT-NOP53 (14–456, numbers refer to Nop53p amino acid residues coded by this cDNA clone) bears the gene encoding the hybrid protein of the GAL4p activation domain and NOP53p. YCpGAL-A–NOP53 was constructed by inserting the BamHI/SalI NOP53-containing fragment obtained from pBTM-NOP53 into Ycp33GAL1-A vector previously digested with the same restriction enzymes. Plasmid pGFP-N-NOP53 was constructed by inserting the fragment XhoI/SalI NOP53 obtained from the Ycp111GAL-HIS–NOP53 vector digested with the same enzymes, into the pGFP-N-FUS vector digested with SpeI/XhoI restriction enzymes. pRS-GAL-His–NOP53 was obtained by inserting the fragment (BamHI–SalI) containing NOP53 sequence and the fragment (EcoRI/BamHI) containing GAL1-HIS sequence into the pRS13 vector digested with EcoRI and SalI. For the construction of pET-NOP53, the PCR amplified NOP53 ORF (BamHI/SalI) was inserted into the pET-28a vector digested with BamHI and XhoI restriction enzymes.

**Yeast transformation and maintenance**

Yeast strains used in this work are listed in Table 2. Yeast strains were maintained in yeast extract-peptone medium (YP) or synthetic medium (YNB) as described previously [47]. Glucose or galactose was added as carbon source to a final concentration of 2% as indicated. Yeast cells were transformed using the lithium acetate method as described previously [47]. A Δnop53 strain was obtained from Euroscarf.

**Yeast two-hybrid screen for proteins that interact with Nop53p**

The host strain for the two-hybrid screen, L40 [46], contains both yeast HIS3 and E. coli lacZ genes as reporters for two-hybrid interaction integrated into the genome. Strain YDG146 is a derivative of L40, bearing plasmid pBTM-NOP53, which encodes a hybrid protein containing the lexA DNA binding domain and the full-length NOP53 ORF. Transformation of YDG146 was performed with plasmid pGAD-NOP17 containing NOP17 ORF fused to the GAL4 activation domain. Alternatively, L40 was transformed with pBTM-NIP7 and pACT-NOP53. Transformants were plated directly onto YNB medium lacking histidine for immediate selection of Nop53p-interacting proteins. His+ clones were tested for lacZ expression by transferring cells to nitrocellulose filters and analyzing β-galactosidase (β-Gal) activity [46]. β-Gal activity of strains analyzed in two-hybrid experiments was quantitated.
using cell extracts generated in buffer Z using ONPG as substrate [41]. Strain L40-41 was used as a positive control and strain YDG-146/pGAD-C2 was used as negative control for two-hybrid interaction [42] (Table 2).

Protein pull-down and immunoblot analysis

Pull-down of His–Nop53p was assayed as follows: whole-cell extracts from E. coli cells expressing either GST or GST–Nop17p were generated in NaCl/Pi buffer and mixed with 500 µL of glutathione-Sepharose beads (Amersham Biosciences). After washing bound material with NaCl/Pi, whole-cell extracts from E. coli cells expressing His–Nop53p were added to the glutathione-Sepharose beads and incubated at 4 °C for 2 h. The glutathione-Sepharose beads were precipitated and washed again with NaCl/Pi, and bound proteins were eluted and resolved on SDS/PAGE and transferred to polyvinylidene difluoride membranes (BioRad Laboratories, Hercules, CA, USA), which were incubated with an anti-(poly histidine) serum (Amersham Laboratories, Hercules, CA, USA), which were probed with [32P]-labeled oligonucleotides complementary to specific regions of the 35S pre-rRNA (Table 3), or with [32P]-labeled DNA fragments corresponding to actin ORF, using the hybridization conditions described previously [9] and analyzed in a Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

Metabolic labeling of rRNA

Metabolic labeling was performed as described previously [9]. Exponentially growing cultures of strains NOP53 and Δnop53 were incubated at 30 °C for 12 h in YNB–glucose medium lacking methionine. Subsequently, cells were pulse-labeled with 100 µCi/mL [methyl-3H]methionine (Amersham Biosciences) for 2 min and chased with 100 µg/mL unlabeled methionine. At various times, samples were taken and quickly frozen in a dry ice-ethanol bath. For metabolic labeling with [3H]uracil, exponential growing cultures of NOP53 and Δnop53 were shifted from galactose to glucose medium and incubated for 12 h. Cells were then pulse-labeled for 3 min at 37 °C with 50 µCi of [3H]uracil per mL and chased for up to 1 h after addition of unlabeled uracil to a final concentration of 300 µg/mL. At various times samples were taken and quickly frozen. Total RNA was isolated, separated by electrophoresis and blotted as described above. Nylon membranes were incubated in En3Hance (NEN) and submitted to autoradiography.

RNA analysis

Exponentially growing cultures of yeast strains were shifted from galactose to glucose medium. At various times, samples were collected and quickly frozen in a dry ice-ethanol bath. Total RNA was isolated from yeast cells by a modified hot phenol method [48]. RNAs were separated by electrophoresis on 1.3% agarose gels, following denaturation with glyoxal [40] and transferred to Hybond nylon membranes (Amersham Biosciences). Membranes were probed with [32P]-labeled oligonucleotides complementary to specific regions of the 35S pre-rRNA (Table 3), or with [32P]-labeled DNA fragments corresponding to actin ORF, using the hybridization conditions described previously [9] and analyzed in a Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

Table 2. List of yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant features</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L40</td>
<td>MATa his3d200 trpl–901 leu2–331 ade2 lys2–801am</td>
<td>[41]</td>
</tr>
<tr>
<td>L40-41</td>
<td>L40, pBTM-NIP7, pACT-NOP8</td>
<td>[28]</td>
</tr>
<tr>
<td>L40-61</td>
<td>L40, pBTM-NIP7, pACT-RRP43</td>
<td>This study</td>
</tr>
<tr>
<td>YFG-131</td>
<td>L40, pBTM-NOP17</td>
<td>[42]</td>
</tr>
<tr>
<td>YFG-247</td>
<td>L40, pBTM-NOP17</td>
<td>[43]</td>
</tr>
<tr>
<td>YDG-146</td>
<td>L40, pBTM-NOP53</td>
<td>This study</td>
</tr>
<tr>
<td>YDG-147</td>
<td>L40, pBTM-NOP53, pGAD-NOP17</td>
<td>This study</td>
</tr>
<tr>
<td>YDG-148</td>
<td>L40, pBTM-NIP7, pGAD-NOP53</td>
<td>This study</td>
</tr>
<tr>
<td>NOP53</td>
<td>MATa/α, his3Δ1/α his3Δ1 leu2Δ1/leu2Δ1 lys2Δ2/lys2Δ2 LYS2 ura3Δ1/ura3Δ1 MET15/meti15Δ0 NOP53::KAN6</td>
<td>Euroscarf</td>
</tr>
<tr>
<td>Δnop53 2n</td>
<td>MATa/α, his3Δ1/α his3Δ1 leu2Δ1/leu2Δ1 lys2Δ2/lys2Δ2 LYS2 ura3Δ1/ura3Δ1 MET15/meti15Δ0 NOP53::KAN6</td>
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</tr>
<tr>
<td>Δnop53</td>
<td>MET15 his3Δ1 leu2Δ1 ura3Δ1 NOP53::KAN6</td>
<td>This study</td>
</tr>
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<td>YDG-149</td>
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<td>NOP53, YCp33GAL-A</td>
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</tr>
<tr>
<td>YDG-153</td>
<td>NOP53, YCp33GAL-A-NOP53</td>
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</tr>
</tbody>
</table>
Primer extension analysis

Total RNA extracted as described above was used for primer extension analysis. Reactions were performed by annealing 1 pmol of [32P]-labeled oligonucleotide to 5 μl of total RNA. Following annealing, extension was performed with 100 U of MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and dNTPs (0.5 mM) for 30 min at 37 °C. cDNA products were precipitated, resuspended in H2O, treated with RNase A, denatured and analyzed on 6% denaturing polyacrylamide gels. Gels were dried and analyzed in a Phosphorimager. Oligonucleotides used in primer extension analyses are listed in Table 3.

Coimmunoprecipitation of RNAs

Total cellular extracts were prepared from strains YDG152 and YDG153 expressing the ProtA or ProtA-Nop53p, respectively, and added to IgG-Sepharose beads (Amer- sham Biosciences) as described previously [49]. Immunoprecipitation was performed at 4 °C for 2 h. IgG-Sepharose beads were washed with buffer A (20 mM Tris/Cl pH 8.0, 0.5 mM magnesium acetate, 0.2% Triton X-100, 150 mM potassium acetate, 1 mM dithiothrietol and protease inhibitors) [49] and 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 6% polyacrylamide or 1.5% agarose gels and transferred to nylon membranes. For comparison, 1% of RNA recovered from total extract was loaded on gel. Hybridization was performed as described above, using probes specific to rRNAs and snoRNAs.

RNA binding assay

DNA fragment corresponding to 5.8S rRNA was cloned into pGEM-T (Promega, Madison, WI, USA) vector and in vitro transcription was performed with T7 RNA polymerase (Invitrogen), in the presence of 50 μCi of [32P]UTP[αP]. One picomole of radiolabeled RNA was incubated with different amounts of purified proteins in the same buffer as used for coimmunoprecipitation of RNAs [49] for 30 min at 37 °C. Cold competitor RNAs were generated by parallel in vitro transcription of pGEM-5.8S (generating 5.8S rRNA) or pBluescript (nonspecific RNA) in the presence of 10 mM NTPs. UV cross-linking was performed by placing RNA–protein complexes on ice and irradiation for 15 min at 260 nm using a Fotodyne transilluminator. They were then treated with 3 μl of RNase A for 30 min at 37 °C, resolved on a 6% denaturing polyacrylamide gel and visualized on a Phosphorimager.

Subcellular localization of Nop53p

The subcellular localization of Nop53p was analyzed by monitoring the fluorescence signal produced by a GFP fusion to the N-terminal of Nop53p. The subcellular localization of Nop1p was analyzed by monitoring the RFP, which was fused to the N-terminus of this protein. GFP, GFP–Nop53p and RFP–Nop1p proteins were expressed from plasmids pGFP-N-FUS, pGFP-N-NOP53 and pRFP-NOP1 (Table 1), respectively, transformed into the strain Δnop53 (Table 2). Δnop53 cells were cotransformed with vectors expressing GFP–Nop53p and RFP–Nop1p fusion proteins. Living cells were immobilized on 1-polylysine coated histological slides, in aqueous medium. The preparations were covered with cover slips, sealed and immediately observed by confocal microscope. Ar (488 nm) and HeNe (543 nm) lasers were used for image acquisition and the confocal software used for image analysis.

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Table 3. DNA oligonucleotides used for northern blot hybridization and primer extension analyses.

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
<td>5'-GGTCTCTCTGCTGCCGAAATG-3'</td>
<td>[9]</td>
</tr>
<tr>
<td>P2</td>
<td>5'-CATGGCTTAATCTTTTGAGAC-3'</td>
<td>[8]</td>
</tr>
<tr>
<td>P3</td>
<td>5'-GCTCTCATGCTCTTGCCAAAAC-3'</td>
<td>[9]</td>
</tr>
<tr>
<td>P4</td>
<td>5'-CGTACGCTTTTCGTCGTTTC-3'</td>
<td>[9]</td>
</tr>
<tr>
<td>P5</td>
<td>5'-CTCCTACTACCAAACAGAATGGTTGAGAAGG-3'</td>
<td>[13]</td>
</tr>
<tr>
<td>P6</td>
<td>5'-GGCTCGCTAGACGGTCTCTCC-3'</td>
<td>[9]</td>
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<tr>
<td>P7</td>
<td>5'-GCGCGTCTACCTGCGTACTAAAGGC-3'</td>
<td>[28]</td>
</tr>
<tr>
<td>anti-U3</td>
<td>5'-ATGGGGCTACTCAACAAAGTTGG-3'</td>
<td>[49]</td>
</tr>
<tr>
<td>anti-U14</td>
<td>5'-CTCAGACATCATGAAAGG-3'</td>
<td>[28]</td>
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<tr>
<td>anti-snR11</td>
<td>5'-GACGAATCGTACTCTG-3'</td>
<td>[20]</td>
</tr>
<tr>
<td>anti-snR37</td>
<td>5'-GATAGTATTAACCACCTAG-3'</td>
<td>[20]</td>
</tr>
</tbody>
</table>
sequencing; Celso R. Ramos for sequence alignment; and José R. Tavares and Mauricio B. Goldfeder for helping with yeast two-hybrid assays; Roberto Cabado for confocal microscopy assistance. DCG, JSL and FC were recipients of FAPESP fellowships, and FAG was recipient of a CNPq fellowship. This work was supported by FAPESP grant (03/06031-3 to CCO).

References

7 Nissan TA, Baßler J, Petfalski E, Tollervey D & Hurt E (2002) 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. EMBO J 21, 5539–5547.
17 Schimmang T, Tollervey D, Kern H, Frank R & Hurt EC (1989) A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. EMBO J 8, 4015–4124.
associates with a subset of small nucleolar RNPs required for peptidyl transferase center modification. Mol Cell Biol 24, 6324–6337.


