Identification of genes that function in the biogenesis and localization of small nucleolar RNAs in Saccharomyces cerevisiae.

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Identification of Genes That Function in the Biogenesis and Localization of Small Nucleolar RNAs in Saccharomyces cerevisiae


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Small nucleolar RNAs (snoRNAs) orchestrate the modification and cleavage of pre-rRNA and are essential for ribosome biogenesis. Recent data suggest that after nucleoplasmic synthesis, snoRNAs transiently localize to the Cajal body (in plant and animal cells) or the homologous nucleolar body (in budding yeast) for maturation and assembly into snoRNPs prior to accumulation in their primary functional site, the nucleolus. However, little is known about the trans-acting factors important for the intranuclear trafficking and nucleolar localization of snoRNAs. Here, we describe a large-scale genetic screen to identify proteins important for snoRNA transport in Saccharomyces cerevisiae. We performed fluorescence in situ hybridization analysis to visualize U3 snoRNA localization in a collection of temperature-sensitive yeast mutants. We have identified Nop4, Prp21, Tao3, Sec14, and Htl1 as proteins important for the proper localization of U3 snoRNA. Mutations in genes encoding these proteins lead to specific defects in the targeting or retention of the snoRNA to either the nucleolar body or the nucleolus. Additional characterization of the mutants revealed impairment in specific steps of U3 snoRNA processing, demonstrating that snoRNA maturation and trafficking are linked processes.

After synthesis in the nucleus, all RNA species undergo a series of maturation steps and transport from the site of synthesis to the site of action, and it is increasingly clear that RNA biogenesis and trafficking are closely linked (54). Whether they are destined to function in the cytoplasm (e.g., rRNA, tRNA, mRNA) or the nucleus (e.g., snRNAs or snoRNAs), all RNAs undergo intranuclear trafficking. It is well established that interactions between trans-acting factors and specific features of RNA substrates can govern which transport pathways will be utilized (38, 59, 73). However, little is known about the intranuclear trafficking factors and how they affect the molecular movement of RNAs within the nucleus.

Small nuclear RNAs (snoRNAs) provide excellent models to study intranuclear RNA transport. snoRNAs remain within the nucleus, where they undergo biogenesis including covalent alterations (e.g., 5’ cap hypermethylation and 3'-end processing), as well as assembly with specific proteins into stable and functional RNP complexes (snoRNPs) (40, 54, 70, 84). Ultimately, snoRNAs are targeted to nucleoli, where they function in rRNA maturation.

The numerous snoRNA species fall into two structurally and functionally distinct classes called the box C/D and box H/ACA snoRNAs (40, 54, 70, 84). Species of both RNA classes interact with pre-rRNA (via base pairing), mainly to guide site-specific nucleotide modification. The majority of box C/D snoRNAs orchestrate 2'-O-methylation (12, 41, 65, 89), while the bulk of the box H/ACA snoRNA species guide pseudouridylation (21, 64). A few RNAs of both classes (e.g., box C/D snoRNA U3 and box H/ACA snoRNA U17 [vertebrates]/snR30 [yeast]) are critical for promoting specific endonucleolytic cleavages of pre-rRNA transcripts required to generate mature 18S rRNA for the ribosome (33, 57).

Box C/D and box H/ACA snoRNAs stably associate with two distinct sets of four proteins. All box C/D snoRNAs bind to the 2'-O-methyltransferase fibrillarin/Nop1 (18, 78, 86, 88), as well as Nop56 (22, 71), Nop58 (47, 53), and 15.5-kDa protein/Snu13 (83, 94), while box H/ACA snoRNAs bind to the pseudouridine synthase dyskerin/Cbf5 (46, 92) and Gar1 (26), Nop10 (28), and Nhp2 (27, 28, 92). The association of core C/D or H/ACA RNP proteins is essential for the stability and/or proper nucleolar targeting of the snoRNAs (28, 46, 91–93).

Several studies have highlighted the role of intranuclear structures called Cajal bodies in snoRNP biogenesis (62, 77, 90). Cajal bodies are spherical structures present in vertebrate, invertebrate, and plant nuclei (13, 19, 20). In addition to containing several Cajal body-specific markers such as colin, survival of motor neuron (SMN) protein, small Cajal body RNAs, and U7 snRNA, Cajal bodies also house many nuclear RNA constituents, including snoRNAs, fibrillarin, and Nop140, and are often localized adjacent to and sometimes within the nucleolus (13, 19, 20). Box C/D snoRNAs injected into Xenopus...
Further analyses indicate that these and proper nucleolar transport of snoRNAs for the first time.

Two YCp50-based (URA3 CEN4 ARS1) yeast genomic libraries (52 his3-1) in Charles Cole’s laboratory (1). The gpr1-2 temperature-sensitive mutant was described previously (60, 98). The antisense deoxyoligonucleotide probes against yeast U3 snoRNA (5’ ATTTCAGGTCCTTTGGAAGTCAAAAAGTACACGCAGTCC TATAGAATGTA3’) and poly(A) mRNA [oligo(dT)30] were synthesized by Operon Technologies with a single Cy3 label at the 5′ end. The aminoaryl-modified snR30 probe (GGTT[AmpC6-dT]ACC AAAA ATG[AmpC6-dT]GCAT GGACCAC[AmpC6-dT]AGGGGCATTTGCA[AmpC6-dT]GTGTTATTGGA[AmpC6-mC6-dT]JAG[T][A] and the aminoaryl-modified U3 probe (TT[AmpC6-dT]GAGTCATGGTG[AmpC6-dT]CTTTTGAAGAG[AmpC6-dT]AAAGAGTGACCAGCA[AmpC6-dT]TCTTATAGA[AmpC6-dT]G) were synthesized by Operon Technologies and labeled with Oregon green 488-X reactive dye according to the manufacturer’s (Molecular Probes/Invitrogen) protocol. The Cy3-labeled U14 probes were fluorescein-labeled (green) and labeled with Oregon green 488-X reactive dye according to the manufacturer’s (Molecular Probes/Invitrogen) protocol. The Cy3-labeled U3 probe was labeled with Oregon green 488-X reactive dye according to the manufacturer’s (Molecular Probes/Invitrogen) protocol.

FISH and immunofluorescence. Fluorescence in situ hybridization (FISH) analysis and immunofluorescence analysis in yeast were performed as previously described (60). The anti-smoothened polyclonal antibodies to Smo5 antibodies (60) (Santa Cruz Biotechnology) were used at a 1:200 dilution. The rabbit anti-p53 1:200 dilution (BD Transduction Laboratories) was used. The mouse anti-FLAG (M2) 1:200 dilution (BD Transduction Laboratories) was used. The anti-FLAG (M2) 1:200 dilution (BD Transduction Laboratories) was used. The anti-phospho-p38 antibody (1:1000 dilution; Cell Signaling) was used. The anti-phosphotyrosine (1:1000 dilution; Cell Signaling) was used. The anti-p53 1:200 dilution (BD Transduction Laboratories) was used. The anti-p53 1:200 dilution (BD Transduction Laboratories) was used. The anti-FLAG (M2) 1:200 dilution (BD Transduction Laboratories) was used. The anti-FLAG (M2) 1:200 dilution (BD Transduction Laboratories) was used. The anti-phospho-p38 antibody (1:1000 dilution; Cell Signaling) was used. The anti-p53 1:200 dilution (BD Transduction Laboratories) was used.

MATERIALS AND METHODS
Yeast strains and plasmids. The yeast temperature-sensitive mutant collection was generated by UV mutagenesis from parental strains FY23 (MATa ara1-32 met15 leu2Δ21) and FY66 (MATa ara1-32 his3Δ200 leu2Δ21) in Charles Cole’s laboratory (1). The gpr1-2 temperature-sensitive mutant was described previously (60, 98). Two YCp50-based (URA3 CEN4 ARS1) yeast genomic libraries (45, 75) were used for complementing the temperature-sensitive mutants. The plasmid overexpressing an artificial box C/D snoRNA (pG14-U14/MS2X2, 2a) was obtained from Edouard Bertrand (91).

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FIG. 1. Aberrant patterns of U3 snoRNA localization detected in temperature-sensitive mutants at the restrictive temperature. Yeast cells were fixed and hybridized with Cy3-labeled anti-U3 probe (green). The nucleolar protein Nop1 was detected by immunofluorescence with an anti-Nop1 antibody (blue). DAPI was used to stain DNA and indicate the location of the nucleoplasm (red). Corresponding DIC (differential interference contrast) images are shown. (A) U3 snoRNA is normally detected in the nucleoli of \textit{S. cerevisiae} by FISH. (B) FISH analysis was performed on yeast temperature-sensitive mutants following a 4-h shift from 25°C to 37°C (37°C panels). Representative phenotypes are shown. The 25°C panels show the nucleolar localization of U3 in the yeast temperature-sensitive mutants at the permissive temperature (25°C). The illustrations on the left show the relationship of the patterns to the nucleolus (crescent) and nucleolar body (small circle) based on the subsequent analysis presented in this work. wt, wild type; NP, nucleoplasm.
TABLE 1. Genes identified from the U3 snoRNA mislocalization yeast mutants

<table>
<thead>
<tr>
<th>Gene name</th>
<th>U3 snoRNA mislocalization pattern</th>
<th>Proposed function(s) of gene product</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOP4</td>
<td>Ring</td>
<td>Nucleolar protein required for cleavage of 35S pre-rRNA (at A0, A1, and A2 sites) and for accumulation of 27S pre-rRNA</td>
<td>8, 81, 82</td>
</tr>
<tr>
<td>PRP21</td>
<td>Ring</td>
<td>Nuclear protein required for spliceosome assembly</td>
<td>2, 79, 95, 97</td>
</tr>
<tr>
<td>TAO3</td>
<td>Ring</td>
<td>Involved in cell morphogenesis</td>
<td>16</td>
</tr>
<tr>
<td>SEC14</td>
<td>Dot</td>
<td>Role in protein secretion from the Golgi body to the plasma membrane</td>
<td>6, 7, 87</td>
</tr>
<tr>
<td>HTL1</td>
<td>Dot</td>
<td>Nuclear protein component of RSC chromatin remodeling complex, which functions in transcription regulation and elongation</td>
<td>51, 74</td>
</tr>
</tbody>
</table>

Sequencing of the nop4, prp21, and sec14 mutant alleles. The relevant genes in the nop4, prp21, and sec14 mutants were amplified by PCR with oligonucleotides approximately 150 nucleotides upstream of the start codon and 50 nucleotides downstream of the stop codon. Genomic DNAs extracted from these mutant strains were used as templates for the PCRs. The genomic DNA extracted from wild-type parental strain FY23 was used as a control. Mutations were confirmed by at least two sequencing reactions with independent PCR products. The wild-type control PCR products were also sequenced and did not contain mutations found in the mutant genes.

Northern analysis. RNA was extracted as previously described (85), separated on denaturing 1.2% agarose-formaldehyde and acrylamide gels, transferred to nylon membranes, and analyzed by Northern blot hybridization. The oligonucleotide probes used were as follows. For U3 analysis (see Fig. 3), the probes used were probe A (CCAAGTTGGATTCAGTGGCTC), probe B (CAAAGCTGCTGCAATG), and a 55 RNA probe (GTTGTTAATCTGTTCA). For RNA analysis (see Fig. 4), we hybridized panels I and VI with GGCCAGCAATTTC (probe A), panel II with TTGTTACCTCTGGGCCC (probe C), panel III with CGGTTTTAATTGTCCTA (probe B), panel IV with CTCCGCTTATTAAGTTA (probe E), panel V with CGGTTTTAATTGTCCTA (probe D), panel VI with GGCCAGCAATTTC (probe A), panel VII with CGGTTTTAATTGTCCTA (probe B), and panel VIII with CTCCGCTTATTAAGTTA (probe E), panel IX with GGCCAGCAATTTC (probe A), panel X with CGGTTTTAATTGTCCTA (probe B), and panel XI with CTCCGCTTATTAAGTTA (probe E).

Electron microscopy (EM). Strains were fixed for 1 h at room temperature in 1.6% glutaraldehyde in 0.1 M Sorensen buffer (pH 6.5), processed by acetylation as previously described (91), and embedded in Epon. Ultrathin sections were mounted on nickel grids and stained with uranyl acetate and lead citrate. Samples were examined in a JEOL CX 100 II transmission electron microscope operated at 60 kV.

RESULTS

Screen for yeast temperature-sensitive mutants defective in U3 snoRNA localization. To identify genes involved in trafficking and assembly of snRNPs, we carried out a large-scale genetic screen of *S. cerevisiae* seeking to identify mutants with altered patterns of U3 snoRNA localization. The localization of U3 snoRNA was detected by FISH with a U3-specific probe (60). In wild-type yeast, U3 snoRNA is predominantly localized in the nucleolus, which, depending on cell orientation, appears as an oblong, curved, or crescent-shaped subnuclear structure adjacent to the nucleoplasm (marked by general DNA staining with DAPI (Fig. 1A). The nucleolar localization of U3 snoRNA was confirmed by its colocalization with a nucleolar marker protein, Nop1 (Fig. 1A).

We analyzed a random collection of ~1,100 temperature-sensitive yeast mutants (1) by FISH in search of phenotypes of U3 mislocalization. As we were primarily interested in fast-acting mutations (and in avoiding secondary effects), mutants were analyzed following a 4-h shift from the permissive temperature (25°C) to the nonpermissive temperature (37°C). Initially, we identified 43 mutants that reproducibly exhibited abnormal patterns of U3 localization specifically at the nonpermissive temperature. We observed four distinct phenotypic classes. Representative U3 mislocalization patterns are shown in Fig. 1B. The classes included mutants where U3 snoRNA localized within the nucleolus to (i) a ring-like structure (ring); (ii) a smaller, spherical structure (dot) which appears to be distinctly separated from the nucleoplasm; (iii) the nucleoplasm at levels higher than observed in the wild type (as well as the nucleolus) (NP); and (iv) double U3 foci within a single cell (suggestive of a cell division defect).

Further characterization was performed on nine mutants with either a ring- or a dot-like U3 localization pattern. Standard genetic analysis (see Materials and Methods) was carried out to confirm that each mutant phenotype was caused by a single recessive mutation that was linked to the U3 localization pattern. Seven of the nine mutants met these criteria and were selected for further analysis. To identify the mutated genes in each of the U3 mislocalization mutants, we transformed two different yeast genomic libraries (*URA3 CEN* (45, 75)) into the selected mutants. This approach led to the identification of the mutant gene in five of the seven mutants, including *NOP4*, *PRP21*, *TAO3*, *SEC14*, and *HTL1* (Table 1 contains a general description of each gene product). We found that the wild-type copy of the relevant genes complemented the U3 mislocalization phenotypes, as well as the temperature-sensitive growth phenotype, of the mutants (data not shown).

Identification of the mutations in the temperature-sensitive mutants. Mutations in the relevant genes in the temperature-sensitive mutants were identified by PCR amplification and sequencing. Our results showed that in the nop4 mutant, there is a mutation (T to C) at position 471368 on chromosome XI causing an amino acid change from leucine to serine at position 478 of the Nop4 protein. This particular leucine lies outside of any obvious identified functional domain but is conserved both within the RRM domain and within RBM28, a CRAL-TRIO domain and is also conserved in human Sec14-like proteins. Because of technical problems, we did not manage to amplify the htl1 or tao3 mutant allele.

U3 mislocalization phenotypes are not the result of global changes in RNA localization. In order to determine whether...
the mutated genes globally affect the transport of cellular RNAs, export of polyadenylated mRNA was analyzed in each of the U3 mislocalization mutants. FISH analysis with an oligo(dT) probe to label poly(A) RNA was performed on the temperature-sensitive mutants after a 4-h temperature shift from 25°C to 37°C (Fig. 2). The wild type and a conditional gsp1-2 mutant that is defective in mRNA export at 37°C (60, 98) were analyzed as controls. As expected, poly(A) mRNA localizes primarily to the cytoplasm in wild-type cells following a 4-h shift to 37°C. In the gsp1 mutant at the nonpermissive temperature (37°C), export is blocked and thus poly(A) mRNA is predominantly localized to the nucleus. For each of the mutants that we identified, poly(A) mRNA is predominantly localized in the cytoplasm, even following a 4-h shift to the nonpermissive temperature. We conclude that mRNA export is not affected in these mutants.

Defects in U3 processing are observed in some mutants. In order to determine if the accumulation and processing of U3 snoRNA are affected by the mutated genes, we performed Northern analysis with two different probes, one specific for a region of the mature U3 protein and one that recognizes only 3'5'-extended U3 precursors (Fig. 3). No significant change in the amount of the mature U3 was detected in any of the mutants (U3 band, probe A panel). Moreover, the processing of U3 is similar to that in the wild type in the nop4 and tao3 mutants (U3-3'5'-ext., probe B panel). In the prp21 mutant, the level of U3 precursor containing the intron (U3 intron) increased and the level of 3'5'-extended precursors (U3-3' ext.) decreased significantly. A significant decrease in the levels of the 3'5'-extended pre-U3 forms with no change in the precursor with the intron was observed for both the sec14 and htl1 mutants. Thus, some of the mutants display defects in U3 snoRNA maturation/processing, as well as localization.

Pre-rRNA processing is affected in the mutants. Given that U3 and other snoRNAs function in rRNA biogenesis, we tested whether the mutated genes resulted in defects in the processing of precursor rRNAs by performing Northern blot assays with RNAs isolated from the strains grown at the per-

FIG. 2. mRNA export is not affected in temperature-sensitive mutants. FISH analysis was performed on temperature-sensitive mutants maintained at the permissive temperature (25°C) or following a 4-h shift to the nonpermissive temperature (37°C). Poly(A) RNA was detected with a Cy3-labeled oligo(dT) probe (green). Poly(A) RNA accumulates within the nuclei of Ran cycle mutant cells (gsp1) but not in wild-type (wt) control cells or any of the mutants identified in the U3 screen. DAPI was used to stain DNA and indicate the location of the nucleoplasm (red).

FIG. 3. Levels and processing of U3 snoRNA in temperature-sensitive mutants. Northern analysis was performed on wild-type (wt) and mutant cells maintained at the permissive temperature (25°C) or following a 4-h shift to the nonpermissive temperature (37°C). The prp21 mutant showed a partial growth defect at the semipermissive temperature of 30°C and was further analyzed at this temperature (*, lane 6). Precursor forms of U3 containing an intron (U3 intron) or a 3'5'-extended species (U3-3' ext.), as well as mature U3 snoRNA (U3), were detected with oligonucleotides A and B, which are specific to different regions of U3, as shown in the schematic (see Materials and Methods for details). 5S rRNA was probed as an internal RNA loading control.
missive and nonpermissive temperatures (Fig. 4). Our probes detected the primary transcript (35S rRNA), processing intermediates (e.g., 32S, 20S, 27S, and 7S rRNAs), or mature rRNAs (18S, 5.8S, and 25S) (Fig. 4A; panel B contains a schematic of the rRNA processing pathway). Compared to wild-type cells, the level of the mature rRNAs (18S, 5.8S, and 25S) did not change significantly after the 4-h temperature shift. However, alterations in the levels of the primary transcripts and specific processing intermediates were observed in each of the mutants, indicating that the mutations do negatively impact pre-rRNA processing or stability. For example, in the nop4, prp21, and tao3 mutants, the relative levels of the 35S pre-rRNA increased while the levels of the processing intermediates, including 27S (pre-25S), 20S (pre-18S), and 7S (pre-5.8S), decreased, indicating that the early processing steps were inhibited. In the sec14 and htl1 mutants, the level of 35S pre-rRNA appeared not to change while amounts of the 27S, 20S, and 7S pre-rRNAs decreased considerably, suggesting a possible destabilization of the processing intermediates.

Localization of an H/ACA snoRNA is affected in dot mutants but not ring mutants. We screened for mutations that affected the localization of U3, a member of the box C/D family of snoRNAs. To address whether the mutants affect the localization of both major families of snoRNAs, we analyzed the localization of an H/ACA snoRNA, snR30 (5, 57). snR30 and U3 snoRNAs were detected simultaneously in the same yeast cells at the permissive and nonpermissive temperatures (Fig. 5). In wild-type cells, snR30 snoRNA colocalizes with U3 in
the nucleolus at both temperatures. However, in mutants where U3 localizes to a ring (nop4, prp21, and tao3), snR30 occupied the whole area of the nucleolus, indicating that the relevant gene products in these ring mutants are not common factors required for proper nucleolar localization of both C/D and H/ACA snoRNAs. The gene product affected in the tao3 mutant likely functions in a pathway common to the C/D RNAs, as we found that another C/D snoRNA, U14, was also observed in a ring in this mutant (data not shown). However, U14 exhibited normal nucleolar localization in both the nop4 and prp21 mutants (data not shown), indicating potential U3-specific effects of these two mutants (see Discussion). In the mutants where U3 is localized in a dot (sec14 and htl1), snR30 colocalized with U3, which might reflect defects common to both C/D and H/ACA snoRNAs. Consistent with this possibility, U14 also localized in a dot in the sec14 and htl1 mutants (data not shown).

**Localization of snoRNP-related nucleolar proteins.** In order to determine whether the mutants also mislocalize nucleolar pro-

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**FIG. 5.** Localization of a box H/ACA snoRNA (snR30) in temperature-sensitive mutants. FISH analysis was performed on wild-type (wt) and mutant strains at the permissive temperature (25°C) or after a 4-h shift to the nonpermissive temperature (37°C). U3 snoRNA and snR30 snoRNA were detected simultaneously with a Cy3-labeled U3-specific probe (green) and an Oregon green-labeled snR30-specific probe (blue).
teins, the subcellular distribution of both U3 and either Nop1 (Fig. 6) or Nsr1 (data not shown) was analyzed in the same set of cells by a combined FISH-immunofluorescence procedure. Nop1 is a core component of C/D snoRNPs (78, 86), and Nsr1 is a nucleolar protein implicated in the snoRNP assembly and transport pathway (52, 91). The three ring mutants showed distinctive phenotypes. For the nop4 mutant, both Nop1 and Nsr1 showed a normal nucleolar localization pattern. For the prp21 mutant, both the Nop1 and Nsr1 proteins were dispersed throughout the cells and did not show accumulation in any specific locus. For the tao3

FIG. 6. Localization of nucleolar protein Nop1 in temperature-sensitive mutants. FISH analysis was performed on the wild-type (wt) and mutant strains at the permissive temperature (25°C) or after a 4-h shift to the nonpermissive temperature (37°C). U3 snoRNA (green) was detected by FISH with a Cy3-labeled U3-specific probe (60). The subcellular localization of Nop1 (blue) was detected in the same cells by immunofluorescence with antibodies specific for Nop1. DAPI was used to stain DNA (red).
mutant, Nop1 was enriched in the area where U3 is absent (i.e., in the hole of the ring) while Nsr1 showed a normal nucleolar distribution. Finally, both the Nop1 and Nsr1 proteins colocalized with U3 in the dot-like structure of the sec14 and htl1 mutants. Thus, several distinct profiles were observed in the mutants with regard to effects on the localization of nucleolar proteins.

The structure of nucleoli is not significantly altered in the mutants. We naturally considered the possibility that the U3 localization (ring and dot) patterns related to an underlying alteration in nucleolar structure. While some of the data presented earlier (e.g., normal patterns of localization of snR30 [Fig. 5] or nucleolar proteins [Fig. 6]) indicated that this was not likely to be the case, we examined nucleolar structure in each of the mutants at the ultrastructural level by EM (Fig. 7). EM analysis revealed that all temperature-sensitive mutants maintained a largely normal nucleolar structure; no major changes were observed in any of the mutants. The prp21, sec14, and htl1 mutants did display a slightly more compact but otherwise normal nucleolus. Collectively, these results argue that the U3 ring and dot patterns are not likely to arise because of a grossly altered nucleolar structure.

Relationship between the U3 mislocalization phenotypes and the nucleolar body. As described earlier, the nucleolar body, a spherical structure housed within the nucleolus, appears to be involved in snoRNP biogenesis or trafficking (90, 91). The U3 patterns in the ring and dot mutants suggested to us that they may relate to the nucleolar body. To directly test this idea, we simultaneously examined the localization of nucleolar bodies (with an established nucleolar body marker [91]) and U3 snoRNA in each mutant (Fig. 8). The nucleolar body is found within the central hole of the ring-like U3 pattern in the nop4, prp21, and tao3 ring mutants (Fig. 8). In the sec14 and htl1 dot mutants, U3 colocalized with nucleolar body (Fig. 8). These results indicate that the ring- and dot-like U3 mislocalization phenotypes physically relate to the nucleolar body and suggest defects in U3 snoRNA transport into or retention within this structure.

DISCUSSION

We screened a large bank of temperature-sensitive yeast mutants for defects in the nucleolar localization of U3 snoRNA. From this screen, we identified five gene products—Nop4, Prp21, Tao3, Sec14, and Htl1—from mutants with two U3 mislocalization patterns—ring-like and dot-like—that are intimately related to the yeast nucleolar body. The gene products identified from the ring mutants (Nop4, Prp21, and Tao3) are apparently important for the accumulation of snoRNAs within the nucleolar body (e.g., reduced delivery or accelerated

FIG. 7. Temperature-sensitive mutants do not exhibit major alterations in nucleolar structure. Cells grown for 4 h at the nonpermissive temperature (37°C) were collected, processed by acetylation, and inspected by EM. Representative nuclei are presented. wt, wild type; F, fibrillar strands; G, granular areas. Scale bar is 0.2 μm.
release), while those from the dot mutants (Sec14 and Htl1) appear to be important for releasing snoRNAs from the nucleolar body to the nucleolus (Fig. 9). This is the first time that these gene products have been implicated in the snoRNA maturation pathway.

Spatial organization of the snoRNA maturation pathway. The pathway of U3 snoRNA maturation is temporally and spatially organized (43, 90, 93). In yeast, newly synthesized U3 transcripts contain a 5' monomethyl cap (m7G), an intron, and extended nucleotides at the 3' end. Conversion of pre-U3 to

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FIG. 8. Spatial relationship of the nucleolar body to U3 in temperature-sensitive mutants. Temperature-sensitive mutants were transformed with a plasmid overexpressing a box C/D snoRNA variant (pG14-U14/MS2X2) that is used as a marker for the nucleolar body (91). FISH analysis of endogenous U3 snoRNA (green) and the ectopically expressed variant snoRNA (red) was performed following a 4-h shift to the nonpermissive temperature (37°C). wt, wild type.
functionally mature U3 involves multiple posttranscriptional events that include (i) hypermethylation of the 5′ monomethyl cap (m^7G) structure to a trimethyl cap (m^3^7^2^G) by the enzyme trimethyl guanine synthase (Tgs1) (58); (ii) precise removal of the intron by the pre-mRNA splicesome; (iii) trimming of nucleotides at the 3′ end by the exosome (43); (iv) assembly with the core box C/D snoRNP proteins, as well as U3-specific binding proteins (93); and (v) intranuclear trafficking to nucleolar compartments where substrate (pre-rRNA) is found. Early U3 maturation steps such as intron removal and assembly with core snoRNP proteins appear to take place in the nucleoplasm (93). Indeed, a growing body of evidence indicates that, in general, the early steps of snoRNP biogenesis are located in the nucleoplasm (93). Nop4 contains four RRMs, each of which is essential for Nop4 function in ribosome biogenesis (81, 82), suggesting that Nop4 may directly bind RNAs such as snoRNAs or rRNAs. However, the precise RNA substrates for Nop4 have not been identified. No stable association between Nop4 and the U3, U14, snR190, and snR30 snoRNAs was identified by coimmunoprecipitation (8, 81), suggesting that if there is any association between Nop4 and snoRNAs, it is transient. Following UV cross-linking, snR190, a box C/D snoRNA (41, 68), was coimmunoprecipitated with the Nop4 antibody (81), consistent with transient snoRNA-binding activity. In our study, we found that site-directed mutagenesis of any of the four RRMs of Nop4 (81, 82) resulted in mislocalization of U3 snoRNA to the same ring-like pattern that was observed in the Nop4 mutant identified in our screen (unpublished data). Additional evidence that Nop4 may interact (at least transiently) with U3 and perhaps other snoRNAs comes from numerous genetic or physical associations that have been observed between Nop4 and snoRNAs. For example, a nop4 allele was identified in a screen for synthetic lethality with a nop1 allele, which suggests that Nop4 functionally interacts with Nop1, a core protein component of U3 and other box C/D snoRNPs (8). Moreover, proteomic studies using large-scale tandem affinity purification and mass spectrometry revealed that Nop4 associates with the C/D snoRNP core proteins Nop1 and Nop56, H/ACA snoRNPs core proteins Chd5 and Gar1, and U3-specific proteins Utp10 and Utp20 (23, 24, 42).

Potential roles of ring genes NOP4, PRP21, and TAO3 in snoRNA accumulation within the nucleolar body. The genes identified from the three ring mutants are NOP4, PRP21, and TAO3, which each encode an essential protein. Nop4 is a nucleolar protein critical for proper processing of pre-rRNA and production of functional ribosomes (8, 81). Depletion of Nop4 results in inhibition of cleavage at A_0, A_1, and A_2 sites of 35S pre-rRNA (8), which are each sites known to require U3 snoRNP activity (33). Nop4 depletion also leads to underaccumulation of 27SA and its processing products (8, 81). The effects of Nop4 depletion on pre-rRNA processing observed by others are quite similar to those that we observed with the temperature-sensitive allele of NOP4 (Fig. 4). The molecular mechanism of Nop4 function is not understood. Our results raise the possibility that Nop4 functions in U3 snoRNA trafficking to (or retention within) nucleolar bodies. Defects in the ability of U3 to accumulate in nucleolar bodies are predicted to impair U3 snoRNA biogenesis and to affect ribosome biogenesis, consistent with the previously observed phenotypes.

Like Nop4, Prp21 was identified in our screen as a protein with transient snoRNA-binding activity. In our study, we found that site-directed mutagenesis of any of the four RRMs of Prp21 (81, 82) resulted in mislocalization of U3 snoRNA to the same ring-like pattern that was observed in the Prp21 mutant identified in our screen (unpublished data). Additional evidence that Prp21 may interact (at least transiently) with U3 and perhaps other snoRNAs comes from numerous genetic or physical associations that have been observed between Prp21 and snoRNAs. For example, a prp21 allele was identified in a screen for synthetic lethality with a prp1 allele, which suggests that Prp21 functionally interacts with Prp1, a core protein component of U3 and other box C/D snoRNPs (8). Moreover, proteomic studies using large-scale tandem affinity purification and mass spectrometry revealed that Prp21 associates with the C/D snoRNP core proteins Prp1 and Prp56, H/ACA snoRNPs core proteins Chd5 and Gar1, and U3-specific proteins Utp10 and Utp20 (23, 24, 42).

Like Nop4, Prp21 was identified in our screen as a protein that, when mutated, gave rise to a U3 ring mislocalization phenotype. Prp21 is an essential nuclear protein involved in the assembly of the presplicesome and is required for intron splicing (2, 24, 79, 95, 97). Consistent with the known role of this protein in splicing, the intron-containing precursor forms of U3 snoRNA selectively accumulated in the prp21 mutant, revealing that intron removal was impaired (Fig. 3). Given that this defect in U3 processing is associated with U3 mislocaliza-
tion to a ring pattern, this finding indicates that the splicing of the U3 intron is important for localization of the RNA in the nucleolar body. Precursors of U3 RNA in wild-type cells are normally not observed in nucleolar bodies, and there is evidence that intron removal of U3 may normally occur at the site of transcription (43, 90). In addition, our finding that the snoRNP-related proteins Nop1 and Nsr1 also become dramatically mislocalized in prp21 mutant cells (Fig. 6A and B) suggests a likelihood of defects in snoRNP assembly as well. Other recent data also indicate that splicing and snoRNP assembly are coupled processes (31, 32, 72).

The third protein in this ring phenotypic class, Tao3, is an essential and conserved protein with unknown molecular functions. The only previous study of Tao3 showed that a temperature-sensitive tao3 mutant displays defects in cell morphogenesis (16). GFP-tagged Tao3 localized in the cytoplasm, as well as the polarized cell periphery (16). On the other hand, proteomic studies have revealed interactions of Tao3 with nuclear proteins and components with potential connections to snoRNPs. For example, Tao3 was identified in complex with Sbp1 (23), a nucleolar, single-strand nucleic acid binding protein that associates with snoRNAs (14). Tao3 was also found to associate with Yra1 (36), a member of the RNA and export factor binding protein family. RNA and export factor binding proteins are nuclear proteins that interact with RNA, and at least some family members are required for mRNA export from the nucleus (69, 80, 102). It is possible that Yra1, together with Tao3, interacts with snoRNAs and plays a role in their intranuclear trafficking. Moreover, Tao3 was identified in complex with Sup45 (24), which (as determined by yeast two-hybrid analysis) physically associates with Nop4 (34) (recall that mutant Nop4 also leads to U3 ring patterns). Our study showed that U3 processing is not affected in the tao3 or nop4 mutant (Fig. 3).

Potential roles of dot genes SEC14 and HTL1 in the release of snoRNAs from the nucleolar body. The two genes identified from the dot mutants are SEC14 and HTL1. U3 processing was similarly affected in both sec14 and htl1 mutants (Fig. 3). Specifically, the 3′-extended U3 precursors were greatly reduced in both mutants after the temperature shift to 37°C without any detectable accumulation of the intron-containing precursor form of U3. The 3′-extended forms of pre-U3 are stabilized by binding of the Lhp1 (La homolog) protein (43), and the Lsm2-8 (like-Sm) complex acts as a chaperone with Lhp1 to stabilize pre-U3 species (44). The stabilization of the 3′-extended precursors is required for the final maturation of U3 (43, 44). It is possible that Sec14 and Htl1 play roles in the stabilization of U3 precursors by functional interaction with Lhp1 and/or the Lsm2-8 complex. Sec14 is essential for viability and was identified as a cytosolic phospholipid transfer protein required for protein secretion from the Golgi body to the plasma membrane (7, 87). However, continuous protein secretion is essential for ribosome synthesis (55), indicating indirect links of Sec14 to possible functions within the nucleus. Htl1 is not an essential protein but is important for yeast growth at 37°C (51). Htl1 is a nuclear protein (74) and is a component of the RSC chromatin remodeling complex, which functions in transcription regulation and elongation from the RNA polymerase II promoter (11, 74). Previous studies indicate that snoRNP assembly initiates at the transcription site, most likely during transcription elongation (4, 72, 99). It is possible that defects in transcription elongation in the htl1 mutant lead to defects in snoRNA 3′-end processing and/or RNP assembly (and ultimately proper nucleolar targeting).

Summary. The findings presented here have increased our understanding of the key factors in snoRNA maturation and transport. We have identified factors required for the accumulation of a snoRNA in the nucleolar body (Nop4, Prp21, and Tao3) and for the trafficking of a snoRNA from the nucleolar body to the nucleolus (Sec14 and Htl1). Some of these proteins affect the localization of members of both major families of snoRNAs (e.g., Sec14 and Htl1 affect the localization of U3, U14, and snR30), and others may be specific for the C/D snoRNAs (e.g., Tao3 affects the localization of U3 and U14) or just U3 snoRNA (e.g., Nop4 and Prp21). A plausible explanation for the specific link between U3 RNA and Prp21 is the unique presence of an intron in this snoRNA. Our results refine the potential function of Nop4 to a role in snoRNA trafficking to or within the nucleolar body. This role would explain the pre-RNA processing phenotypes previously observed with alterations in this protein (8, 81). The observation that Prp21, Sec14, and Htl1 are required for both snoRNA processing and nucleolar localization indicates a strong link between the maturation and transport pathways. Specifically, based on the prp21 phenotype, accumulation of the snoRNA in the nucleolar body appears to depend on intron removal (which is thought to take place in the nucleoplasm [90]). The phenotypes produced by sec14 and htl1 suggest that exiting the nucleolar body is linked to later snoRNA maturation steps. More detailed molecular characterization of the identified proteins will now allow further delineation of the mechanisms by which these proteins influence the intranuclear trafficking of snoRNAs.

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