

Analysis of Pre-Ribosomal Processing and Assembly Factors in Yeast

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When I was looking for labs to do my PhD in, a former colleague of mine, Henrik Daub, suggested David Tollervey's lab in Edinburgh to me since he knew I was interested in everything RNA. When I came for an interview to David's lab, I definitely liked the lab, however, being mad about London, I was not so sure of Edinburgh. Now, three years on, I certainly got surer of Edinburgh, although London is still my love, and I know I could not have chosen a better lab or supervisor than David Tollervey for my PhD.

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Abstract

The synthesis of ribosomes is a major metabolic pathway in all cells. In eukaryotes, a polycistronic pre-ribosomal RNA transcript is processed to the mature 18S, 5.8S and 25S/28S rRNAs, whilst undergoing extensive covalent nucleotide modifications and assembling with the ~80 ribosomal proteins. More than 140 known factors are required for post-transcriptional steps in ribosome synthesis in yeast and, although the processing of ribosomal RNA precursors is fairly well understood, the relationship between ribosomal processing, ribosome assembly, subunit export and subnucleolar structures is still largely unclear. To gain insights into these processes, I analysed three previously uncharacterised ribosomal factors. To study their involvement in the ribosomal RNA processing and assembly, conditional alleles were generated of the essential genes *NOP7*, *RRP12* and *NOP15*, as well as epitope-tagged versions of their protein products.

The nucleolar protein Nop7p has multiple roles in the synthesis of the 60S ribosomal subunit and is required for efficient 5' to 3' exonuclease digestion that generates the 5' end of the major, short form of the 5.8S rRNA, assembly and export of the 60S ribosomal subunit. Nop15p is also needed for normal 5' to 3' exonuclease digestion and, additionally, for processing of residual 27SB to 7S pre-rRNA. Nop15p contains an RNA recognition domain and is therefore likely to associate directly with the pre-rRNA to facilitate its processing. Rrp12p is the only *trans*-acting factor known to be required during late maturation of both ribosomal subunits. The protein appears to fulfil very different roles in the different subunits, as assembly factor and facilitator of exosome activity in the large subunit, and during export of the small subunit. Rrp12p is predicted to be composed of HEAT-repeats and is therefore structurally related to importin β -like family of export and import receptors

Several ribosomal processing factors, including Nop7 and Nop15p, seem to be multifunctional, with roles in different cellular processes suggesting that major metabolic processes within the cell may be linked.

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Abbreviations

Amp	ampicillin
ARM	<i>armadillo</i>
ATP	adenosine triphosphate
BRCT	breast cancer carboxy-terminal domain
bp	base pair
BSA	bovine serum albumin
Ci	Curie
cm	centimetre
d	deoxy-
DEPC	diethylpyrocarbonate
DFC	dense fibrillar component
DNA	deoxyribonucleic acid
dNTP	desoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra acetic acid
EtOH	ethanol
ETS	external transcribed spacer
FC	fibrillar centre
FISH	fluorescent <i>in situ</i> hybridisation
g	gram
GAL	galactose
GC	granular component
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate
h	hour
³ H	tritium, isotope 3
HEPES	4-(2-hydroxyethyl)-1-piperazineethansulfonic acid
HIS	histidine
H ₂ O	water, double distilled, sterile
ITS	internal transcribed spacer
IPTG	isopropyl-D-thiogalactopyranoside
kb	kilobase pair
kDa	kilo Dalton
l	litre
LB	luria broth
M	molar
μ	micro (10 ⁻⁶)
m	milli (10 ⁻³)
min	minute
mRNA	messenger ribonucleic acid

MRP	mitochondrial RNA processing
n	nano (10^9)
NE	nuclear envelope
NPC	nuclear pore complex
nt	nucleotide
nup	nucleoporin
OD	optical density
o/n	overnight
ORF	open reading frame
p	pico (10^{-12})
³² P	phosphorus, isotope 32
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonylfluoride
RNA	ribonucleic acid
RRM	RNA recognition motif
rRNA	ribosomal RNA
rpm	rotations per minute
r-protein	ribosomal protein
RT	room temperature
³⁵ S	sulphur, isotope 35
SD	synthetic dropout
SDS	sodium dodecyl sulphate
sec	second
snoRNA	small nucleolar RNA
snoRNP	small nucleolar ribonucleoprotein
TAP	tandem affinity purification
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)
tRNA	transfer RNA
TRP	tryptophan
ts	temperature sensitive
TTP	thymidine triphosphate
U	unit
URA	uracil
UTP	uracil triphosphate
UV	ultraviolet
wt	wild-type
VRC	vanadyl ribonucleoside complex

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Chapter One

Introduction

1.1. The nucleolus and ribosome biogenesis

In all eukaryotic cells ribosomal RNAs are matured in a special nuclear compartment, the nucleolus. The nucleolus is often viewed as an accumulation of actively transcribed rRNA genes, which is surrounded by transcription products in form of pre-ribosomal particles at various stages during their maturation. However, it was shown that the nucleolus is a structure in dynamic equilibrium, consisting of three subregions, fibrillar centers (FCs), a dense fibrillar component (DFC) and a granular component (GC) (Leger-Silvestre et al., 1999; Scheer and Hock, 1999; Phair and Misteli, 2000). The relationship between the observed subnucleolar structures and the different steps of ribosome biogenesis is not well established. The FCs contain RNA polymerase I and transcription factors making it likely that transcription of rDNA occurs in the FCs or at the interface between FCs and the DFC (Schwarzacher and Wachtler, 1993; Cheutin et al., 2002). In the DFC, the pre-rRNA may assemble with the pre-rRNA processing and modification machinery, followed by rRNA modification and early processing steps, with late processing steps and ribosome assembly occurring in the GC (Leger-Silvestre et al., 1999). Several components required for early processing and modification machinery, including Nop1p/fibrillarin and the U3 snoRNP, have been found within the DFC but not the GC, whereas other non-ribosomal proteins that are specifically associated with the pre-60S particles, like Rlp7p, have been found only in the GC (Gadal et al., 2002). Final maturation of the subunits is believed to occur after their release from the nucleolus and export to the cytoplasm via the nucleus and the nuclear pores.

1.2. Pre-ribosomal RNA processing in Yeast

Processing of ribosomal RNA is a major metabolic activity of every eukaryotic cell. Due to the availability of molecular genetic techniques, this process is best understood in yeast (Venema and Tollervey, 1999). In *Saccharomyces cerevisiae* 100 to 200 copies of

the rDNA genes are tandemly arranged on the right arm of chromosome XII. The structure of a single rDNA unit, which is 9.1kb in length, is shown in Figure 1A. Three out of the four mature rRNAs, 18S, 5.8S and 25S, are transcribed by RNA polymerase I (Pol I) as a single large precursor, the 35S pre-rRNA, which contains the mature sequences separated by two internal transcribed spacers, ITS1 and ITS2, and flanked by two external transcribed spacers, the 5' ETS and 3' ETS (Figure 1). The remaining part of the rDNA repeat is formed by two non-transcribed spacers, NTS1 and NTS2, which are separated by the 5S rRNA gene. This is transcribed independently in the opposite direction by RNA polymerase III (Pol III) (Figure 1). The organisation of eukaryotic rRNA operons is conserved in evolution and only the presence of the 5S rRNA gene within the rDNA repeats is an unusual feature of *S.cerevisiae*.

Due to the high copy number of the rDNA units, *cis*-acting elements, which are specific primary and secondary structural features with regulatory function, could not initially be analysed genetically within the 35S pre-rRNA. One of the first approaches to study the role of the conserved and variable regions of eukaryotic rRNA was based on the introduction of a complete, plasmid-encoded rDNA-unit into yeast cells. Prior to introduction, these rDNA-units were subjected to *in vitro* mutagenesis and, to distinguish (pre-) ribosomes derived from the mutated rDNA from endogenous ones, they were furthermore tagged within mature rRNA sequences using short oligonucleotides that did not perturb biosynthesis and function of the ribosome (Musters et al., 1989). Since the cells were not dependent on the mutated rRNA, recessive-lethal mutations and their effect on pre-rRNA processing, assembly and function could be studied *in vivo*. A later approach used strains that carried a temperature sensitive mutation within the largest subunit of Pol I and in which rRNA was transcribed from a plasmid under an inducible Pol II promoter at non-permissive temperature (Nogi et al., 1991a; Henry et al., 1994; Venema et al., 1995a; Allmang et al., 1996a). In these cells the mutant rRNA forms were the only source of cellular rRNA, thus the effect of rRNA mutations in respect to processing, assembly and cell viability could be studied, irrespectively of the severity of the defect. Identification and analysis of *trans*-acting factors involved in pre-rRNA processing was particularly successful in yeast due to the

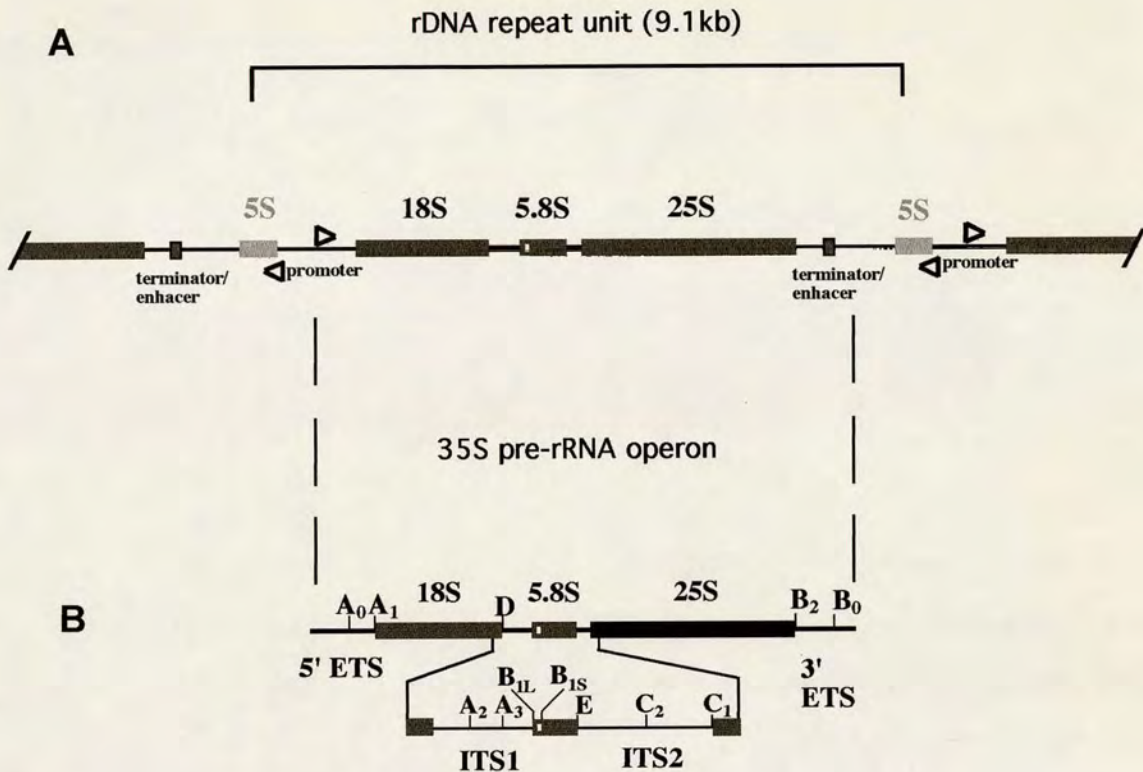


Figure 1: Structure of a *S.cerevisiae* rDNA unit and 35S pre-rRNA operon .

(A) The structure of a single rDNA unit, which is 9.1kb in length. This consists of three mature rRNA sequences transcribed as a single operon and two non transcribed spacers separated by the 5S rRNA gene, which is transcribed in the opposite direction.

(B) Structure of pre-rRNA and location of processing sites. In the pre-rRNA the regions encoding the 18S, 5.8S and 25S rRNAs are flanked by the 5' and 3' external transcribed spacers, 5'ETS and 3'ETS, and separated by internal transcribed sequences 1 and 2, ITS1 and ITS2.

in vivo depletion of selected proteins under the control of an inducible promoter, which was introduced into the chromosome immediately upstream of the transcription initiation site by homologous recombination (Scherer and Davis, 1979; Orr-Weaver et al., 1981; Fieschko et al., 1987).

1.2.1. *Pre-rRNA modification and early processing steps*

The longest detectable pre-rRNA transcript in yeast, the 35S pre-rRNA, extends from the transcription initiation site to a position a few nucleotides beyond the 3' end of the mature 25S rRNA (Figure 2). The actual transcription termination site is predicted to be around 200 nucleotides further downstream but the intermediates are not detectable in wild-type cells due to rapid processing events in the 3' ETS carried out by the endoribonuclease Rnt1p (Kempers-Veenstra, 1986; Kufel et al., 1999). The 3' ETS cleavage sites lie on both sides of a predicted stem-loop structure and Rnt1p cleaves structurally related stems flanking several small RNA species showing that Rnt1p directly recognises and cleaves this sequence/structure motif in the nascent pre-rRNA transcript (Chanfreau et al., 1998). In strains lacking Rnt1p or carrying mutations in the stem-loop structure in the 3'ETS, this cleavage does not occur and 3'-extended 35S species can be detected (Allmang and Tollervey, 1998; Kufel et al., 1999).

The major pre-rRNA processing and modification activities do not commence until after cleavage in the 3' ETS is completed. It was therefore assumed that signals required for the initiation of pre-rRNA processing might be located in the 3'ETS. However, an extensive search did not reveal such elements but showed that deletion of the Rnt1p cleavage site, or indeed the entire 3'ETS, did not have any effect on the initiation of upstream pre-rRNA processing (Allmang and Tollervey, 1998). Prior to the continuation of pre-rRNA processing, the 35S RNA precursor undergoes a large number of covalent modifications, mostly 2'-O-ribose methylation and pseudouridilation (ψ), which predominantly take place within the universally conserved core secondary structure of the rRNA (Klootwijk et al., 1972; Brand et al., 1977; Maden, 1990; Ofengand and Fournier, 1998).

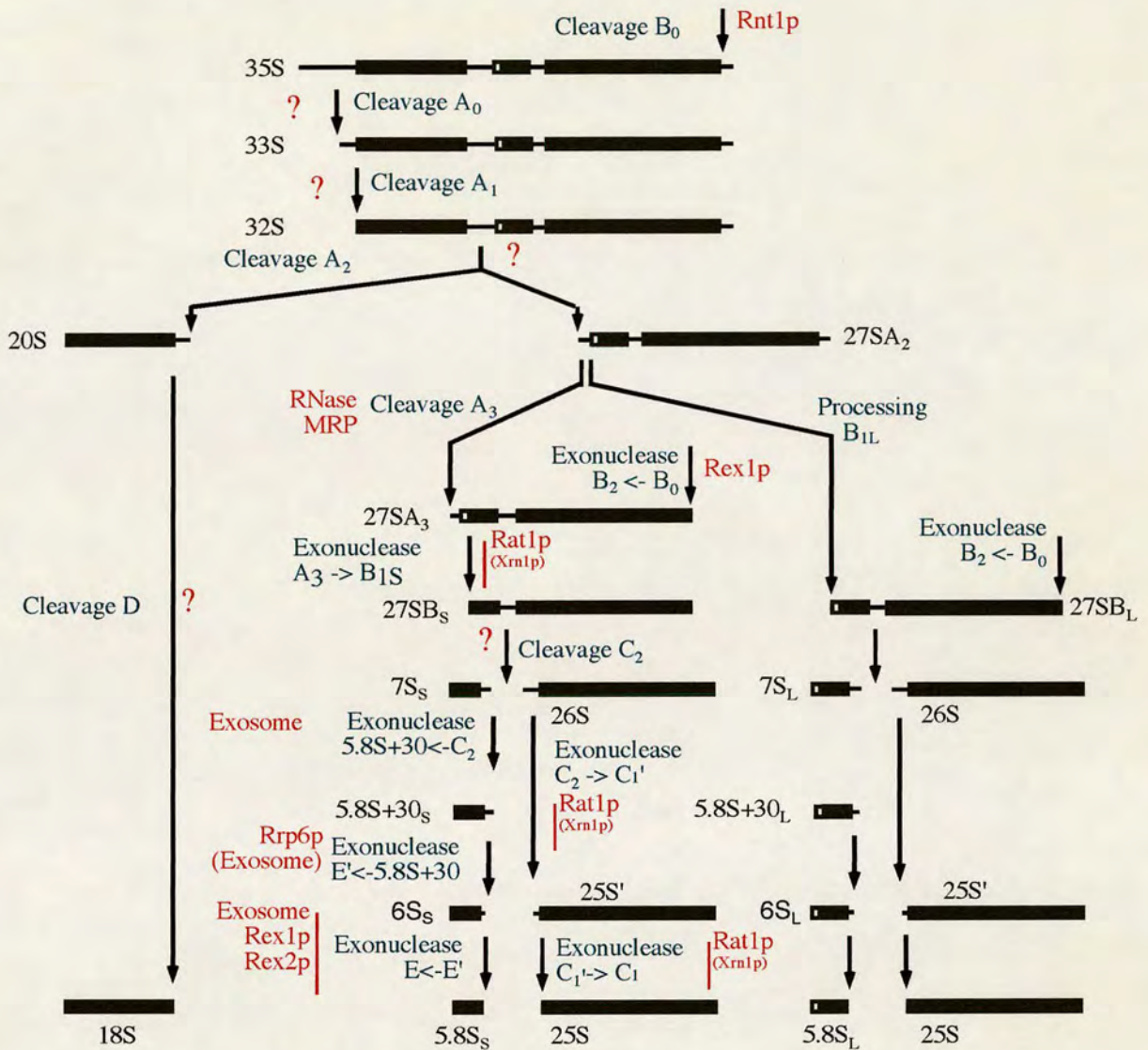


Figure 2: The pre-rRNA processing pathway in *Saccharomyces cerevisiae*. Known processing enzymes are indicated in red. Unidentified enzymes are indicated as question marks. Processing events are indicated in blue.

One specific class of *trans*-acting factors has been implicated in these modifications, the small nucleolar RNAs (snoRNAs). The snoRNAs base pair to specific sites in the pre-rRNA and so guide the rRNA-modifying enzymes to the sites of modification (Ganot et al., 1997; Kiss-László et al., 1998). These RNAs can be divided into two classes, designated box C/D and box H/ACA, based on the presence of conserved sequence and structural elements, and are associated with specific sets of proteins forming the snoRNPs. All box C/D snoRNPs share the proteins Nop1p, the yeast homologue of the vertebrate fibrillarin, Nop56p, Nop5/58p and Snu13p, whereas the box H/ACA snoRNPs share Cbf5p, Gar1p, Nhp2p and Nop10p (Balakin et al., 1996; Ganot et al., 1997; Henras et al., 1998; Lafontaine et al., 1998b; Watkins et al., 1998; Watkins et al., 2000). Due to its homology to known methyltransferases, Nop1p is believed to be the 2'-O-methyltransferase that functions within box C/D snoRNPs (Niewmierzycka and Clarke, 1999). Moreover, in cells depleted of Nop1p or carrying the temperature sensitive (ts) lethal allele *nop1-3*, 2'-O-methylation is inhibited (Tollervey et al., 1991; Tollervey et al., 1993). Based on sequence homology, the box H/ACA snoRNP protein Cbf5p is strongly predicted to be the rRNA pseudouridine synthase (Lafontaine et al., 1998b), however, the other box H/ACA components are likely to be needed for snoRNP stability and genetic depletion of any H/ACA snoRNP protein component prevents global pseudouridine formation (Henras et al., 1998; Lafontaine et al., 1998b). The box C/D snoRNAs U3 and U14 as well as the H/ACA box snoRNAs snR30 and snR10 are also required for pre-rRNA processing where they are assumed to establish or maintain the correct structure of the RNA by base-pairing to the pre-rRNA (Tollervey, 1987; Morrissey and Tollervey, 1993; Liang and Fournier, 1995; Sharma and Tollervey, 1999).

After modification, the 35S pre-rRNA is rapidly processed. The earliest cleavage takes place at site A₀ in the 5'ETS, 90 nucleotides upstream of the 5' end of mature 18S rRNA (Figure 1B). Cleavage at A₀ yields the 33S precursor, which is then cleaved at the 5'-end of mature 18S rRNA, site A₁, producing the 32S pre-rRNA. Cleavage at site A₂ in ITS1 splits the 32S pre-rRNA into 20S and 27SA₂ pre-rRNAs, destined to form the mature rRNA species of the small and large ribosomal subunit,

respectively. So far no enzyme has been identified that is responsible for the endonucleolytic cleavage of A₀, A₁ or A₂. The U3 snoRNP is strictly required for A₀ cleavage, and conditional mutations in the U3 snoRNP revealed that this cleavage requires base-pairing between the U3 snoRNA and a site within the 5' ETS (Hughes and Ares, 1991; Beltrame et al., 1994). Processing at site A₀ is not, however, essential for cell viability (Venema et al., 1995b). U3 snoRNP is also involved in processing at sites A₁ and A₂ and this requires its base-pairing to a sequence within the mature 18S rRNA. Truncated versions of the U3 snoRNP components Dhr1p and Mpp10p were also shown to specifically inhibit cleavage at those sites (Lee and Baserga, 1997; Colley et al., 2000).

The position of cleavage at site A₁ was shown to be determined by two partially separate *cis*-acting signals. One mechanism involves recognition of conserved nucleotides immediately adjacent to A₁ in the 5'ETS. The second mechanism, places the cleavage site at an exact distance from a conserved stem-loop structure within the 5'-end of the mature 18S rRNA (Sharma et al., 1999). An apparently similar spacing mechanism also functions at site A₂ in ITS1, where both recognition of a sequence at the site of cleavage and a stem-loop structure 3' to the cleavage site are used to position the site of cleavage (Allmang et al., 1996b). Processing at A₁ and A₂ are both endonucleolytic events, since spacer fragments released by these steps can be detected in wild-type cells. As both cleavages appear to be positioned by similar mechanisms, it was speculated that processing at those sites might be carried out by the same endoribonuclease but such an enzyme has not yet been identified. However, it was shown that cleavage at the two sites is coupled (Venema and Tollervey, 1999). No mutation has been identified that allows processing at site A₂ without prior cleavage at A₁. Mutations in the pre-rRNA that inhibit A₁ cleavage cause accumulation of aberrant 22S rRNA, which extends from A₀ to A₃, indicating that processing is inhibited at site A₂ but not at A₀ or A₃ (Venema et al., 1995b). Furthermore, strains depleted of many different *trans*-acting factors involved in synthesis of 18S rRNA, accumulate precursors that have been cleaved directly at A₃ without prior cleavages at A₀, A₁ or A₂ (Venema and Tollervey, 1999). The resulting 3' product is the normal 27SA₃ pre-rRNA that is

processed to mature 5.8S and 25S rRNA. In many, but not all strains, the aberrant 5' intermediate, 23S rRNA, is apparently not a substrate for the processing machinery, and is degraded by a complex of 3'->5' exonucleases, known as the exosome (Allmang et al., 2000).

1.2.2. Synthesis of 18S rRNA

As previously mentioned, four snoRNAs, U3, U14, snR10 and snR30, were shown to be required for synthesis of 18S rRNA. U3 and U14 are necessary for cleavage reactions and function through direct base pairing with the pre-rRNA. U3 snoRNA base pairs to regions within the 5'ETS and a 5'terminal region of the mature 18S rRNA sequence. Sequence mutations to prevent interaction with the 5'ETS lead to loss of 18S rRNA and inhibition of cleavages at sites A₀, A₁ and A₂, mimicking the effect of U3 snoRNA depletion (Beltrame and Tollervey, 1995). In contrast, prevention of base pairing of U3 snoRNA to the 18S rRNA region inhibits cleavage at sites A₁ and A₂ but not A₀ showing that each interaction promotes distinct steps during early processing (Sharma and Tollervey, 1999). U14 snoRNA also base pairs to sequences within the 18S rRNA and compensating mutations demonstrated this interaction to be necessary for ribosome synthesis (Liang and Fournier, 1995). SnR10 and snR30 are the only box H/ACA snoRNAs for which a function in pre-rRNA processing has been demonstrated, but how these snoRNAs interact with the rRNA precursor is still unknown.

Since these four snoRNAs are associated with sets of snoRNP proteins according to their families, box C/D or H/ACA respectively, depletion of any of those proteins inhibits 18S synthesis. However, the essential function of these proteins seems to be mediated by their association with the snoRNPs (Henras et al., 1998; Venema and Tollervey, 1999; Dragon et al., 2002). Compared to the box C/D and H/ACA snoRNPs, the U3 snoRNP is distinctly different. It not only differs in structure from other box C/D snoRNAs but is also associated with additional factors, beside the common box C/D core proteins. Recently it was reported that the U3 snoRNP contains around 28 proteins, all of which are required for 18S rRNA synthesis, forming a large RNP complex. This

will be discussed in further detail in section 1.3.3. (Dragon et al., 2002). However, a subsequent report suggested this complex actually represents the 90S pre-ribosome (Grandi et al., 2002). Another group of *trans*-acting factors that are required for synthesis of mature 18S rRNA are the putative ATP-dependent RNA helicases. These proteins share several conserved motifs and in a few cases have been shown to possess ATP-dependent translocation and the ability to dissociate double-strand nucleic acids. These activities are assumed to be involved in establishment or dissociation of snoRNA:pre-rRNA interactions, since some of these involve over twenty consecutive base pairs. Depletion of several different helicases resulted in phenotypes similar to those observed on snoRNA depletion (Venema and Tollervey, 1999).

Yeast cells are unusual amongst eukaryotes concerning the last step of 18S rRNA maturation. After cleavage at A₂, the 20S pre-rRNA is exported from the nucleolus as part of a pre-40S ribosomal particle and subsequently cleaved in the cytoplasm at site D, the 3' end of mature 18S rRNA and the 5' end of ITS1 (Udem and Warner, 1973; Trapman and Planta, 1976; Stevens et al., 1991). Prior to this cleavage further modifications are carried out on the cytoplasmic 20S pre-rRNA, during which the methyltransferase Dim1p, dimethylates two adjacent adenosine residues at positions 1779 and 1780 in the loop closing the 3' terminal stem of the 18S rRNA (Klootwijk et al., 1972; Udem and Warner, 1973; Brand et al., 1977). Detection of a D to A₂ fragment indicates that this cleavage is endonucleolytic but the enzyme responsible has not yet been identified. Processing at site D appears to require sequences located immediately downstream of the cleavage site (van Beekvelt et al., 2001). Site-specific mutations within this element entirely abolish processing at site D indicating a sequence specific recognition mechanism for the endonuclease (van Beekvelt et al., 2001).

1.2.3. Synthesis of 5.8S and 25S rRNAs

The pathway of synthesis of 5.8S and 25S rRNA is more complex than that of 18S rRNA synthesis, requiring both endonuclease cleavage and exonuclease digestion, and several enzymes involved in this pathway have been identified.

The synthesis of large subunit rRNA is initiated by cleavage at site A₃, carried out by a ribonucleoprotein complex, RNase MRP, which consists of one RNA molecule and nine protein components (Chamberlain et al., 1998). Mutations in the RNA or any of the protein components of the complex except Pop8p, inhibit cleavage at site A₃ (Schmitt and Clayton, 1993; Chu et al., 1994; Lygerou et al., 1994; Chu et al., 1997; Dichtl and Tollervey, 1997). The cleavage has also been reproduced *in vitro* using naked RNA and the purified enzyme, suggesting that RNase MRP does not require cofactors to recognise the site of cleavage (Lygerou et al., 1996). Interesting, however, is the fact that RNase MRP is essential for viability although cleavage at site A₃ is not required for rRNA synthesis (Henry et al., 1994). Rrp5p, a non-ribosomal protein, is also required for pre-rRNA processing at site A₃, as well as sites A₀, A₁, A₂, and is believed to mediate interaction between RNase MRP and the rRNA precursor during these cleavages (Tollervey, 1996; Venema and Tollervey, 1996; Torchet et al., 1998; Eppens et al., 1999).

Following cleavage at site A₃, the 5' end of the major form of the 5.8S rRNA is generated by 5'→3' exonucleolytic digestion carried out by the exonuclease Rat1p and Xrn1p. Strong accumulation of 5'-extended forms of 5.8S rRNA can be observed in double mutant strains, with lower accumulation in each single mutant, showing that both enzymes participate in processing (Henry et al., 1994). One complication, however, seemed to be the fact that Xrn1p is a cytoplasmic protein while its substrate, the 27SA₃ pre-rRNA is nucleolar. An explanation could be that processing at this step is normally carried out by Rat1p, with Xrn1p playing a major role in processing the 5'-end of 5.8S rRNA only in its absence (Johnson, 1997). A small fraction of 5'-unprocessed 5.8S rRNA may normally escape to the cytoplasm and be processed there by Xrn1p.

In the absence of A₃ cleavage an alternative and normally minor ITS1 processing pathway, through processing at site B_{1L} continues to synthesize the 5.8S_L and 25S rRNAs (Figure 2) and can support growth. It can therefore be assumed that RNase MRP, which is essential, has other substrates in the cell in addition to the pre-rRNA. Nothing is known about the 5'-end formation of 5.8S_L rRNA, since no *trans*-acting

factors for B_{IL} processing have been identified yet and mutations around site B_{IL} do not inhibit processing.

Formation of the 5'-end of 5.8S rRNA, B_{IS} and B_{IL}, also occurs at the same time as processing of the 3'-end of 25S rRNA at site B₂, which is carried out by the 3'->5' exonuclease Rex1p (van Hoof et al., 2000a). This was demonstrated by the fact that 27SA, but not 27SB, pre-rRNA can be detected with an oligonucleotide complementary to a 10-nt extension downstream of site B₂ (Kufel et al., 1999). However, it was observed that deletions in the 3'ETS inhibited cleavage at site A₃, thus preventing synthesis of 5.8S_s rRNA in the absence of correct 3'-end formation of 25S rRNA. Processing at site B_{IL} was also blocked although to a lesser extent, indicating that, unlike the early cleavages at sites A₀ to A₂, the 60S specific cleavages in ITS1, are coupled to processing in the 3' ETS (Allmang and Tollervey, 1998).

Processing events in the ITS2 at last separate the precursors of the large ribosomal subunit RNAs. The cleavage reaction at site C₂ generates the 7S and 26S pre-rRNAs (Figure 2). Mature 25S rRNA is finally generated through exonuclease digestion from site C₂ to C₁, which is carried out by Rat1p and Xrn1p (Geerlings et al., 2000). Processing at C₁ and C₂ occurs almost simultaneously, and substitutions in the sequences surrounding either of the two cleavage sites abolish processing at both sites, suggesting that they may be coupled (van Nues et al., 1995a; van Nues et al., 1995b). No enzyme has so far been identified for the cleavage at C₂, which is most likely an endonucleolytic event. In rat cells an endonucleolytic activity that cleaves in the centre of the ITS2 has been reported for the nucleolar protein B23, but no obvious homologue could be identified from search of the yeast database (Savkur and Olson, 1998). The 3'-end of the 5.8S rRNA is generated by exonuclease digestion from site C₂ mediated by a complex of 3'->5' exonucleases called the exosome and which consists of 11 components (Mitchell et al., 1996; Mitchell et al., 1997). The 3'-end formation of 5.8S rRNA is a multistep process and two intermediates in the processing of 7S pre-rRNA to mature 5.8S rRNA that are 3'-extended by 30nt (5.8S+30) and 8nt (6S pre-rRNA) are detectable in wild-type cells. The 6S intermediate accumulates strongly in strains carrying conditional mutations in several of the exosome components, whereas the 5.8S+30 intermediates

accumulates specifically in strains lacking one specific exosome component, Rrp6p. This indicates that 3' end processing of mature 5.8S rRNA involves at least two changes in the nuclease that is active within the exosome complex (Mitchell et al., 1996; Allmang et al., 2000). Further processing of the 6S pre-rRNA requires the 3'->5' exonuclease Rex1p, which can be substituted by Rex2p, resulting in the 5.8S that is 3'-extended by 5nt (5.8S+5) (van Hoof et al., 2000a). Recently it was shown that the last few nucleotides of this 5.8S+5 rRNA are removed by the potential endonuclease Ngl2p (Faber et al., 2002). Furthermore, based on observations in *ngl2Δ* strains and *rex1Δ/rex2Δ/rex3Δ* triple mutants, it was suggested that this last step in the 3'-end formation of the 5.8S rRNA is enzyme specific whereas the preceding steps are carried out by a preferred enzyme, but can be taken over by another enzyme, albeit not with the same efficiency (van Hoof et al., 2000a; Faber et al., 2002).

3' end synthesis of 5.8S rRNA also requires the DEAD-box putative RNA helicase Dop1p/Mtr4p, a cofactor of the exosome. Depletion of Dop1p results in phenotypes similar to those observed in the absence of Rrp6p and in conditional mutants for exosome components (de la Cruz et al., 1998; Allmang et al., 2000). However, it is not known whether Dop1p is merely needed to unfold the secondary structure of the pre-rRNA to make it accessible to the exosome, or if it has a more specific function in targeting the exosome to its substrate.

1.3. Pre-ribosomal RNA processing in other organisms

1.3.1. Pre-rRNA processing in prokaryotes

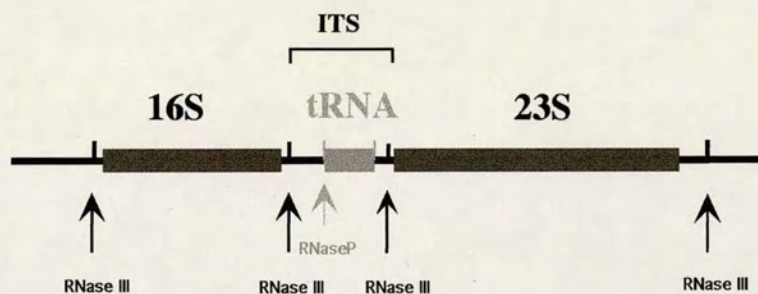
Escherichia coli is the only organism in which rRNA processing is understood in comparable detail to yeast. Comparison between those two systems have pointed out some interesting parallels and differences which make it evident that some features of the enzymology and organisation of pre-rRNA processing have been conserved across evolution.

The organisation of bacterial and eukaryotic pre-rRNAs is very similar with the small subunit rRNAs, 16S or 18S rRNA, and the large subunit rRNAs, 23S or 5.8S/25S rRNA, being co-transcribed. The 5.8S rRNA is homologous to the 5' end of the bacterial 23S rRNA. In most eukaryotes this has been separated by the insertion of the internal transcribed spacer 2 (ITS2), creating distinguishable 5.8S and 25S rRNAs. *E.coli* lacks the ITS2 and therefore does not require the machinery used by yeast to process the ITS2, notably the exosome complex. Although *E.coli* homologues of exosome components do exist, they are not organised into an equivalent complex.

A second difference is the presence of a tRNA in the ITS region of most bacterial and indeed archaeal pre-rRNAs (Figure 3A). The 5' end of this tRNA is cleaved by RNase P, providing an alternative processing pathway, which can take over when the major pre-rRNA endonuclease RNaseIII is inhibited (King et al., 1984). In eukaryotes the equivalent position is cleaved by the homologous enzyme RNase MRP, which appears to be a form of RNase P that has become specialised for pre-rRNA processing (Morrissey and Tollervey, 1995). In yeast, cleavage by RNase MRP is part of the major processing pathway but, as discussed above, an alternative pathway is retained only for this step in rRNA synthesis. The major 5' end of yeast large subunit rRNAs is generated by the 5'→3' exonucleases Rat1p and Xrn1p but no 5'→3' exonuclease has yet been identified in *E.coli*.

Similar to yeast, pre-rRNA processing in *E.coli* is initiated with an endonucleolytic cleavage by RNaseIII. The bacterial enzyme cleaves across the stem structures that are formed by the sequences flanking the mature 16S and 23S rRNAs. Also, the 3' end of the large subunit rRNA is generated by 3'→5' exonuclease digestion from the RNaseIII cleavage site and trimming of the 3' end of 23S rRNA specifically requires RNaseT that also processes the 3' end of 5S rRNA (Li and Deutscher, 1995; Li et al., 1999a). 5' processing of the 16S and 23S rRNAs in *E.coli* is carried out by two homologous endonucleases, RNaseE and RNaseG (Li et al., 1999b). In yeast 5' processing of 18S rRNA is endonucleolytic, and this is also likely to be the case for processing at B_{1L}. However, it might be tempting to suggest that these cleavage reactions might be carried out by homologues of RNaseE and RNaseG, database

A-*Escherichia coli*



B-vertebrate

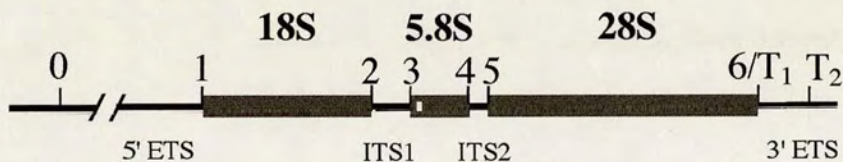


Figure 3: Ribosomal pre-RNAs in *E. coli* and vertebrates. Shown are the pre-rRNAs from *Escherichia coli* (A) and vertebrates (B).

searches have not yield any clear candidates and no enzymes have been identified for these steps as yet.

1.3.2. Pre-rRNA processing in higher eukaryotes

The overall organisation of pre-rRNA in vertebrates is comparable to the yeast pre-rRNA (Figure 1). The pre-rRNAs of *Xenopus laevis* (40S or 7.5kb) and mammals (47S or 13kb) are considerably larger as are the mature 28S rRNAs, which are homologues to 25S rRNA in yeast (Figure 3B). In contrast to the knowledge gained for pre-rRNA processing in yeast, much less detailed information is available concerning processing events in the transcribed spacer regions of higher eukaryotes. Most of the available information is derived from analyses of processing events that were reproducible *in vitro* and from RNase H mediated *in vivo* depletion of snoRNAs after microinjection of antisense DNA into *Xenopus* oocytes.

The 5'ETS region in mammals is 3 to 4kb in length. The first processing event on mouse pre-rRNA was found to take place at position +650, approximately 3kb upstream of the 5' end of 18S rRNA (Miller, 1981). Both, mouse and human pre-rRNA substrates were processed using a mouse *in vitro* system. A region of 200 nucleotides that is highly conserved between mammals lies 3' to the processing site and was shown to be necessary and sufficient for processing (Craig et al., 1987; Kass et al., 1987). 5'ETS processing in *Xenopus* is similar to mouse cells and *Xenopus* pre-rRNA is recognised by the mouse processing machinery (Mougey et al., 1993) although very little of the *Xenopus laevis* pre-rRNA is actually cleaved at this site *in vivo*. This processing region was found to associate with a number of polypeptides to form a 20S complex that also contained the U3 snoRNA and fibrillarin (Kass et al., 1990a; Kass and Sollner-Webb, 1990b). Vertebrate pre-rRNA transcripts also extend considerably beyond the 3' end of the 28S rRNA to one of several tandem RNA polymerase I termination sites. In mouse, 3' end formation of the primary transcripts is directed by the first of these termination sites, located 560 nucleotides downstream of the 28S rRNA 3' end (Grummt et al., 1985).

In addition to the U3 snoRNA, the U8 and U22 snoRNAs are required for vertebrate pre-rRNA processing. Moreover, it was suggested that U3 is not only needed during processing of the 5' ETS but also for cleavage of pre-rRNA processing at the boundary of ITS1 and 5.8S rRNA in *Xenopus* (cleavage site 3, Figure 3B) (Savino and Gerbi, 1990).

In many systems including *Xenopus* oocytes and yeast, actively transcribed pre-rRNA can be spread to reveal a peculiar formation, termed "Christmas trees", which can be observed under the electron microscope (Miller and Beatty, 1969; Osheim and Beyer, 1989). Closely packed branches of nascent transcripts extend from a rDNA 'trunk' and are decorated with at the their ends with terminal 'balls' or 'knobs' that are believed to contain the 5'ETS processing machinery (Mougey et al., 1993). Electron microscopy performed in yeast indicate that their nascent rRNA transcripts also possess 5' terminal 'balls', which are lost upon depletion of U3 snoRNA (Dragon et al., 2002). These data suggests that in *Xenopus* as well as in yeast, the U3 snoRNA is required for coupled 5'ETS and ITS1 processing and is part of a larger snoRNP complex that co-ordinates cleavage in both spacer regions (Beltrame et al., 1994; Dragon et al., 2002). In *Xenopus*, U22 also appears to be part of this complex since its depletion inhibits processing of the pre-rRNA at both ends of 18S rRNA (Tycowski et al., 1994).

ITS2 processing in yeast involves an endonucleolytic cleavage and exonucleolytic digestion to form the mature 5.8S and 25S rRNAs. In vertebrates, there are indications that pre-rRNA can also undergo internal processing within the ITS2. In rat cells, the nucleolar protein B23 was reported to have endonucleolytic activity and was shown to cleave in the centre of the ITS2 (Nashimoto et al., 1988; Savkur and Olson, 1998). The mechanism for 3'end formation of 5.8S rRNA and 5'end formation of 28S rRNA has not been studied in vertebrates. However, in human cells a complex homologous to the yeast exosome complex has been identified (Allmang et al., 1999b). This human exosome was originally reported to contain 11 to 16 components most of which were subsequently shown to be homologous to yeast exosome components and are predicted to exhibit 3'->5' exonuclease activity (Mian, 1997; Briggs et al., 1998; Brouwer et al., 2001a; Raijmakers et al., 2002). Moreover, there are two forms of the human exosome

in the nucleus and in the cytoplasm that, like in yeast, can be distinguished by the presence of hRrp6p (PM-Sc1100) (Allmang et al., 1999b; Brouwer et al., 2001b). A function for the cytoplasmic form of the human exosome in mRNA degradation was recently demonstrated. It seems very likely that the nuclear form carries out similar functions to the yeast complex, but a role for the human exosome during 3' end formation of 5.8S rRNA has yet to be established (Mukherjee et al., 2002).

1.4. Protein *trans*-acting factors

Many different *trans*-acting factors, RNAs as well as proteins, have been implicated in ribosome biogenesis. Most of these can be grouped into classes according to their experimental or predicted enzymatic functions and their physical or functional interactions with the rRNA precursor, snoRNAs and/or other proteins. These classes include components of snoRNPs, rRNA-modifying enzymes, endo- and exonucleases and putative RNA helicases. Most *trans*-acting factors do not fall into such simple groups and many of these contain particular conserved motifs or domains that may mediate predicted functions such as RNA-protein or protein-protein interactions during ribosome biogenesis.

1.4.1. Ribosomal RNA modifying enzymes

Posttranscriptional modifications of the mature rRNAs occur on the pre-rRNA and include pseudouridine formation (ψ), 2'-O-ribose methylation and base methylation (Maden, 1990; Ofengand and Fournier, 1998). Cbf5p, a common component of all box H/ACA snoRNPs, is believed to function as the ψ -synthase since it is homologous to a family of putative ψ -synthases (Koonin, 1996). Mutations in Cbf5p block ψ formation in the pre-rRNAs due to the loss of its enzymatic function and the co-depletion of box H/ACA snoRNAs (Lafontaine et al., 1998b; Zebarjadian et al., 1999). In contrast to the presence of a ψ -synthase in box H/ACA snoRNPs, the putative methyl transferase in

box C/D snoRNPs has not clearly been identified. However, it is presumed that Nop1p fulfils this function due to its homology to known methyltransferases shown by X-ray structure (Niewmierzycka and Clarke, 1999). Moreover, in cells depleted of Nop1p and a temperature sensitive strain carrying a point mutation, 2'-O-methylation is blocked (Tollervey et al., 1991; Tollervey et al., 1993).

Base methylation is not believed to involve guide snoRNAs and much less is known about the enzymes involved in eukaryotes. The only well-studied case in yeast is the conserved methylation of two adjacent adenosines at the 3' end of 18S rRNA, which is carried out by the essential dimethylase Dim1p (Lafontaine et al., 1995). Dim1p is also involved in pre-rRNA processing at sites A₁ and A₂ that generate the 20S pre-rRNA (Lafontaine et al., 1994). However, dimethylation occurs on the 20S pre-rRNA and mutations were isolated in *DIMI* that inhibit dimethylation but do not affect pre-rRNA processing. This indicates that processing at site A₁ and A₂ does not require dimethylation, which is carried out after these cleavages on the 20S pre-rRNA (Lafontaine et al., 1995; Lafontaine et al., 1998a). Processing at sites A₁ and A₂, however, takes place in the nucleus whereas earlier data shows that formation of m₂⁶Am₂⁶A is a late event in ribosome synthesis occurring in the cytoplasm (Salim and Maden, 1973; Brand et al., 1977). Dim1p may therefore bind to the pre-rRNA in the nucleus and is exported with the pre-40S particle into the cytoplasm.

1.4.2. The U3 processome

The U3 snoRNP is a distinctive box C/D snoRNP. It not only differs in structure from other box C/D snoRNAs but is also associated, beside the common box C/D core proteins, with 24 unique proteins that are not present in other box C/D snoRNPs (Jansen et al., 1993; Dunbar et al., 1997; Lee and Baserga, 1997; Wiederkehr et al., 1998; Lee and Baserga, 1999; Venema et al., 2000; Dragon et al., 2002). The U3 snoRNA lacks the complementarity to the pre-rRNA across the methylation sites but is required for pre-rRNA processing reactions.

The U3 snoRNP is the only *trans*-acting factor found to be involved in A₀ cleavage, and conditional mutations in the U3 snoRNA revealed that this cleavage requires base-pairing between the U3 snoRNA and a site within the 5'ETS (Hughes and Ares, 1991; Beltrame et al., 1994). U3 snoRNP is also involved in processing at sites A₁ and A₂ and base pairs to a 5'terminal region of the mature 18S rRNA sequence. Truncated versions of the U3 snoRNP component Mpp10p were shown to specifically inhibit cleavage at those sites (Lee and Baserga, 1997). Sequence mutations in the pre-rRNA to prevent interaction with the 5'ETS also lead to loss of 18S rRNA and inhibition of cleavages at sites A₀, A₁ and A₂, mimicking the effect of U3 snoRNA depletion (Beltrame and Tollervey, 1995).

Previously it was believed that the U3 snoRNP consisted of the U3 snoRNA, four box C/D core proteins, Nop1p, Nop56p, Nop58/5p and Snul3p, and six U3-specific proteins, Sof1p, Imp3p, Imp4p, Mpp10p, Dhr1p and Rrp9p (Jansen et al., 1993; Dunbar et al., 1997; Lee and Baserga, 1997; Wiederkehr et al., 1998; Lee and Baserga, 1999; Venema et al., 2000). However, a recent study showed that the U3 snoRNP is a much larger complex than so far anticipated (Dragon et al., 2002). Seventeen additional proteins, Utp1p to Utp17p, were shown to associate with the U3 snoRNP as well as the non-ribosomal *trans*-acting factor Rrp5p (Venema and Tollervey, 1996). Depletion of each of these essential proteins resulted in reduction of 18S rRNA and inhibition of the early cleavages but not in depletion of the U3 snoRNA (Dragon et al., 2002). It was therefore suggested that reduction of 18S rRNA upon depletion of the U3 snoRNP protein components might be due to a specific protein deficiency and not a general defect in U3 snoRNP biogenesis.

1.4.3. *Endo and exonucleases*

Several endo- and exonucleases carry out different processing reactions during maturation of pre-rRNAs. The first two endonucleolytic cleavages take place on the primary transcript at the nucleotide positions +15 and +45 relative to the 3' end of mature 25S rRNA and is followed by a shortening of the pre-rRNA up to position +7 (Kempers-

Veenstra, 1986). These cleavages are made across a stem structure and are dependent on Rnt1p, a double strand specific endonuclease, which is homologous to bacterial RNase III (Abou Elela et al., 1996). In yeast, deletion of *RNT1* was shown to affect growth and causes accumulation of the primary transcript (Chanfreau et al., 1998). Moreover, purified Rnt1p can cleave a stem-loop structure located immediately 3' of the mature 25S rRNA *in vitro* (Abou Elela and Ares, 1998). Rex1p, a putative 3'-5' exonuclease, the product of the *RNA82* gene is also required for 3'ETS processing at site B₂, and strains carrying a mutation in the *RNA82/REX1* gene were reported to contain transcripts that extend around 10 nucleotides beyond the 3'end of mature 25S rRNA (Kempers-Veenstra, 1986; van Hoof et al., 2000a), predominantly extending to the Rnt1p cleavage site.

RNase MRP is an endo-ribonuclease that cleaves pre-rRNA at site A₃ (Chu et al., 1994; Lygerou et al., 1996). RNase MRP consists of one snoRNA and nine associated proteins. MRP RNA, encoded by the *RRP2* gene, is a unique snoRNA that does not contain complementary sequences to the pre-rRNA and its mechanism thus presumably does not rely on extensive base pairing (Schmitt and Clayton, 1992). MRP RNA is structurally related to the RNA component of another nuclease, RNase P, and eight of the nine proteins, Pop1p, Pop3p, Pop4p, Pop5p, Pop6p, Pop7p and Pop8p, of RNase MRP are shared with RNase P (Chamberlain et al., 1998). The only protein known to be unique to RNase MRP is Snn1p. Mutation or depletion of the RNA or any of the proteins, except Pop8p, leads to co-depletion of MRP RNA and the inhibition of cleavage at site A₃ (Schmitt and Clayton, 1993). As a consequence, synthesis of 5.8S_S rRNA is impaired. However, processing at site B_{1L} and 5.8S_L rRNA synthesis continues via the alternative pathway. No endonuclease specific for cleavage at site B_{1L} has yet been identified, or for cleavages at sites D and C₂.

In contrast, several enzymes responsible for exonucleolytic pre-rRNA processing have been identified. The essential Rat1p and the nonessential Xrn1p are homologous and functionally related proteins (Johnson, 1997). Both proteins have processive 5'->3' exonuclease activity *in vitro* and while Xrn1p functions predominantly in the cytoplasm, Rat1p is found in the nucleus. The roles of Rat1p and Xrn1p in pre-rRNA processing

are the 5' → 3' exonucleolytic digestion of the 27SA₃ pre-rRNA up to site B_{1S}, generating the 5' end of 5.8S_S rRNA and processing from site C₂ to the 5' end of mature 25S rRNA (Amberg et al., 1992; Henry et al., 1994; Geerlings et al., 2000). 5' extended forms of 5.8S rRNA accumulate in *Δxrn1* single mutants and even stronger in *Δxrn1/rat1-1* double mutants (Henry et al., 1994). Xrn1p and Rat1p are also required for the degradation of several excised pre-rRNA spacer fragments, namely A₀-A₁, D-A₂ and A₂-A₃, each of which results from endonucleolytic cleavage (Stevens et al., 1991; Petfalski et al., 1998). Moreover, both proteins were found to be involved in posttranscriptional processing of snoRNAs as well as mRNA and pre-mRNA degradation (Amberg et al., 1992; Caponigro and Parker, 1996; Petfalski et al., 1998; Bousquet-Antonelli et al., 2000).

Two nonessential putative 3' → 5' exonucleases, Rex1p and Rex2p, which are homologues to members of the RNase D family of exonucleases, were found to be required for specific functions during pre-rRNA processing (van Hoof et al., 2000a). Both proteins are functionally redundant in the 3' end formation of the 5.8S rRNA, namely processing of 6S pre-rRNA, generating a 5.8S rRNA, which is 3' extended by 5nt. Accumulation of 6S pre-rRNA was only observed in *rex1Δ/rex2Δ* double mutants but not *rex1Δ* single mutants, indicating that Rex2p can functionally replace Rex1p (van Hoof et al., 2000a). Interestingly, triple mutants of *REX1*, *REX2* and *REX3*, another putative exonuclease, showed even higher accumulation of 6S pre-rRNA than the *rex1Δ/rex2Δ* double mutant strain, suggesting that Rex3p can also process 6S pre-rRNA in the absence of Rex1p and Rex2p. As mentioned above, Rex1p is also involved in the 3' end formation of mature 25S rRNA at site B₂. All three Rex-proteins were furthermore implicated in processing of U4 and U5 snRNA, MRP RNA, RNase P as well as formation of mature 5S rRNA (van Hoof et al., 2000a).

1.4.4. The exosome complex

Formation of the 3' end of mature 5.8S rRNA from the 7S pre-rRNA occurs via a 3' → 5' exonucleolytic processing mechanism which requires the exosome (Mitchell et al.,

1996). The exosome is a complex of 3'->5' exonucleases. The nuclear form of the exosome is composed of 11 components, all of which except Csl4p were shown to be 3'->5' exonucleases *in vitro* or are predicted to have this activity based on sequence homology (Mitchell et al., 1997; Allmang et al., 1999a; Burkard and Butler, 2000). Six of the exosome components, Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p and Mtr3p, are homologous to the *E.coli* exonuclease RNase PH. Rrp44p is homologous to *E.coli* RNase R and Rrp6p is homologous to *E.coli* RNase D. Rrp4p and Rrp44p are homologous to each other and contain a predicted S1 RNA-binding motif, as does Csl4p (Mian, 1997; Allmang et al., 1999a).

All components of the exosome are essential for viability with the exception of Rrp6p the absence of which causes temperature-sensitive lethality (Briggs et al., 1998). Mutation or depletion of most components inhibits 3' end formation of 5.8S rRNA and leads to the accumulation of 3' extended forms of 5.8S rRNA, which extend to heterogeneous positions between 5.8S+30 and site C₂ (Mitchell et al., 1997; Allmang et al., 1999a). Depletion of any exosome component also affects the early cleavages at sites A₀, A₁ and A₂, however, these are each endonuclease cleavages and no direct role for an exonuclease complex can be readily envisaged. As noted above, many mutations that lead to defects in 60S subunit synthesis also delay these early cleavages, apparently via indirect effects, and this is also likely to be the case for the exosome mutations. This is presumably part of a quality control mechanism that ensures only correctly processed and assembled pre-rRNAs are matured (Allmang et al., 2000). The exosome is also required for degradation of the 5' ETS-A₀ spacer fragment as well as aberrant and truncated RNAs that are products of blocked or delayed pre-rRNA processing events. In addition to its role in 3' end formation of 5.8S rRNA, the nuclear exosome also functions in pre-snRNA and pre-snoRNA processing and nuclear pre-mRNA turnover (Allmang et al., 1999a; Bousquet-Antonelli et al., 2000; van Hoof et al., 2000b; Torchet et al., 2002).

In all of its nuclear activities the exosome appears to be assisted by the DEAD-box putative RNA helicase, Dop1p/Mtr4p (de la Cruz et al., 1998). Depletion of Dop1p leads to the same phenotype observed in mutants of various exosome components.

Dop1p therefore appears to function as a cofactor of the exosome in its functions in pre-rRNA processing and degradation of spacer fragments.

Nuclear and cytoplasmic forms of the exosome can be distinguished by the presence of Rrp6p, which is found exclusively in the nuclear complex (Allmang et al., 1999a). The cytoplasmic exosome complex was shown to function in mRNA turnover and mRNA deadenylation with Ski2p replacing Dop1p (Jacobs et al., 1998). Furthermore, different point mutations in the exosome core component Csl4p/Ski4p were shown to affect either pre-rRNA processing or mRNA degradation, indicating that the distinct functions of the exosome might be separated genetically (van Hoof et al., 2000c). Recently, the cytoplasmic exosome was also implicated in the recognition and degradation of mRNAs lacking a translation termination codon (van Hoof et al., 2002). This degradation of “non-stop” mRNAs, which are unstable in eukaryotic cells, requires the exosome-associated protein Ski7p, a GTP binding protein (van Hoof et al., 2000c).

1.4.5. ATP-dependent RNA helicases

A large class of readily defined *trans*-acting protein factors involved in ribosome biogenesis comprises the RNA helicases of the DEAD-box family. Many of these proteins are involved in numerous metabolic processes and possess a RNA-dependent ATPase activity, which is contained within nine highly conserved amino acid regions required for ATP-binding (AXXXGKT), ATP-hydrolysis (DEAD), helicase activity (SAT) and RNA-binding (C-terminal region) (Pause and Sonenberg, 1992; Pause et al., 1993). During ribosome synthesis, these proteins could be envisaged to fulfil different functions including unwinding of the pre-rRNA, establishment of snoRNA-pre-rRNA interactions and accessibility of pre-rRNA substrates to endo- and exonucleases, as well as recruitment, rearrangement and dissociation of *trans*-acting factors during ribosomal processing and assembly but very few of the helicases have actually been shown to have this activity (de la Cruz et al., 1999).

So far 16 putative RNA helicases have been directly implicated in ribosome biogenesis in yeast, seven of which are required for the early pre-rRNA cleavages at

sites A₀ to A₂. Depletion of any of these causes similar processing defects, but these proteins are not redundant, showing that they each carry out different functions. These phenotypes could, however, be indirect consequences of early assembly defects. The remaining nine RNA helicases act at different steps during pre-60S ribosomal subunit synthesis. For example, Dbp3p, which is required for efficient pre-rRNA processing at site A₃, and Dop1p/Mtr4p, which is involved in the 3' end processing of 5.8S rRNA and acts as a cofactor to the exosome complex (de la Cruz et al., 1998; de la Cruz et al., 1999).

1.4.6. Other nucleolar non-ribosomal proteins

A large number of otherwise unclassified protein *trans*-acting factors has been implicated in ribosome biogenesis and have been characterised over recent years (Kressler et al., 1999). Some of these proteins have been identified genetically, while others were obtained by genetic screens, which yielded mutants that preferentially affected ribosome biogenesis. Depletion of many of these proteins results in inhibition at specific steps of either 18S or 25S/5.8S rRNA synthesis. Despite this evidence for the step at which they are likely to be involved, the precise function of most of them is not yet understood. Some *trans*-acting factors, including Nop3p, Nsr1p, Mrd1p, Nop12p and Nop4p, contain one or several RNA recognition motifs and are therefore likely to interact with the pre-rRNA (Lee, 1991; Russell and Tollervey, 1992; Kressler et al., 1999; Wu et al., 2001; Jin et al., 2002). Others are part of conserved protein families, for example the Brix-family proteins and contain a domain specific for this group of proteins (Lee and Baserga, 1999; Eisenhaber et al., 2001; Mayer et al., 2001; Fatica et al., 2002).

One of the unusual *trans*-acting factors is Rrp5p. Rrp5p is the only identified factor that was found to be simultaneously required for pre-rRNA processing at sites A₀, A₁, A₂ and A₃, and therefore for the synthesis of both 18S and 5.8S_s rRNAs (Venema and Tollervey, 1996). Rrp5p is a large non-ribosomal protein containing twelve S1-RNA-binding motifs (S1-RM) and seven tetratricopeptide repeats (TPRs) (Torchet et al.,

1998; Eppens et al., 1999). S1-RM is involved in binding of single strand RNA and the TPR motif mediates protein-protein interactions suggesting that Rrp5p is able to interact with both the pre-rRNA and other *trans*-acting factors (Lamb et al., 1995; Bycroft et al., 1997). It was therefore proposed that Rrp5p might act as a bridging factor between the machineries required for the cleavages at sites A₀ to A₂, as well as A₃ processing, RNase MRP, and the rRNA precursor (Tollervey, 1996). Deletion analyses of Rrp5p showed that the N-terminal S1 RNA binding motifs within the protein are important for the 5.8S_S/5.8S_L rRNA ratio. In these deletion mutants processing at site A₃ was reduced but appeared to occur at a site just upstream of A₃ and downstream of A₂, termed A₄ (Eppens et al., 2002).

Recently, Rrp5p was found to associate with the 90S pre-ribosome or U3 processome, which supports its role as a bridging factor for this complex but no evidence for a direct interaction with RNase MRP has yet been reported (Dragon et al., 2002).

1.5. Ribosome assembly

During ribosome synthesis not only does the pre-rRNA have to undergo processing but it must also assemble with the 80 ribosomal proteins and the 5S rRNA, which is independently transcribed and processed. Moreover, since a large number of *trans*-acting factors is involved in pre-rRNA processing it seems indispensable that a pathway exists for their ordered assembly and disassembly from the pre-rRNAs and rRNAs. While many features of the pre-rRNA processing pathway are relatively well understood, the knowledge about ribosome assembly has been lagging behind. An outline of the ribosome assembly pathway became apparent from sucrose gradient analyses in the 1970s (Udem, 1972). These studies identified three major pre-ribosomal particles. An early 90S particle is subsequently processed into 66S and 43S pre-ribosomes, the precursor to mature 60S and 40S ribosomal subunits (Figure 4) (Trapman et al., 1975). Despite the identification of many putative ribosome assembly factors over

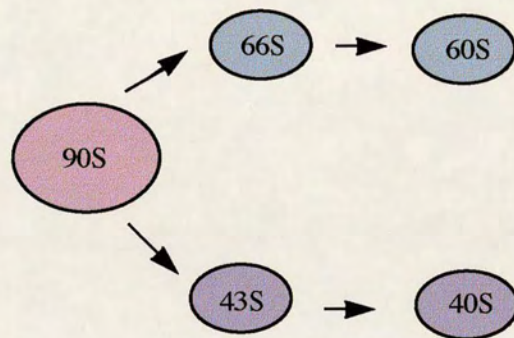


Figure 4: Model for 40S and 60S subunit biogenesis.

Outline pathway of biogenesis of 60S and 40S ribosomal subunits based on the sucrose gradient experiment by Udem and Warner in 1972.

the last three decades there was little advance in detailed understanding of the ribosome assembly pathway (Venema and Tollervey, 1999).

In the past year this has changed due to advances in proteomic analyses that made it possible to analyse complete sets of proteins and RNA components associated with epitope-tagged proteins of known function, and, most recently, by the release of data from large-scale, high-throughput proteomic analyses (Bassler et al., 2001; Harnpicharnchai et al., 2001; Milkereit et al., 2001; Saveanu et al., 2001; Fatica et al., 2002; Gavin et al., 2002; Grandi et al., 2002; Nissan et al., 2002). These experiments made it possible to look more closely into the composition of the pre-ribosomal particles and revealed a much more complex picture of ribosome maturation and, as part of it, ribosome assembly.

1.5.1. The 90S pre-ribosomal particle

The 90S pre-ribosome was originally identified by metabolic labelling (Udem, 1972). Using epitope-tagged forms of proteins known to be involved in the early steps of pre-rRNA processing, this early pre-ribosomal complex was purified and analysed (Grandi et al., 2002). The 90S complex appeared to contain, next to the 35S pre-rRNA, a large number of non-ribosomal proteins including the U3 snoRNP and factors specifically required for 40S synthesis, among them Nop14p, Krr1p, Rrp5p and the putative GTPase Bms1p (Venema and Tollervey, 1996; Gelperin et al., 2001; Liu et al., 2001; Wegierski et al., 2001).

The U3-associated processing machinery has previously been proposed to correspond to the terminal knobs visualised in electron microscopy spreads at the 5' end of nascent pre-rRNA transcripts (Miller and Beatty, 1969; Mougey et al., 1993; Dragon et al., 2002). With a calculated weight around 2.7 MDa, the purified 90S complex could represent or be part of these terminal knobs (Grandi et al., 2002).

Notably absent from the 90S complex were factors that are known to be involved in 60S synthesis as well as most ribosomal proteins of the large subunit (Grandi et al., 2002). Furthermore, recently characterised early pre-60S complexes contained the

27SA₂ precursor but little or no 35S pre-rRNA, and lacked all known 40S processing factors (Fatica et al., 2002). This indicates that the processing factors involved in 40S synthesis assemble first onto the 35S pre-rRNA prior to assembly of the 60S synthesis machinery, clearly characterising the assembly of 40S and 60S subunits as biphasic event (Figure 5). It was suggested that this reflects a 5' to 3' synthesis of the pre-rRNA during the early transcription of the primary transcript binding site in the 5'ETS might first become available and initiate assembly of the 40S subunit processing factors to the nascent transcript. Only after several minutes, when the 3' sequences of the transcript have been synthesised, can the processing and assembly factors of the 60S subunit also be recruited (Grandi et al., 2002).

1.5.2. The 40S pre-ribosome

Cleavage at site A₂ generates the 20S pre-rRNA as well as the 27SA₂ precursor, which is part of the pre-60S particle. The 20S pre-rRNA is exported to the cytoplasm where it is processed to mature 18S rRNA.

Unexpectedly, many factors known to be required for 40S subunit synthesis were associated with the 35S pre-rRNA but showed little or no co-precipitation of 20S pre-rRNA. This indicates that they dissociate from the pre-rRNA at or immediately following A₂ cleavage or are released with the excised 5' ETS, possibly still in a complex corresponding to the terminal balls (Grandi et al., 2002). Late processing of 20S to mature 18S rRNA is known to require Rrp10p/Rio1p, Tsr1p and the dimethylase Dim1p, which modifies the 20S pre-rRNA in the cytoplasm (Lafontaine et al., 1994; Gelperin et al., 2001; Vanrobays et al., 2001). Moreover, two smaller complexes have been reported that contained Dim1p and also included the non-ribosomal proteins Tsr1p, Rio2p, Rrp12p and Yor145p, out of which only Tsr1p has been characterised (Gavin et al., 2002).

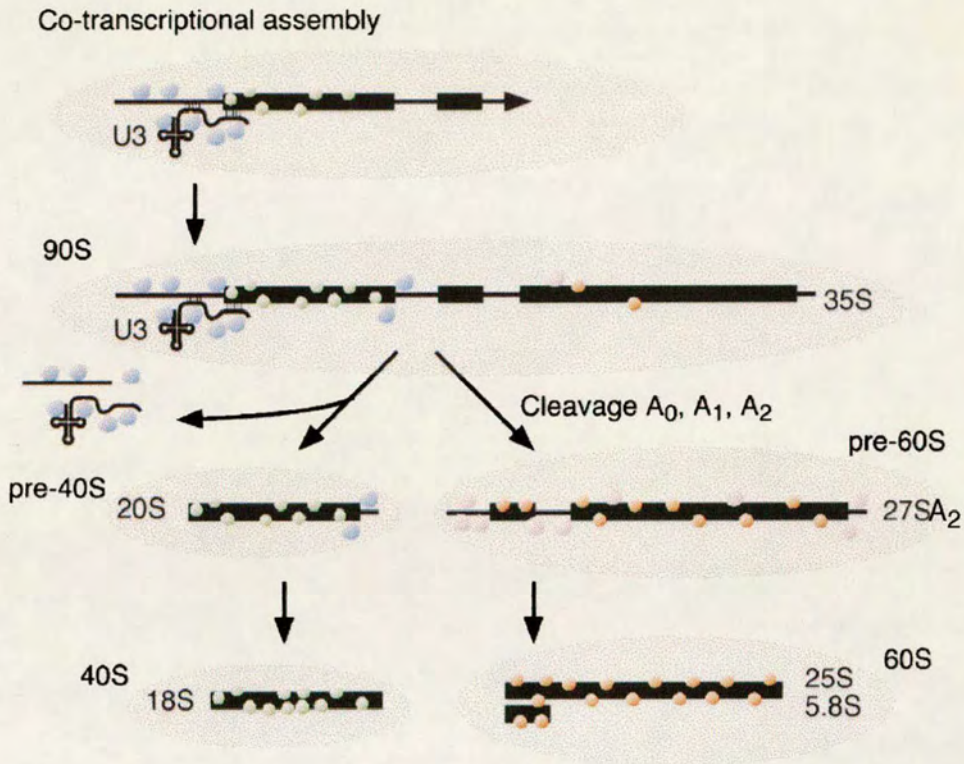


Figure 5: Model of biphasic assembly of 40S and 60S machinery. Model of the 90S pre-ribosome with associated factors and r-proteins reflecting the largely separate assembly of 40S and 60S processing factors and r-proteins onto the pre-rRNAs in a 5' to 3' direction according to synthesis of the rRNA precursor. Model adapted from Grandi et al., 2002.

1.5.3. *The 60S pre-ribosome*

The first indication of the dynamics of pre-60S processing factors came through the analysis of the Noc1/Noc2 and Noc2/Noc3 complexes (Milkereit et al., 2001). The Noc1/Noc2 complex associates with the 90S and 60S pre-ribosomal particles and is enriched in the nucleolus, whereas Noc2/Noc3 associates with the 60S pre-ribosome only and is mainly nucleoplasmic. During the maturation of 90S to pre-60S particles, Noc1p dissociates from Noc2p and is replaced by Noc3p. This change in the complex might trigger the release of the pre-60S particles from the nucleolus to the nucleoplasm.

Following this analysis, several research groups have studied the 60S pre-ribosome in more detail and found a number of distinct pre-60S complexes that contained pre-rRNA at different stages of processing and differed in content of protein components. Comparison of the pre-rRNA and proteins associated with these complexes led to the identification of four distinct pre-60S particles, which can be placed in a tentative pre-60S assembly pathway (Figure 6) (Fatica et al., 2002). The earliest pre-60S particle, E₁, assembles soon after the cleavage at site A₂ to the 27SA₂ pre-rRNA. It remains associated during the cleavage at site A₃ and processing to B_{1S}. The related pre-60SE₂ complex associates with the 27SB precursor and remains associated during cleavage at site C₂, when 7S and 26S pre-rRNAs are formed. Within the next complex, pre-60SM, exonuclease digestion to the 5' end of 25S rRNA is predicted to occur, together with formation of 3' end of 5.8S rRNA through, probably transient, association with the exosome complex. Pre-60SM complex is associated with a number of proteins depletion any of which inhibits release of the complex from the nucleolus/nucleus to the cytoplasm. This suggests that these proteins are required for the complex to obtain export competence and may serve as a control mechanism in order to designate the pre-60S particle as being processed and assembled correctly and ready to be exported (Bassler et al., 2001; Milkereit et al., 2001; Gadal et al., 2001b). The final characterised complex, pre-60SL, contains only three non-ribosomal proteins, Nug1p, Nug2p/Nog2p and Rlp24p, and may be an intermediate in the disassembly of the processing machinery before the subunit is exported to the cytoplasm (Bassler et al.,

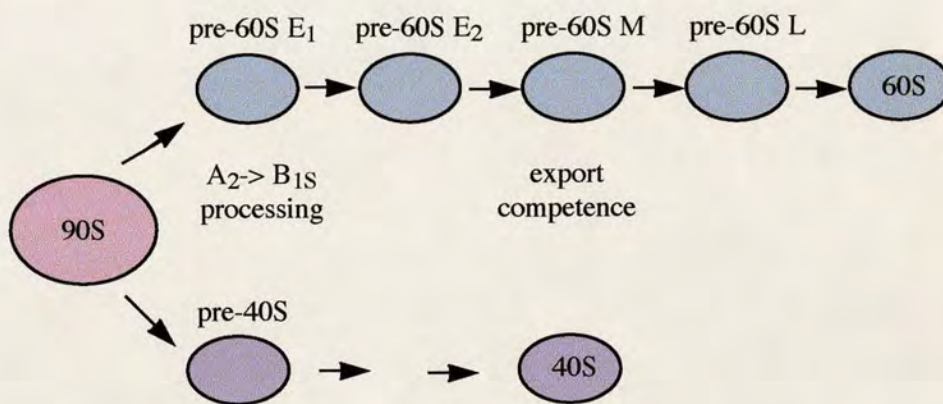


Figure 6: Revised model for 40S and 60S subunit biogenesis.

Outline pathway of biogenesis of 60S and 40S ribosomal subunits. Pre-60SE₁ contains the 27SA₂ pre-rRNA. In this particle occurs processing at sites A₃ and B_{1S}. The related pre-60SE₂ complex associates with the 27SB precursor and remains associated during cleavage at site C₂, when 7S and 26S pre-rRNAs are formed. In the pre-60SM, exonuclease digestion to the 5' end of 25S rRNA is predicted to occur and 3' end formation of 5.8S rRNA. This complex contains numerous factors required for 60S subunit export and acquisition of export competence is likely to be obtained within this complex. Pre-60SL, contains only three non-ribosomal proteins and may be an intermediate in the disassembly of the processing machinery before the subunit is exported to the cytoplasm.

2001; Saveanu et al., 2001). Nug1p and Nug2p are putative GTPases, which were shown to function during rRNA maturation but also affected export of pre-60S subunits from the nucleus to the cytoplasm. It was therefore suggested that GTP-hydrolysis might be important for export of pre-60S particles. The non-ribosomal protein Rlp24p belongs to the archaeal and eukaryotic Rpl24e family of ribosomal proteins and is the conserved nuclear counterpart of the cytoplasmic ribosomal protein Rpl24p (Saveanu et al., 2001). Since deletion of the *RPL24* gene did not affect ribosome synthesis, it was suggested that Rpl24p associates to the 60S subunit only after export from the nucleus to the cytoplasm, replacing Rlp24p, which associates with the pre-60SE₂ particle in the nucleolus and might have an essential function during 60S synthesis (Kruiswijk et al., 1978; Saveanu et al., 2001).

Furthermore, it became apparent that some protein factors, including the putative GTPase Nog1p, are associated with several pre-60S particles, while others, for example Ssf1p, are associated with only a single characterised particle (Park et al., 2001; Fatica et al., 2002). It is likely that further distinct pre-60S particles remain to be characterised since several known 60S synthesis factors have not yet been detected in pre-ribosomal complexes. Most obvious is the absence of the processing enzymes RNase MRP, Rat1p, Xrn1p and the exosome complex. These may associate only transiently with the pre-60S particles *in vivo* or dissociate during purification *in vitro*.

1.6. Nucleocytoplasmic transport

In eukaryotic cells, the nucleus is separated from the cytoplasm by a double membrane system, the nuclear envelope. DNA replication and RNA biogenesis occur in the nucleus, whereas protein synthesis occurs in the cytoplasm. RNAs transcribed in the nucleus must therefore be transported out of the nucleus into the cytoplasm. On the other hand, proteins that are needed during transcription, ribosome synthesis or any other process within the nucleus need to be imported into the nucleus. Proteins, RNPs, RNAs and pre-ribosomes, are transported through the nuclear pore complexes (NPCs) to

cross the nuclear envelope. This transport requires partly distinct factors for different cargoes as well as energy. Many of these factors have been intensively studied over the recent years by utilisation of *in vitro* assays and yeast genetics to gain more insight into nucleocytoplasmic transport.

1.6.1. *The nuclear pore complex and nuclear transport receptors*

The nuclear envelope consists of a double lipid layer with an intervening lumen. The envelope is penetrated by the nuclear pore complexes, which mediate bi-directional transport between the cytoplasm and the nucleus (Pante and Aebi, 1995; Adam, 2001). Each NPC consists of a basic framework of three rings, nuclear, central and cytoplasmic (Rout et al., 2000). The NPC is composed of thirty distinct polypeptides, called nucleoporins (nups) that are present in multiple copies (Rout and Blobel, 1993; Bastos et al., 1995; Rout et al., 2000). Many nucleoporins contain regions with multiple FG-rich repeats (Phe, Gly), FXFG, or GLFG (Gly, Leu, Phe, Gly). These FG-repeats have been shown to interact with transport factors and are the docking regions for translocation through the NPC (Iovine et al., 1995; Rexach and Blobel, 1995; Stutz et al., 1996; Iovine and Wentz, 1997; Strawn et al., 2001). Moreover, it was observed that subsets of FG-nups were concentrated to one side of the nuclear pore or distributed asymmetrically suggesting that differences between the nuclear and cytoplasmic NPC docking sites may be important for transport (Blobel, 1995; Rout et al., 2000).

Nuclear transport is a signal-mediated process. Different cargoes carry a nuclear localisation signal (NLS) or a nuclear export signal (NES) to be able to either enter or leave the nucleus. Different NLS have been identified, ranging from mono or bi-partite short amino acid sequences to much more complex regions in the case of most ribosomal proteins (Dingwall and Laskey, 1991; Makkerh et al., 1996; Annilo et al., 1998; Rosorius et al., 2000). Leucine-rich NES have been identified in a variety of cellular and viral proteins (Fischer et al., 1995; Michael et al., 1995). Since all RNAs are believed to associate with proteins in the nucleus, it has been suggested, that RNA export events are mediated by proteins containing appropriate export signals (Nigg,

1997). Many transport signals are recognised by import or export receptors of the importin β -like family, which shuttle between the nucleus and cytoplasm (Mattaj and Englmeier, 1998; Conti and Izaurralde, 2001).

All importin β homologues or karyopherins consist of 15 to 19 tandemly repeated modules known as HEAT-repeats, while importin α consists of *armadillo* (ARM) repeats (Andrade et al., 2001). HEAT-repeats are sequences of approximately 40 residues of a loose consensus pattern of conserved hydrophobic amino acids (Figure 7) folded into a bent helix and a straight helix (Andrade et al., 2001; Conti and Izaurralde, 2001). ARM-repeats form three helices where the second and third are separated by one glycine residue, causing a bend in the structure. The multiple repeats pack side by side in a parallel fashion, forming elongated molecules with a superhelical twist (Chook and Blobel, 1999; Cingolani et al., 1999). This structural framework is shared by a number of proteins within the family of importin β -like karyopherins (Conti et al., 1998; Chook and Blobel, 1999; Cingolani et al., 1999; Andrade et al., 2001). In yeast, fourteen karyopherins have been identified, amongst them the exportins Los1p, Msn5p, Cse1p, Kap120p and Xpo1p (Crm1p) (Stade et al., 1997; Hellmuth et al., 1998; Kunzler and Hurt, 1998; DeVit and Johnston, 1999). Out of these, only Kap120p and Xpo1p have been implicated in ribosomal subunit export (Moy and Silver, 1999; Neville and Rosbash, 1999; Stage-Zimmermann et al., 2000; Moy and Silver, 2002). All tested transport factors of this family were shown to interact with FG nucleoporins, however, different factors favoured different FG nups (Rout et al., 1997; Marelli et al., 1998; Aitchison and Rout, 2000).

Importins and exportins can transport macromolecules across the NPC against a gradient of chemical activity, a process, which requires energy. The metabolic energy that needs to be fed into the transport cycle is solely gained through the RanGTPase system (Weis et al., 1996). Almost all transport factors have the ability to interact with Ran, yeast Gsp1p (Nigg, 1997), which is a highly abundant protein of the *ras* superfamily of small GTPases (Goldfarb, 1994). The energy required for nuclear transport is provided through the maintenance of a gradient of Ran-GTP:GDP across the nuclear envelope in the cell (Mattaj and Englmeier, 1998). In the nucleus, Ran is

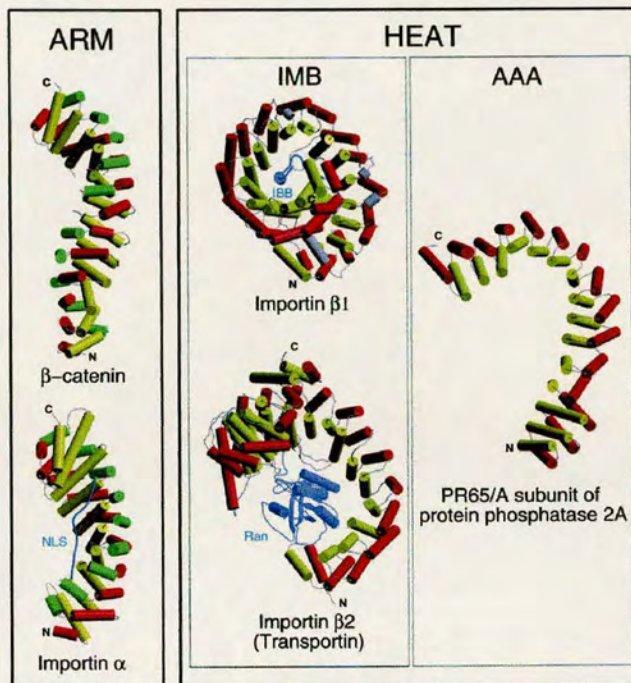


Figure 7: Models for the structure of HEAT and ARM-repeats.

H1, H2 and H3 helices of ARM repeats are shown in green, red and yellow, respectively. A and B helices of HEAT repeats are in red and yellow, respectively. Bound ligands are in blue. The structures are shown with their N-termini at the bottom and their C-termini at the top. Figure adapted from Andrade et al., 2001.

maintained in its GTP-bound state, while the cytoplasmic pool is largely in its GDP-bound form. This distribution is used by transport factors to determine which side of the nuclear envelope they are on. In yeast, the high concentration of RanGTP in the nucleus is maintained by the guanine-exchange factor Prp20p/Srm1p/Mtr1p (RCC1) and the cytoplasmic pool of RanGDP is generated by the GTPase-activating protein Rna1p (Amberg et al., 1993; Becker et al., 1995). Formation of an import complex is stable in the presence of cytoplasmic GDP but in the nucleoplasm Ran-GTP triggers its disassembly. In contrast, formation of an export complex is stabilised in the nucleus by Ran-GTP but when the complex reaches the cytoplasm, the Ran-bound GTP is hydrolysed and the complex disassembles (Gorlich et al., 1996; Koepp and Silver, 1996; Fornerod et al., 1997; Kutay et al., 1997; Mattaj and Englmeier, 1998). Interestingly, no nucleoporins appear to contain Ran binding sites suggesting that Ran does not need to be bound to the NPC and therefore that GTP hydrolysis is not required for the translocation steps across the NPC (Schwoebel et al., 1998; Englmeier et al., 1999).

However, nuclear transport is not the only cellular process during which Ran GTPase functions. It is furthermore involved in mitotic spindle assembly, the regulation of cell cycle progression and nuclear assembly, suggesting that it plays a central role in spatial and temporal organisation of eukaryotic cells (Dasso, 2002).

1.6.2. Mechanism and regulation of nucleocytoplasmic transport

In nucleocytoplasmic transport the NPCs function as highly selective gates and allow passage of material in two ways, passive diffusion and facilitated translocation (Mattaj and Englmeier, 1998; Rabut and Ellenberg, 2001). Passive diffusion is fast for metabolites and small proteins, but limited dependent on size by the pores. Translocation of larger macromolecules requires transport factors, the karyopherins, and, as mentioned above, substrate loading and release guided by a concentration gradient of RanGTP:GDP across the nuclear envelope.

The question as to how interaction of transport receptors with FG-repeats can promote NPC passage led to different models over the years. A recent model, the

Brownian affinity gate model (Figure 8A), pictured the NPC as a narrow, aqueous channel surrounded by filamentous nucleoporins, which attract transport receptors to the pore (Rout et al., 2000). The central channel is assumed to be so narrow that particles above the passive diffusion limit are unlikely to enter. The model predicts that binding to the FG-repeats is necessary for facilitated translocation and that the rate of translocation is determined by efficient targeting of receptor-cargo complexes to the pores. However, the model does not explain how large macromolecules or complexes larger than the physical diameter of the channel can cross the NPC, even with the aid of transport receptors.

A different approach was suggested by the latest, the selective phase model (Figure 8B) (Ribbeck and Gorlich, 2002). This model assumes the central channel to be wide enough to accommodate the largest receptor-cargo complexes known to cross NPCs. The channel would not be fully open but filled with a selective phase, formed by the hydrophobic FG-repeats of nucleoporins. Through weak hydrophobic interaction between each other the nucleoporins are suspected to create a hydrophobic mesh across the nuclear pore. For hydrophilic macromolecules, this would represent a sieve-like structure whose mesh size determines the passive diffusion limit of the pores, which was found to be altered in certain nucleoporin mutants (Shulga et al., 2000). Passage of larger macromolecules would require breaking of the inter-repeat interactions, which is energetically unfavourable for hydrophilic molecules. However, transport receptors were shown to be collectively more hydrophobic than most cytosolic proteins due to their HEAT-repeats (Andrade et al., 2001; Conti and Izaurralde, 2001; Ribbeck and Gorlich, 2002). Structural studies of importin β also revealed details of interaction between transport receptors and nucleoporins, which showed that the FG-repeats intrude into the hydrophobic pockets of HEAT-repeats (Bayliss et al., 2000). Using a hydrophobic interaction column to mimic the nucleoporin mesh of NPCs, it was furthermore shown that under such conditions only the known transport receptors were bound to the column, creating a protein pattern that resembled that previously retrieved from a RanGTP column (Ribbeck and Gorlich, 2002). This leads to the conclusion that the permeability barrier across the nuclear pore channel functions based on hydrophobic



1. Initial targeting 2. Mid-stage translocation

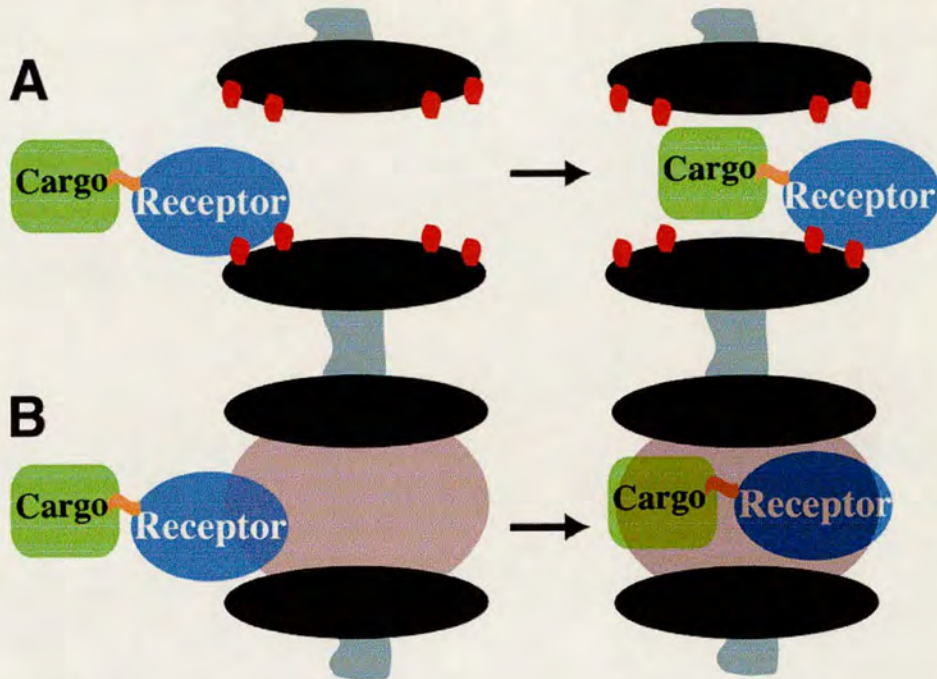


Figure 8: Diagram of the nuclear pore complex (NPC) illustrating two models for nucleocytoplasmic transport.

(A) Brown gating model. The receptor is attracted and bound by the filamentous nucleoporins (red dots) at the periphery of the channel, which triggers a movement through the aqueous and rigid channel.

(B) The selective phase model. Receptor cargo-complexes must partition completely into a tightly sealing selective hydrophobic phase, which implies that the receptor (which is attracted by the phase) as well as the cargo (which is repelled by the phase) become exposed to the permeability barrier.

Figure adapted from Ribbeck and Gorlich., 2002.

exclusion, which can be overcome by nuclear transport receptors through binding to the nucleoporin FG-repeats and thereby competing the repeat-repeat interactions. The barrier can “seal” around transport receptor and the translocating complex “dissolves” through the pore. Moreover, it was shown that while nuclear transport receptors promote NPC passage, exposed hydrophilic domains of cargo molecules are able to counteract the process and even weaken the hydrophobicity of the barrier (Ribbeck and Gorlich, 2002). Consequently, nucleocytoplasmic transport also depends on a sufficient receptor: cargo ratio to prevent hydrophilic interaction with the FG-repeats during translocation (Ribbeck and Gorlich, 2002).

Furthermore, this model provides an explanation for the recycling of nuclear transport receptors, which due to their hydrophobic nature would not require active translocation across the NPC, but could simply “dissolve” through the nucleoporin barrier on their own.

1.6.3. Import of ribosomal proteins

All ribosomal proteins are potentially small enough to passively diffuse through the NPC into the nucleus. However, as ribosomal proteins concentrate 50-fold in the nucleus within 5 minutes of their synthesis, and free cytoplasmic ribosomal proteins are degraded with a half-life of two to three minutes, they are expected to require active nuclear import (Warner et al., 1985; Warner, 1989). The presence of a classical tetrapartite NLS in the human ribosomal protein S6 suggested that ribosomal proteins are imported by the classical import pathway, involving the importin α/β heterodimer (Schmidt et al., 1995). NLS found in several other ribosomal proteins in *S.cerevisiae*, however, did not resemble a bi- or tetrapartite consensus sequence, but rather complex sequences, which in the case of Rpl25p make up to fifty percent of the mass of the protein (Schaap et al., 1991), indicating the possibility of an alternative pathway for the import of ribosomal proteins. In yeast two importin β -like karyopherins, Kap123p and Kap121p/Pse1p, but not Kap95p or Kap104p to which they are homologues, were found to bind to ribosomal proteins (Rout et al., 1997). Out of these two, Kap123p shows a

higher affinity for ribosomal proteins, and nuclear accumulation of a reporter construct fused to a NLS found in r-proteins as well as import of Rpl10p and Rpl25p proved to be Kap123p-dependent (Rout et al., 1997; Gadai et al., 2001b). However, since deletion of Kap123p does not affect viability of the cells, nuclear accumulation of a reporter construct containing an r-protein NLS was tested in *kap123Δ/GAL::pse1* cells, showing that different levels of Pse1p lead to different levels of nuclear accumulation of the construct (Rout et al., 1997). Furthermore, addition of Kap123p to those cells caused the nuclear import of the reporter construct to become Pse1p-independent. It was therefore suggested that Kap123p specifically mediates the nuclear import of ribosomal proteins and in its absence is replaced by Pse1p. Kap123p and Pse1p have furthermore been implicated in the nuclear import of transcription factors and histone proteins, the import of hydrophobic proteins into mitochondria as well as nuclear export of mRNAs (Seedorf and Silver, 1997; Kaffman et al., 1998; Isoyama et al., 2002).

Alternative pathways for the nuclear import of ribosomal proteins have also been identified in mammalian cells, where next to importin β , transportin, RanBP5 and RanBP7 were found to bind and import the ribosomal proteins L23a, S7 and L5 (Jakel and Gorlich, 1998). A possible reason for the existence of multiple separate import pathways could be the reduction of competition for import between major classes of import substrates and furthermore, to separate their import processes.

1.6.4. Export of pre-ribosomal subunits

In growing cells, around 4000 ribosomal subunits are predicted to leave the nucleus every minute (Warner, 1999). At the same time ribosomal proteins that have been translated in the cytoplasm, are imported to the nucleoplasm in order to associate with the nascent ribosomal RNA (Rout et al., 1997). As discussed earlier in this chapter, the pre-ribosomes undergo modifications and are matured and assembled as two separate subunits, the 40S and 60S. Maturation of the pre-60S subunit happens for the most part in the nucleolus, except from the final maturation step, which is believed to take place in the cytoplasm (Tollervey et al., 1993). The 40S subunit is exported to the cytoplasm as

a pre-ribosomal complex and after its maturation in the cytoplasm, the small subunit binds to the 5' end of mRNA and starts to scan for the translation initiation complex, waiting for the large subunit (Aitchison and Rout, 2000). Until recently it was completely unknown how ribosomes are transported from the nucleus.

A new assay to monitor export of the 60S ribosomal subunit in yeast, following the export of a GFP-tagged ribosomal protein that is known to assemble to the pre-60S complex in the nucleus, was used to gain more insight into the export of the large ribosomal subunit (Hurt et al., 1999; Stage-Zimmermann et al., 2000). This assay has helped to define a late pre-ribosomal particle, the pre-60S M, all tested components of which are required for 60S subunit export, including Nug1p, Nug2p, Noc2p, Noc3p and Rix1p (Bassler et al., 2001; Milkereit et al., 2001; Gadai et al., 2001a; Gadai et al., 2001b). Mutations in any of these proteins leads to defects in export suggesting a requirement for the intact structure of this pre-ribosomal particle. Since multiple components of this complex are required for subunit export, it was predicted that export-competence is established within this particle. This suggests that the maturation of ribosomes is closely linked to its transport. Furthermore, it was demonstrated that 60S subunit export is dependent on Ran/Gsp1p and temperature-sensitive mutants defective in the Ran cycle lead to aberrant pre-rRNA processing (Cheng et al., 1995; Suzuki et al., 2001). In addition, studies identified subsets of nucleoporins, including Nup49p, Nup82p, Nup159p, Nic96p and Nsp1p, which appear to be required for ribosome export (Hurt et al., 1999; Gleizes et al., 2001).

A transport receptor that appeared to be required for 60S subunit export is the exportin Crm1p/Xpo1p for which Nmd3p was shown to function as an adapter protein (Ho et al., 2000b; Gadai et al., 2001b) and experiments using a drug-sensitive version of Xpo1p provided direct evidence for its involvement in ribosomal export. Leptomycin B (LMB) is an antibiotic that binds to metazoan Xpo1p and inhibits its function in binding to NES-containing proteins (Fornerod et al., 1997). In yeast, Xpo1p is usually LMB-insensitive, but introduction of a single cysteine into the protein renders it susceptible to the drug (Neville and Rosbash, 1999). Addition of LMB to the LMB-sensitive Xpo1p-strain, but not the insensitive control, led to accumulation of both Nmd3p and large

subunit reporter Rpl25p-GFP in the nucleus (Ho et al., 2000b). However, accumulation of neither was detected in the temperature-sensitive *xpo1-1* strain after shift to restrictive temperature, which could be due to a transient inhibition of ribosome biogenesis caused by the temperature shift (Hurt et al., 1999; Warner, 1999; Ho et al., 2000b). Nmd3p is a non-ribosomal protein that is essential during the late stages of pre-60S maturation and contains a NLS as well as a putative NES (Ho et al., 2000a). Nmd3p also associates with the ribosomal protein Rpl10p/Grc5p, as was shown by two-hybrid analysis, which assembles late to the 60S subunit (Karl et al., 1999; Gadad et al., 2001b). Furthermore, deletion of the Nmd3p NES localises the usually cytoplasmic protein to the nucleus and accumulates pre-60S ribosomal subunits within the nucleus (Ho et al., 2000a; Gadad et al., 2001b). Thus a proposed model suggested that Nmd3p associates with the pre-60S ribosomes during the late stages of subunit maturation, possibly with the pre-60SL complex, via binding to Rpl10p. Once the subunit is matured, the Nmd3p NES is then recognised and bound by Xpo1p, which also binds RanGTP. Xpo1p chaperones the mature 60S subunit through the NPC to the cytoplasm where the RanGTP is hydrolysed causing the dissociation of Xpo1p and Ran and the release of the 60S subunit and Nmd3p (Figure 9). Nmd3p is expected to dissociate from the large subunit prior to its assembly onto the mRNA (Aitchison and Rout, 2000). Although it is clear that Xpo1p is an export receptor for Nmd3p and 60S subunits, the lack of accumulated Nmd3p and Rpl25p-GFP in the *xpo1-1* mutant strains suggests that there might be an alternative, Xpo1p-independent pathway that can mediate 60S subunit export at elevated temperatures and bypass a Xpo1p-dependent pathway (Hurt et al., 1999; Ho et al., 2000b). Such a pathway could involve Mtr2p, an essential mRNA export factor, which is linked to 60S subunit export in an allele-specific manner (Santos-Rosa et al., 1998; Bassler et al., 2001). The temperature sensitive mutant allele *mtr2-33*, but not *mtr2-21*, accumulated Rpl25p-GFP within the nucleus after shift to restrictive temperature. This defect was furthermore strongly enhanced in *mtr2-33/ecm1Δ* double mutant strains in which nuclear Rpl25p accumulation already occurred at permissive temperature, indicating that a role of Mtr2p in 60S export depends on Ecm1p, a nucleolar protein that has previously been implicated in cell wall biogenesis (Lussier et al., 1997;

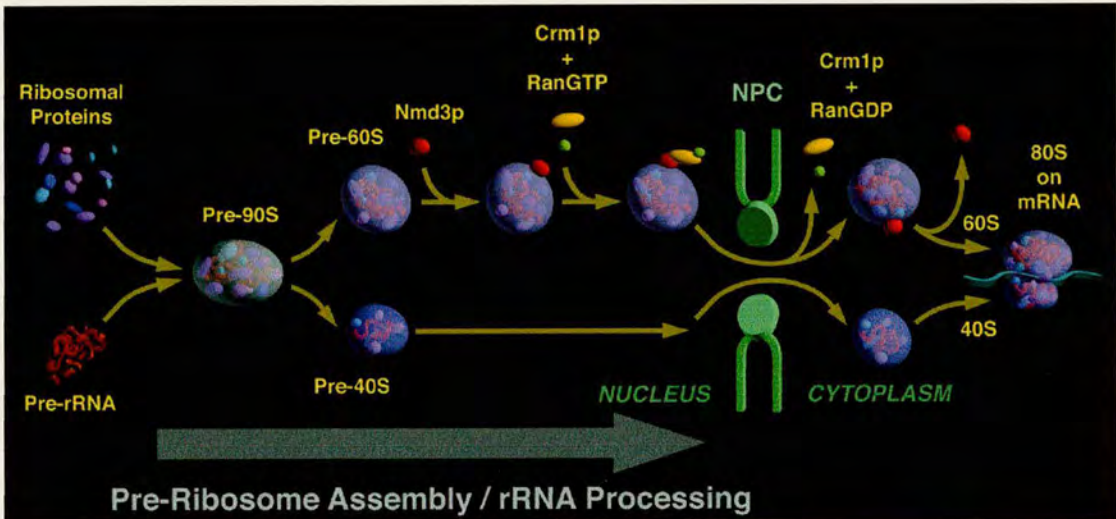


Figure 9: Diagram of ribosome biogenesis and model for 60S subunit export in *S.cerevisiae*.

Pre-ribosomal RNA is modified, processed and assembled with the ribosomal proteins. Nmd3p associates with the pre-60S subunit via Rlp10p. The Nmd3p NES is bound by Crm1p/Xpo1p, which binds to RanGTP, and the mature 60s subunit is transported through the NPC to the cytoplasm. The pre-40S subunit is transported to the cytoplasm where it is matured by dimethylation and processing of the 20S pre-rRNA to mature 18S rRNA.

Model adapted from Aitchison and Rout, 2000.

Bassler et al., 2001). Moreover, *mtr2-33* was found to be synthetically lethal in the presence of a *nmd3* mutant allele, which provides further evidence for a function of Mtr2p as a possibly alternative mediator of 60S subunit export.

The export pathway of the small ribosomal subunit is less well understood than 60S subunit export. Recent advances were made due to an assay developed to study the release of pre-40S subunits from the nucleus to the cytoplasm. The assay uses fluorescent *in situ* hybridisation of short oligonucleotides complementary to the 5'ITS1 in yeast and allows determination of the localisation pattern of 20S pre-rRNA (Moy and Silver, 1999). The assay was originally performed in strains lacking the Xrn1p exonuclease, which degrades the ITS1 5'-fragment after cleavage at site D. In *xrn1Δ* cells, the ITS1-5'RNA is cleaved off and accumulates in the cytoplasm. In this strain background it is possible to detect both defects in small ribosome subunit export and assembly, since the ITS1-5'RNA accumulates in the nucleus or the nucleolus, respectively (Moy and Silver, 1999). However, the assay can also be carried out in *XRNI*⁺ wild-type yeast strains, where the ITS1-5'RNA primarily localises to the nucleolus with a very small amount in the cytoplasm. This background is more suitable to detect mild nuclear export defects that can be obscured in the *xrn1Δ* strains by the excess ITS1-5' RNA. However, ribosome assembly defects cannot be detected in *XRNI*⁺ cells, since the ITS1-5'RNA remains localised to the nucleolus (Moy and Silver, 2002).

A study using this assay revealed that in temperature-sensitive *xpo1-1* strains and LMB-treated LMB-sensitive *XPO1* strains 20S pre-rRNA, and therefore pre-40S subunits, accumulated in the entire nucleus in both *xrn1Δ* and *XRNI*⁺ strain backgrounds (Moy and Silver, 1999; Moy and Silver, 2002). Similar effects were observed in temperature-sensitive mutants of Ran-GTP suggesting that export of the 40S ribosomal subunit depends on Ran GTP and the karyopherin Xpo1p. No adapter protein has been identified so far although the export of the small subunit was delayed in cells deleted of the non-essential yeast Ran binding protein 2, Yrb2p (Maurer et al., 2001; Moy and Silver, 2002). However, this was only detected in strains deleted of the exonuclease

Xrn1p but not *XRNI*⁺ strains, and a role of Yrb2p as an adaptor protein to Xpo1p during the export of the small subunit remains to be determined.

1.7. Aims of this Thesis

In eukaryotic cells the mature ribosomal RNAs (rRNAs) are released from a large precursor molecule, the pre-rRNA, by a complex series of endo- and exonucleolytic processing reactions. A large number of non-ribosomal *trans*-acting factors has been implicated in the maturation of the ribosomal subunits and characterised over recent years. Although pre-rRNA processing is fairly well understood this is not the case for ribosome assembly and it was predicted that many factors involved in ribosomal subunit assembly and transport of ribosomal subunits to the cytoplasm remain to be identified.

A biochemical purification of human nucleoli led to the identification of 271 nucleolar proteins many of which had not yet been characterised (Andersen et al., 2002). From these, three essential proteins of unknown biological function were selected and their yeast homologues, Ygr103p, Ypl012p or Ynl110p, analysed for involvement in ribosome biogenesis. The criteria for selection were based on essentiality of the gene product for cell viability, presence of protein or RNA interaction domains as well as data obtained in previous studies. Cells deleted of *Ygr103w* or *Ynl110c* had recently been shown to arrest in the cell cycle during G1 before passing “Start”, a phenotype that is characteristic for defects in ribosome synthesis (Kinoshita et al., 2001; Capozzo et al., 1999). Ynl110p furthermore contained a RNA recognition motif and displayed homologies to proteins that are known to be involved in processing of ribosomal RNA precursor suggesting a potential role during ribosome synthesis. No preliminary data exists regarding Ypl012p, its presence in the nucleolus, however, made the protein a potential candidate for this study.

Previous analyses of yeast strains depleted for specific proteins have helped to identify the role of many *trans*-acting factors. Analysis of strains depleted of Ygr103p, Ypl012p or Ynl110p, will make it possible to define sites of function for these proteins. Moreover, biochemical analysis using epitope-tagged forms of these proteins

may provide information concerning interactions with other *trans*-acting factors and enzymes involved in rRNA processing as well as rRNA precursors. Furthermore, it might help to gain insight into the ribosome assembly pathway and ribosomal transport.

Chapter Two

Materials and Methods

2.1. Enzymes and Chemicals

Restriction enzymes and other modifying enzymes were obtained from New England Biolabs, Boehringer Mannheim, Roche, Promega and Stratagene. Standard laboratory reagents were purchased mainly from Sigma, other suppliers were Gibco BRL, Difco, and Fluka. Reagents obtained from specific sources are indicated.

2.2. Culture media

E.coli cultures were grown in Luria-Bertani medium (LB): 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl, adjusted to pH 7.2 with NaOH (for 1 litre). Ampicillin was added to media immediately prior to use (100µg/ml). For agar plates, bacto-agar was added to 2% (w/v) prior to autoclaving at 15lb/sq.inch for 20 minutes in a liquid cycle.

Standard *S. cerevisiae* growth and handling techniques were employed (Sherman, 1991). Yeast strains were grown either in rich (YPD and YGSR) or in minimal media (SD and SGSR). 1 litre of the following media was composed as follows:

YPD: 10g bacto-tryptone, 10g bacto-yeast extract, 20g D-glucose;

YGSR: 10g bacto-tryptone, 10g bacto-yeast extract, 20g D-galactose, 20g raffinose, 20g sucrose;

SD: 0.67g yeast nitrogen base without amino acids, 20g D-glucose, supplemented with amino acids as required;

SGSR: 0.67g yeast nitrogen base without amino acids, 20g D-galactose, 20g raffinose, 20g sucrose, supplemented with amino acids as required;

To make agar plates, bacto-agar was added prior to autoclaving at 15lb/sq.inch for 20 minutes in a liquid cycle.

2.3. Bacterial Strains

E. coli B strain XL1-blue (*recA end A1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lac^rZΔM15Tn10 (Tet^r)]*) (Stratagene) was used for propagation of all plasmids.

2.4. Yeast Strains

The yeast strains used in this study are shown in table 2.1.

Table 2.1. Yeast strains

Strain	Genotype	Reference
YDL401	MATa <i>his3Δ200 leu2Δ1 trp1 ura3-52 gal2 galΔ108</i>	(Lafontaine and Tollervey, 1996)
BMA64	MATa <i>ade2-1 his3-11,-15 leu2-3,-112 trp1Δ1 ura3-1</i>	F. Lacroute
YMO1	as YDL401 but <i>GAL10::nop7-HIS3</i>	This work
YMO2	as YDL401 but <i>GAL10::nop7-TAP-TRP1</i>	This work
YMO3	as YMO2 but +[pUN100-DsRednop1 LEU1]	This work
YMO4	as YMO1 but +[pRS316-Rpl25-eGFP]	This work
YMO5	as BMA64 but +[pA3ura3]	This work
YMO6	as BMA64 but <i>GAL10::nop7</i> + [pA3ura3]	This work
YMO7	as YDL401 but <i>GAL10::rrp12-HIS3</i>	This work
YMO8	as YMO7 but <i>rrp12-TAP-TRP1</i>	This work
YMO9	as YMO8 but +[pUN100-DsRednop1 LEU1]	This work
YMO10	as BMA64 but <i>GAL10::rrp12</i> , pA3ura3	This work
YMO11	as YDL401 but <i>GAL10::nop15-HIS3</i>	This work
YMO12	as YMO11 but <i>nop15-TAP-TRP1</i>	This work
YMO13	as YMO12 but +[pUN100-DsRednop1 LEU1]	This work

YMO14	as BMA64 but <i>GAL10::nop15</i> +[pA3ura3]	This work
YMO15	as YMO12 but +[pRS316- <i>Rpl25</i> -eGFP]	This work
YCA31	as YDL401 but <i>GAL10::prot.A-RRP4</i> , <i>RRP6:(Kl)TRP1</i>	(Allmang et al., 1999)
GAL::DOB1	MATa <i>ura3-1 ade2-1 his3-11,-15 leu2-3,-112 trp1-1 Dob1::HIS3MX6</i> +[pAS24- <i>DOB1</i>]	(de la Cruz et al., 1998)
P170	as YDL401 but GAL:: <i>cs14-HIS3</i>	(Allmang et al., 1999)

2.5. Oligonucleotides and primers

Oligonucleotides used in this study for hybridisation of Northern blots and primer extension analyses are shown in table 2.2.

Table 2.2. Oligonucleotides

Oligonucleotide	Sequence	Remarks
002 (20S-2)	GCTCTTTGCTCTTGCC	Complementary to pre-rRNA 5' of site A ₂
003 (27SA ₃)	TGTTACCTCTGGGCCC	Complementary to pre-rRNA between sites A ₃ and B ₁
004 (20S)	CGGTTTTAATTGTCCTA	Complementary to 3' end of 18S rRNA
006 (27SB)	AGATTAGCCGCAGTTGG	Complementary to pre-rRNA 5' of site C ₂
007 (25S)	CTCCGCTTATTGATATGC	Complementary to pre-rRNA 5' of mature 25S

		rRNA
008 (18S)	CATGGCTTAATCTTTGAGAC	Complementary to mature 18S rRNA
013 (RNA2.1)	GGCCAGCAATTTCAAGT	Complementary to pre-rRNA 5' of site C ₂
017 (5.8S)	GCGTTGTTTCATCGATGC	Complementary to mature 5.8S rRNA
020 (ITS2-5'B)	TGAGAAGGAAATGACGCT	Complementary to pre-rRNA 3' of mature 5.8S rRNA
026 (5'ETS+911)	CCAGATAACTATCTTAAAA G	Complementary to pre-rRNA across site A ₁
033 (5'ETS+278)	CGCTGCTCACCAATGG	Complementary to pre-rRNA 278 nt downstream of site A ₀
041 (5S)	CTACTCGGTCAGGCTC	Complementary to mature 5S rRNA
5'ITS1-1	TGGA CTC5CCATCTCTTGAC 5TCTTGCC CAG5AAAAGCTC 5CATGCTC5T	Complementary to pre-rRNA 3' of site D; with 5 amino-C6-dT modifications

Primers used in this study for amplification by Polymerase Chain Reaction are shown in table 2.3.; sequences complementary to tagging-cassettes are shown in upper case, chromosomal sequences in lower case.

Table 2.3. Primers

Primer	Sequence
Ygr013w-HisF	cctgaaaaaattaaagaaaatTTTTgcgatgagatgagatgagaCTCTTGGCCTCCTC

	TAG
Ygr103w-Gal10	tacctctggtgtttttctcttgattctcattgtataagtaggtatCGAATTCCTTGAATTT TCAAA
Ygr103w-TAP	attgccaaacaaaaagctaaactgaataaactagattccaagaaaTCCATGGAAAAGA GAAAG
Ygr103w-Trp1	tgttgctataaccgaggaattataaagtaataaagatgttaaattaTACGACTCACTATA GGG
Ygr103w-BMA5'	aagcccttgtagtttcttagcaaaaagtcttgtgttctcta
Ygr103w-BMA3'	tttatgcctaaatattcgtggacggaccgtacaggtcttg
Ypl012w-HisF	agaacttctcggtcgcagttaattaattctctcagagaagagtttagCTCTTGGCCTCC TCTAG
Ypl012w- Gal10	ctaaaagaaaagcaactttgtcttgatccatgatgaactctgagatCGAATTCCTTGAA TTTTCAAA
Ypl012w-TAP	aaacataataagaaaggtccaaagtcaaactagaaaaaattaTCCATGGAAAAGA GAAG
Ypl012w-Trp1	aatatacagtattataatattcttatatacatatctgattttTACGACTCACTATAGGG
Ypl012w-BMA5'	cttaggcgaataatgaaatatcagatctagaagatatgta
Ypl012w-BMA3'	tttcttccaaatctagctaaccttattcgtactagacca
Ynl110c-HisF	tgaaaaatttccgccataagaaaaaatgaagtagtgcacgcacCTCTTGGCCTCCTC TAG
Ynl110c- Gal10	tccttggtggaagttttgcttgtgactttaccatcgcacgcacCGAATTCCTTGAATTT TCAAA
Ynl110c-TAP	gaaaggataaaaaaattagccaagtcaggtatcgaattcaaatggTCCATGGAAAAG AGAAG
Ynl110c-Trp1	aattaatgatataaatggttcccaagtttgctatatctcctattTACGACTCACTATAGG G
Ynl110c-BMA5'	aacgaattctaagaagccataatgtctggaatttcagtt
Ynl110c-BMA3'	atacatatgtcaaagtctagtgtaaattgatagaattaac

2.6. Radiolabelled compounds

All radiolabelled compounds were purchased from Amersham (UK):

[γ - ^{32}P]ATP (5000 Ci /mmol); [α - ^{35}S]ATP (Ci /mmol); [^3H]Uracil (1200Ci /mmol);

2.7. Plasmids

The plasmids used in this study are listed in table 2.4.

Table 2.4. Plasmids

Plasmid	Description	Reference/Remarks
pLT26	CEN – HIS3 –pGAL10	(Lafontaine and Tollervey, 1996)
pRS1147-TAP	CEN-TAP-TRP1	(Rigaut et al., 1999)
pRS316-Rpl25eGFP	CEN – URA3-ADE2	(Hurt et al., 1999)
pUN100-DsRedNOP1	CEN-LEU2	(Gadal et al., 2001b)
prDNA	rDNA	Dr. D. Tollervey
pA3	CEN-URA3	Dr. D. Lafontaine
pAS24- <i>DOB1</i>	CEN – <i>DOB1</i> - URA3	(de la Cruz et al.,1999)

2.8. Antibodies

Antibody	Description	Supplier
Peroxidase-anti-Peroxidase (PAP)	rabbit IgG	Sigma, UK
anti-Protein A	rabbit IgG, primary	Sigma, UK
anti-rabbit-FITC	goat, secondary	Sigma, UK

2.9. Bacterial techniques

2.9.1. Preparation of competent cells

Cells were prepared for transformation by the following procedure. *E.coli* were grown in LB to an optical density of 0.4 at 600nm. The cells were pelleted at 4000rpm for 15 minutes in a Model Avanti™ J-25 Centrifuge (Beckman), resuspended in ice-cold sterile 0.1M CaCl₂ and left on ice for an hour. The bacteria were recovered by centrifugation at 4000rpm and resuspended in ice-cold, sterile 0.1M calcium chloride/10% (v/v) glycerol (2ml suspension for each 50ml original culture). This suspension was incubated on ice for 18 hours, aliquoted, then frozen and stored at -80°Celsius.

2.9.2. Transformation of competent cells

Competent *E.coli* were transformed with DNA plasmid by the following method. 2µl plasmid [500ng/µl] were added to 100µl competent cells. The mixture was incubated on ice for 30 minutes, heat shocked at 42°C for 2 minutes. After the addition of 900µl of pre-warmed LB medium (37°C), the mixture was incubated for 1 hour at 37°C on a rotary shaker at 225rpm before plating on LB plates containing ampicillin.

2.10. Yeast techniques

2.10.1. Transformation of Yeast and selection

Yeast strains were transformed using a lithium acetate method (Gietz et al., 1992). An overnight yeast culture was grown YPD or YGRS to an optical density of 1.0 at 600nm (OD₆₀₀). The cells were diluted to an OD₆₀₀ of 0.1 in 50ml medium and grown to an OD₆₀₀ of 0.5 (10⁷ cells/ml). After harvesting, the cells were washed with 50ml sterile

water, centrifuged again and resuspended in 1ml of water. Then the cells were washed in 1ml LiTE (10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, 100mM lithium acetate). The cells were centrifuged again and, after two more washes in 500µl LiTE, resuspended in 500µl of LiTE. 1 to 5µg transforming DNA was added to 50µl of yeast suspension, as well as 5µl carrier DNA (herring sperm DNA, Boehringer) and 300µl fresh PEG solution (40% Polyethylenglycol 4000 in LiTE). The suspension was thoroughly mixed and incubated at 30°C under constant shaking for 30 minutes. The cells were heat shocked at 42°C for 15 minutes without shaking, centrifuged for 5 seconds and resuspended in 100µl 1xTE (10mM Tris-HCl pH 7.5, 1mM EDTA, pH 8.0) which were plated on selective plates and incubated at 30°C.

2.10.2. Recovery of plasmids from Yeast

This method was performed according to (Robzyk and Kassir, 1992). 3 ml of saturated yeast culture grown under selective conditions were harvested and resuspended in 0.1ml of STET (8% sucrose, 50mM Tris-HCl pH 8.0, 50mM EDTA pH 8.0, 5% Triton-X100). 0.2g of glass beads (Ø 0.45mm) were added and the suspension was vortexed for 5 minutes. After adding 100µl of STET, the mixture was briefly vortexed and boiled for 3 minutes. The suspension was cooled on ice and centrifuged at 4°C for 10 minutes. 100µl of supernatant were precipitated at -20°C with 50µl 7.5M ammonium acetate and 200µl of cold 100% EtOH for 1 hour and then centrifuged for 30 minutes at 4°C. The pellet was washed once with 70% EtOH, air-dried and resuspended in 20µl of sterile water. 5µl of DNA were used for transformation into yeast.

2.11. Recombinant DNA techniques

Standard recombinant DNA techniques were carried out according to (Sambrook et al., 1989). This includes agarose gel electrophoresis of DNA, ethidium bromide fluorescence, phenol/chloroform extraction and alcohol precipitation.

2.11.1. Restriction enzyme digests

All restriction enzymes and molecular weight markers were obtained from New England Biolabs, UK.

Restriction digests were performed in total volumes of 20µl, using approximately 0.5µg DNA per digest, and appropriate amounts of enzyme (usually 1µl) in buffer [1:10] and BSA (bovine serum albumine) [1:100] if required'. The buffer used was the one most suitable for the particular digest, according to the manufacturer's instructions. Digests were incubated at 37°C for 1-2 hours and then analysed on an agarose gel with appropriate molecular weight markers.

2.11.2. Plasmid preparation

Medium scale preparations of DNA were performed using the Quiagen plasmid preparation kit and following manufacturer's instructions for midi/maxi preparations.

2.11.3. Primer purification

Primers longer than 20 nucleotides were purified prior to use. 200µl of primer stock were loaded onto an 8 % polyacrylamide urea gel (1x TBE: 0.9M Tris-borate pH 8.3, 20mM EDTA) and run at 300V for an appropriate length of time. The primer band was cut out under UV light using a thin layer chromatography plate and, cut into small pieces, incubated with 400µl elution buffer (500 mM NH₄OAc, 0.1 mM EDTA, 0.1 % SDS) at RT overnight. The buffer was recovered and precipitated with 3 volumes of ethanol, washed, and after drying resuspended in appropriate volume of water.

2.11.4. Manual DNA sequencing

Dideoxy sequencing was used to sequence DNA constructs (Sanger et al., 1977). All sequencing reactions were performed on double strand plasmid DNA using the United States Biochemical (USB) Sequenase kit and following the manufacturer's instructions.

2.11.5. Polymerase Chain Reaction

PCR was used to amplify regions of DNA plasmids and regions of the yeast genome for cloning, to produce recombinant constructs, to generate DNA fragments and to identify recombinants. All applications followed the general protocol described below or modifications upon it.

PCR reactions in a volume of 50 to 100 μ l contained 1.5mM magnesium chloride, 0.2mM of each dNTP, 0.25 μ M of two oligonucleotide primers, 1x of the supplied reaction buffer, 10 U/ μ l thermostable polymerase and template DNA. The reactions were subjected to temperature cycling in a Hybaid Thermal Reactor (Biometra) with hotlid function. A typical temperature profile was:

94°C for 5 min, 1 cycle

94°C for 30 sec, 45°C for 1 min, 72°C for 3 min, 35 cycles

72°C for 10 min, 1 cycle

2.11.6. Polyacrylamide gel electrophoresis

Standard PAGE techniques were employed (Sambrook et al., 1989). Gels were normally 6 – 8% acrylamide-bis-acrylamide (30:1 cross-linking), 8M urea, 1x TBE. DNA sequencing gels were 40cm long and 0.4 mm thick; gels for separating low molecular weight RNAs were 20cm long and 1.5mm thick. Following electrophoresis sequencing gels were dried on Whatman 3MM paper before exposure to X-ray film.

2.12. DNA techniques

2.12.1. Preparation of total genomic yeast DNA

50ml of a yeast culture were grown to saturation, harvested and washed once with 50ml water. The cell pellet was then resuspended in 0.3ml of water and mixed with the following solution (2 % Triton-X100, 1 % SDS, 100mM sodium chloride, 10mM Tris-

HCl pH 8.0, 1mM EDTA pH 8.0). 0.3ml of phenol/chloroform were then added as well as 0.3g glass beads (\emptyset 0.45mm). The suspension was then vortexed for 3 minutes and centrifuged for 5 minutes. The supernatant was mixed with 1.2ml EtOH and incubated at -20°C for 10 minutes. After centrifugation for 30 minutes at 4°C the pellet was resuspended in water. RNase A (10mg/ml in water) was added and incubated at 37°C for 40 minutes. The solution was phenol/chloroform extracted twice and 3M sodium acetate was added to the final aqueous phase at a concentration of 1 in 10 μl . After addition of 3 volumes EtOH the DNA was pelleted and resuspended in 20 μl H_2O .

2.13. RNA techniques

2.13.1. RNA extraction

RNA extraction was essentially carried out as described in (Tollervey and Mattaj, 1987). 20 OD₆₀₀ of exponentially growing cells were harvested. 500 μl GTC (4M Guanadinium Thiocyanate, 0.1M Tris-HCl pH 7.5), 500 μl of phenol and 500 μl glass beads (\emptyset 0.45mm) were added and the mixture vortexed for 5 minutes at 4°C . 7.5ml of GTC and 7.5ml of phenol were added and the tube incubated at 65°C for 5 minutes. 7.5ml chloroform and 4ml sodium acetate (100mM sodium acetate in 1x TE) were added. The tube was centrifuged and the supernatant extracted twice with phenol/chloroform before the RNA was precipitated with 3 volumes of 100% EtOH. The pellet was washed with 70% EtOH, briefly air-dried and resuspended in H_2O . This procedure was scaled down as appropriate to the experimental requirements.

2.13.2. RNA gel electrophoresis and Northern blotting

Polyacrylamide and agarose electrophoresis was performed as has previously been described (Sambrook et al., 1989).

Low molecular weight RNAs (shorter than 500nt) were separated on 8% polyacrylamide urea gels (1x TBE: 0.9M Tris-borate pH 8.3, 20mM EDTA). The gels were stained with ethidium bromide in 0.5x TBE and transferred to Hybond N⁺ (Amersham) membranes by electroblotting in 0.5x TBE buffer at 50V for 4 hours.

High molecular weight RNAs (longer than 500nt) were separated on an agarose formaldehyde gel (1.2% agarose, 6 % formaldehyde, 1x Hepes). The gels were run in 1x Hepes buffer (50mM Hepes, 1mM EDTA, pH 7.8) overnight (16 hours at 60V) with circulation of the buffer. After migration was completed the gel was rinsed with water and washed with 75mM sodium hydroxide for 20 minutes followed by two washes with 0.5M Tris/ 1.5M sodium chloride to neutralise the gel and one wash with 10x SSC (1.5M sodium chloride, 150mM tri-sodium citrate pH7.0) for 20 minutes. The RNA was transferred in 10x SSC by capillary force to a Hybond N⁺ membrane (Amersham). The loading buffer for polyacrylamide gels was 47% formamide, 10mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol and for the agarose gels 50% formamide, 6% formaldehyde, 0.1x Hepes, 0.025% bromophenol blue, 0.025% xylene-cyanol and 0.2mg/ml ethidium bromide.

2.13.3. Hybridisation of Northern blots

The membranes were hybridised with 5'end-labelled oligonucleotides. The oligonucleotides used are listed in section 2.5. 10pmol of oligonucleotide was labelled in a 15µl reaction containing 70mM Tris-HCl pH 7.5, 10mM MgCl₂, 10mM DTT, 4pmol [γ^{32} -P]ATP, 10U polynucleotide kinase (Stratagene) at 37°C for 30 minutes. The filter was pre-hybridised for 30 minutes at 37°C in 6x SSPE (0.9M NaCl, 60mM NaH₂PO₄, 6mM EDTA, pH7.4), 5x Denhardt's solution (0.1% ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.5% SDS, 200µg/ml herring sperm DNA (Boehringer). The labelled probe was added to the hybridisation mix and hybridised, shaking, overnight at 37°C. Following hybridisation, the filter was washed twice in 6x SSPE at temperatures ranging from 37°C to 42°C, according to the T_m of the oligonucleotide. The filter was wrapped in saran-wrap and exposed to X-ray film.

2.13.4. *Primer extension analysis*

Primer extension analysis was performed as previously described (Beltrame and Tollervey, 1992). 4pmol of oligonucleotide primer was incubated in a volume of 10µl in 70mM Tris-HCl pH 7.6, 10mM magnesium chloride, 10 mM DTT, 12pmol [γ -³²P]ATP, 2U polynucleotide kinase (Stratagene) for 30 minutes at 37°C. The oligonucleotide was precipitated by the addition of 20µg glycogen, ammonium acetate to a concentration to 2M and 2 volumes of EtOH. The precipitated oligonucleotide was washed with 70% EtOH, briefly air-dried and resuspended in 50µl of water. 2µl of labelled oligonucleotide were incubated with 5µg of template RNA in 300 mM NaCl, 10 mM Tris-HCl pH 7.5, 2 mM EDTA pH 8.0 in a volume of 10µl, denatured at 80°C for 5 minutes and annealed at 46°C for 90 minutes. 40µl of pre-warmed buffer (1.25mM dATP, 1.25mM dCTP, 1.25mM dGTP, 1.25mM dTTP, 12.5mM DTT, 12.5mM Tris-HCl pH 8.4, 7.5mM magnesium chloride), 30U RNasin (Promega) and 7.5U AMV reverse transcriptase (Promega) were added and the reaction incubated for 45 minutes at 46°C. The reaction was stopped by addition of 50mM EDTA, 100mM sodium hydroxide and incubation at 55°C for 1 hour. The sodium hydroxide was neutralised by adding hydrochloric acid to a final concentration of 100mM and the RNA precipitated with EtOH/glycogen/ammonium acetate. The pellet was washed once with 70% EtOH, air-dried and resuspended in formamide loading buffer, diluted with water (1:2). Half of this reaction was run on a 6% PAGE urea sequencing gel. The same oligonucleotide which had been phosphorylated with [α -³⁵S]ATP was used to obtain a sequencing ladder that was run alongside the primer extension sample to enable the position of primer extension stops to be determined.

2.13.5. *Pulse-Chase analysis*

Pulse-chase labeling of pre-rRNA was performed as previously described (Tollervey et al., 1993) using 100µCi [5,6-³H]uracil (Amersham) for 2 minutes at 30°C. Unlabeled uracil was added to a final concentration of 240µgml⁻¹. Samples (1ml) were taken after 0, 1, 2.5, 5, 10, 20 and 60 minutes, transferred to micro centrifuge tubes at room

temperature and spun for 10 seconds at full speed in an Eppendorf centrifuge. Cell pellets were frozen in liquid N₂. Total RNA was extracted with phenol/chloroform and ethanol precipitated. [³H]-labeled pre-rRNA and rRNA was resolved on 1.2% agarose formaldehyde gels for high molecular weight and 8% acrylamide-8M urea gels for low molecular weight RNAs. RNA was transferred to HybondTM-N⁺ Nylon membranes (Amersham), dried and exposed to X-ray film for 30 days at -80°C with an intensifying screen.

2.14. Epitope tagging of proteins and production of conditional alleles

2.14.1. Construction of conditional GAL10 alleles

To construct conditional GAL10 alleles of specific genes a HIS3-pGAL cassette was amplified from the plasmid pTL26 using two primers containing flanking sequences specific to the sequence upstream of the target gene (Lafontaine and Tollervey, 1996). The amplified cassette was transformed into a yeast strain (YDL401) carrying a deletion in both, the HIS3 and the GAL locus, and integration was selected for on SGSR-HIS plates. The obtained transformants were repeatedly streaked onto SD-HIS plates to select for conditional alleles. Integration of the cassette was also confirmed by PCR.

2.14.2. Construction of TAP – tagged proteins

To construct proteins expressing a C-terminal tandem affinity purification tag (TAP-tag) a TAP-TRP1 cassette was amplified from a plasmid, pRS1147-TAP, using two primers containing flanking sequences specific to the sequence just upstream of the stop codon of the target gene (Rigaut et al., 1999). The amplified cassette was transformed into a yeast wild-type strain (D250) carrying a deletion in the TRP1 locus, and integration was selected for on SD-TRP plates. Integration of the cassette was confirmed by PCR and

expression of the TAP-tag was established by Western blot analysis using PAP antibodies. The tag contains a calmodulin-binding domain linked to the sequence of *Staphylococcus aureus* protein A by a cleavage site recognised by a proteinase from the tobacco etch virus (TEV). The TAP-tag can be detected in cells lysates using peroxidase-anti-peroxidase, anti-Protein A (Sigma) or anti-calmodulin (Invitrogen) antibodies (Rigaut et al., 1999).

2.15. Protein and immunological techniques

2.15.1. Preparation of Yeast extracts

Yeast cells were grown in 2 litres of YPD or YGSR to an optical density of 1.5 to 2.0 at 600nm. Cells were harvested by centrifugation, transferred into a 50ml-Falcon tube, washed once with 50ml cold, sterile water and once with 50ml of lysis buffer (100mM sodium chloride, 0.05mM Tris-HCl pH 7.5, 1.5mM magnesium chloride, 0.15 % NP40) containing protease inhibitor cocktail (Sigma) and 5mM PMSF. The cell pellet was resuspended in 10ml of lysis buffer containing 5mM PMSF, 1mM DTT and 2.5mM vanadyl-ribonucleoside complexes (VRC) (Sigma) and 5ml of acid-washed, siliconised glass beads were added. The cells were lysed by vortexing six times for 30 seconds with 30-second intervals on ice. The lysate was centrifuged for 5 minutes at 5krpm to pellet the glass beads and any unbroken cells. The lysate was then transferred into a sterile Beckman 50ml-polycarbonate tube for the rotor JA-25.50 (Beckman). After centrifugation at 14krpm for 1 hour at 4°C the cleared extract was removed from underneath the floating lipid layer and transferred to a 50ml-Falcon tube with glycerol to a final concentration of 5%. The extract was frozen at -80°C.

2.15.2. Immuno - precipitation/Affinity purification

TAP – tagged yeast proteins were precipitated using total rabbit IgGs and calmodulin-binding protein immobilised on agarose (Sigma) (Lygerou et al., 1994; Rigaut et al., 1999). 500µl of 50% suspension of rabbit IgG agarose beads were washed three times in lysis buffer and then added to 3000-4000 OD₆₀₀ equivalents of extract. After incubation for 1 hour at 4°C on a rotating-wheel, the IgG agarose beads were washed once with lysis buffer. The beads were taken up in 1ml lysis buffer containing 0.5mM DTT and transferred to Econo-column (Bio-Rad). 2U recombinant TEV protease were added and incubated over night at 4°C. The supernatant containing immuno-precipitated proteins and RNAs was drained from the column and collected. For analysis of co-precipitated RNAs, the supernatant was, after extraction with phenol/chloroform and precipitation with ethanol, used for Northern blots and primer extension analysis.

For analysis of co-precipitated proteins, the supernatant was diluted 1:4 with lysis buffer containing 4mM calcium chloride and 1mM DTT and added onto an Econo-column (Biorad) containing equilibrated calmodulin-binding beads. The column was incubated 1 hour at 4°C on a rotating wheel. The supernatant was discarded and the beads washed with 5ml lysis buffer containing 2mM calcium chloride. The proteins were eluted from the beads by incubation with 600µl elution buffer (10mM Tris-HCl pH 8.0, 5mM EGTA) at 37°C for 10 minutes. The eluate was taken off and after precipitation with 10% tri-chloroacetic acid, the proteins were analysed by Western blot and Coomassie staining.

2.15.3. Sucrose density gradient centrifugation

For the isolation of ribosomes under low salt conditions by sucrose gradient centrifugation 500 ml of a yeast culture was grown in YPD medium to an OD₆₀₀ of 0.5–0.8 before cycloheximide was added to a final concentration of 100µg/ml. Cells were harvested by centrifugation and washed in 10ml ice-cold lysis buffer (100mM sodium chloride, 0.05mM Tris-HCl pH 7.5, 1.5mM magnesium chloride, 0.15 % NP40) containing protease inhibitor cocktail (Sigma), 5mM PMSF, 1mM DTT and 2.5mM

VRC (Sigma). 1.4g glass beads (\emptyset 0.45mm) were added to 0.5ml of cell suspension. Cells were broken by vortexing for 5 minutes at 4°C. The suspension was centrifuged for 5 minutes at 14krpm. The supernatant (300 μ l) was loaded onto a 10%–50% sucrose gradient in lysis buffer without DTT and cycloheximide and centrifuged for 15 hours at 27krpm in an Ultracentrifuge SW40-Ti rotor (Beckman). 0.5ml-fractions were collected, of which 250 μ l were precipitated with TCA and analysed by SDS-PAGE and Western blot analysis. 250 μ l were used for RNA extraction and analysis by Northern hybridisation.

2.15.4. SDS-Polyacrylamide electrophoresis

Proteins were separated on polyacrylamide gels containing SDS as described by (Laemmli, 1970). Samples were denatured by adding an equal volume of protein loading buffer (125mM Tris-HCl pH 6.8, 200mM DTT, 4% SDS, 40% glycerol, 0.025% bromophenol blue), heating to 100°C for 5 minutes and centrifugation for 30 seconds. Pre-cast 4 – 12% gradient gels or 10% gels (Invitrogen) were run in 1x MOPS buffer (50mM 3-(N-morpholino) propane sulphonic acid, 50mM Tris base, 3.5 % SDS, 1.25mM EDTA) at 200V for 1 hour. Protein bands were visualised by silver staining.

2.15.5. Silver staining of SDS-Polyacrylamide gels

The gel was fixed in destain (5% acetic acid, 45% methanol) for 30 minutes. To remove the acid the gel was rinsed with water for 1 hour and then sensitised in 0.02% sodium thiosulphate solution for 2 minutes. The gel was stained with 0.1% silver solution (AgNO_3) for 5 minutes. After rinsing twice with water, the gel was developed with developing solution (0.04% formalin, 2% sodium thiosulphate) until bands were sufficiently visible. The solution was replaced when turning yellow. The development was stopped with 1% acetic acid and the stained gel stored in 1% acetic acid solution at 4°C.

2.15.6. Western blotting

Following separation of proteins by SDS-PAGE the proteins were transferred to a nitro-cellulose membrane (Schleicher & Schuell) in a Semi-Dry blot apparatus (Bio-Rad).

The gel was assembled on the nitro-cellulose membrane between 3 sheets of Whatman paper on each side, soaked in transfer buffer (25mM Tris base, 40 mMglycine, 0.05% SDS, 20 %methanol) and taking care to remove air bubbles from between the layers. Transfer was performed at 150mA for 2 hours. After the transfer the membrane was blocked in 3% low-fat dried milk (w/v) in PBS for 30 minutes to 1 hour shaking at room temperature.

Depending on the protein to be detected the blot was decorated with different immunological reagents. For detection of Protein A or TAP-tagged proteins the membrane was incubated with a 1:1000 dilution of rabbit IgG antibody coupled to horseradish peroxidase-anti-peroxidase (PAP, Sigma). After incubation for 2 hours at room temperature or overnight at 4°C, the blot was washed four times 15 minutes with 1x phosphate buffered saline (PBS; 137mM sodium chloride, 3mM potassium chloride, 10mM di-sodium phosphate, 2 mM potassium di-phosphate) and proteins were detected using an enhanced chemiluminescence (ECL) kit (Amersham), following the manufacturers instructions.

2.16. Immunofluorescence and Microscopy

2.16.1. Visualisation of epitope-tagged proteins

Cells were grown to an optical density of 1.0 at 600nm and fixed with 4% formaldehyde for 10 minutes at room temperature. The culture was centrifuged and the cells spheroplasted by resuspension in 1ml solution A (1.2M sorbitol, 100mM potassium phosphate, pH 7.4) 0.2mg/ml zymolyase (Seikagaku Kogyo) and incubation at 30°C for 8 minutes. The cells were washed with solution A, spotted onto L-polylysine-coated coverslips and incubated for 10 minutes in a humid chamber. Unbound cells were

removed with 1x PBS/1.2M sorbitol. Polylysine-bound cells were dehydrated in 100% methanol at -20°C for 6 minutes and flattened by incubation with ice-cold acetone for 30 seconds. The cells were washed twice with 1x PBS and after blocking with 5% milk/1x PBS for 30 minutes, were incubated with an antibody against the epitope diluted in 5% milk/1x PBS (1:1000) over night at 4°C . Following incubation with the primary antibody, the cells were washed twice with 5% milk/1x PBS and incubated with a secondary antibody at a dilution 1:200 in 5% milk/1x PBS for 1 hour in a dark, humid chamber. The cells were washed twice with 1x PBS and mounted onto slides using Vectashield (Vector Laboratories, USA), containing DAPI, and sealed with nail-varnish (Boots).

For visualisation of fluorescent epitopes cells were fixed in 4% formaldehyde for 10 minutes at room temperature and viewed using a Zeiss Axioskop upright fluorescence microscope.

2.16.2. Fluorescent labelling of RNA oligonucleotides

Fluorescent labelling of RNA probes for In Situ hybridisation were performed using an Amersham Cy3 ULS labelling kit following manufacturer's instructions.

2.16.3. RNA Fluorescent In-Situ Hybridisation

Cells were grown to an optical density of 0.5-1.0 at 600nm and fixed with 2% formaldehyde/5% acetic acid for 10 minutes at room temperature. The culture was centrifuged and the cells spheroplasted by resuspension in 1ml solution A (1.2M sorbitol, 100mM potassium phosphate, pH 7.4) containing 28mM β -mercaptoethanol, 0.5mM PMSF, 0.9 μM aprotinin, 0.5 μM leupeptin, 0.7 μM pepstatin A, 20mM VRC (all Sigma) and 0.2mg/ml zymolyase (Seikagaku Kogyo), and incubation at 30°C for 8 minutes. The cells were washed with solution A, spotted onto L-polylysine-coated coverslips and incubated at 4°C for 30 minutes. Unbound cells were removed with ice-cold solution A. Polylysine-bound cells were dehydrated in 100% EtOH at -20°C for 30

minutes and re-hydrated in 2x SSC (300mM sodium chloride, 30mM sodium citrate, pH 7.0). After cells had been washed once with 2x SSC containing 40% formamide the coverslips were incubated with hybridisation buffer (2x SSC, 40% formamide, 2.5mg/ml BSA, 10mM VRC) containing 100ng Cy3-labelled RNA oligonucleotide, 100µg herrings sperm DNA (Boehringer) and 100µg *E.coli* tRNA (Boehringer) for 16 hours at 37°C.

After incubation cells were washed twice with 2x SSC/40% formamide at 37°C, twice with 1x SSC (150mM sodium chloride, 15mM sodium citrate, pH 7.0) at room temperature and once with 1x PBS at room temperature. Cover-slips were mounted onto slides using Vectashield (Vector Laboratories, USA), containing DAPI, and sealed with nail-varnish (Boots). The fluorescent signals were visualised in the rhodamine channel of a Zeiss Axioskop upright fluorescence microscope.

2.16.4. Fluorescence Microscopy

Fluorescent images were observed by using a Zeiss Axioskop upright fluorescence microscope and were captured with a Coolsnap HQ device camera with Smart capture IP software. Images were assembled with Adobe Photoshop 5.5.

Chapter Three

Nop7p is required for multiple activities during 60S ribosomal subunit synthesis.

3.1. Introduction

A proteomic analysis of the human nucleolus identified 271 putative nucleolar proteins including Pes1 (Andersen et al., 2002). A database search clearly identified YGR103w as the probable yeast homologue with 40% identity to human Pescadillo. YGR103w was originally published under the name of *YPH1* (Yeast Pescadillo Homologue) (Kinoshita et al., 2001) but has been designated as *NOP7* (Nucleolar Protein) by the Saccharomyces Genome Database.

The Pescadillo gene was initially identified in Zebrafish as the site of a retrovirus insertion, which resulted in defects in embryonic development (Allende et al., 1996). Pescadillo mRNA showed widespread expression in developing mouse embryo brain with increased protein levels in replicating cells (Kinoshita et al., 2001). Protein levels were also increased in malignant cells (Charpentier et al., 2000; Kinoshita et al., 2001), which could possibly relate to increased nucleolar size and ribosome synthesis in such cells. In addition, Pescadillo was observed to contain a BRCT domain (Haque et al., 2000), which was originally identified in the breast and ovarian cancer gene BRCA1 and has been identified in several proteins involved in cell-cycle checkpoints and DNA repair (Bork et al., 1997). Pescadillo is localized to the nucleolus in HeLa cells (Kinoshita et al., 2001) and the *S.pombe* homologue, SPBC19F5.05c, has also been found to be nucleolar in a high throughput screen for sub-cellular localization of GFP fusion proteins (Ding et al., 2000).

The Yeast pescadillo homologue was first analyzed for defects in cell cycle progression since the human pescadillo (Pes1) shares significant similarities (40%) with the *Saccharomyces cerevisiae* homologue, including the BRCT domain and putative SUMOylation sites (Figure 10A and B) (Kinoshita et al., 2001). Yph1p/Nop7p is essential for viability and two temperature sensitive (ts) lethal mutant alleles have been reported to block growth at different steps in the cell-cycle; *yph1-24* led to arrest in G1, while the *yph1-45* allele caused G2 arrest (Figure 10) (Winzeler et al., 1999; Kinoshita et al., 2001). G1 arrest is expected for mutations defective in ribosome

A

human	1	MGGLEKKKYERGSATNYITRNKARKKLOLSLADFRRLCILKGIYPHEPKHKKKVNKGSTA	60
zebrafish	1	MGGLQKKKYESGSATNYITRNKARKKLSLSLADFRRLCILKGIYPHEPKHKKKVNKGSTA	60
yeast	1	MRIKKKNTRGNARNFITRSQAVRKLQVSLADFRRLCIFKGIYPREPRNKKKANKGSTA	58
human	61	ARTFYLIKDIRFLLHEPIVNFREYKVFVRKLRKAYGKSEWNTVERLKDKNPNYKLDHII	120
zebrafish	61	ARTYLLKDIRFLLHEPIVNGFREYKIFVRKLRKAYGKAWSAVERLKENKPGYKLDHII	120
yeast	59	PTTFYAKDIQYLMHEPVLAKFREHKTFAKLRALGRGEVSSAKRLEENRDSYTLDHII	118
human	121	KERYPTFIDALRDLDLDDALSMCFLFSTFPRTGKCHVQTIOLCRRLTVEFMHYIIAARLRK	180
zebrafish	121	KERYPTFIDALRDVDDALSMCFLFSTFARTGKCHVQTIOLCRRLSVEWMNYIISSRLRK	180
yeast	119	KERYPSFPDAIRDIDDALNMLFLFSLNLPSTNQVSSKIINDAQKICNQWLAYVAKERLVRK	178
human	181	VFLSIKGIYYQAEVLGQPIVWITPYAFSHDHPDQVYRVMATFTEFYTTLLGFVNFRLYO	240
zebrafish	181	VFLSIKGIYYQAEVLGQTIWIVPYQFAHNHPTDQVYRVMATFTELYTTLLGFVNFRLYO	240
yeast	179	VFVSIKGVVYQANIKGEEVRLVPPKFPENIPSDVDFRIMLTFLEFYSTLLHFVLYKLYT	238
human	241	LLNLHYPPKLEGOAQ-----AEAKAG-EGTYALDSESCMEKLAALSASLARVVVP	289
zebrafish	241	TLNLVYPPKLDGQGE-----LSLKAEFEDYALESESYTEKLSALSASLARVMAS	290
yeast	239	DSGLIYPPKLDLKKDKIISGLSSYILESROE-DSSLKLDPTETEDVKVESLDASTLKSA	297
human	290	ATEEEAEVDEFFPTDGEMSAQEE-DRRKEL-----EAQEKHK-----	324
zebrafish	291	VEEEEAELDHPFTEGEDQEKME-VREKME-----QQOSKOK-----	325
yeast	298	LNADAEANTDETEKEEKEQKQEKQEKQNEETELDTFEDNNKNGDILIQPSKYDSPVA	357
		-----BRCT Domain-----	
human	325	KLFEGLKFFLNREVPREALAFIIRSFGEVSWDKSL-CIGATYDVTDSRITHQIVDRPQG	383
zebrafish	326	KLFEGLKFFLNREVPRESLAFVIRCFGGEVSWDKSL-CIGSTYRATDETITTHVDRPQM	384
yeast	358	SLFSAFVYVSREVPIDILEFLILSCGGNVISEAAMDQIENKKDIDMSKVTHTQIVDRPVL	417

human	384	QTSVIGRCYVOPWVFDVSNARLLLPVAEYFSGVQLPPLHSPFVTEKEGDIVPPEKLLK	443
zebrafish	385	DKQYINRYIOPWVYDSVNAKIQLPVEEYFLGVTLPPHLSPFVEETEGDIVPPEKLLK	444
yeast	418	KNKVAGRTYIQPQWIFDCINKGELVPAKYLPGEALPPLHSPWGDATGIDYPTAP-----	471
	Acidic Domains.....	
human	444	ALQRGEDPGLNLSSEEEEEEDDNEGDGDEGENEEEEEDAEAGSEKKEEARLAALAEQR	503
zebrafish	445	ALQRGEKP----QAEDEEEEGEEED-DEE--DEEDDEQSEDEEEBAEENLAEMEKR	497
yeast	472	VEE-GEEE----ESESESESEDQVEEE-DOEVVAGEEDDDDEELQAQKELEEAQGIKY	525
		SUMO Nuclear Localization Signals	
human	504	MEGK--KPRVMAGTLKLEDKQRLAQEESEAKRLAIMMMKKREKYLQKIMFGKRRKIRE	561
zebrafish	498	SQGSLSVKVTPGKAKAENRARAEEBEKABEKRLAIMMMKKREKYLQKIMFGKRRKIRE	557
yeast	526	SETS----EADKDVNKSNNKRRKVDDEE-BEKKLMIMMSNKKKLYKMKMYSNAKKEEQ	580
human	562	ANKLAEKRK--AHDEAVRSEKKAKKARPE	588
zebrafish	558	ANKLAAKRK--AHDDASKADKKKKKCC	582
yeast	581	AENLKKKKQIAKQKAKLNKLDSSK	605

B

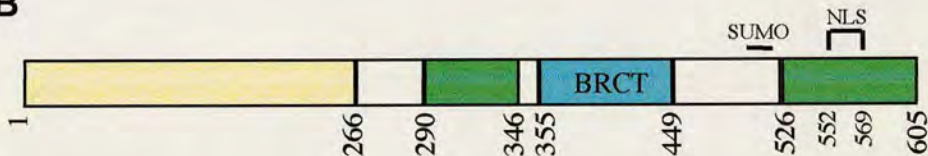


Figure 10: Sequence conservation among pescadillo homologues and a schematic diagram of Nop7p.

(A) A sequence alignment showing the conservation among human, zebrafish and yeast pescadillo proteins. Identical aminoacids are shaded dark grey and similar amino acids are shaded light grey. The schematic illustrates the location of several structural motifs including the BRCT domain, the two acidic domains, the presumptive nuclear localisation signal and the most conserved site for SUMOylation. The asterisks denote the localisation of the *yph1-24* and *yph1-25* point mutations (Kinoshita et al., 2001).

(B) Diagram of Nop7p. Two putative coiled-coiled domains are marked in green, the BRCT domain is marked in blue and the conserved region in yellow.

synthesis, which are unable to pass the “Start” checkpoint control, but G2 arrest would not normally be predicted for a ribosome synthesis defect. Based on these observations, Pescadillo and Yph1p/Nop7p were proposed to perform some cell-cycle specific function. Very recently, this conclusion was confirmed by the finding that Yph1p/Nop7p interacts with the origin recognition complex (ORC) and associates with replication and checkpoint proteins in proliferating cells (Du and Stillman, 2002).

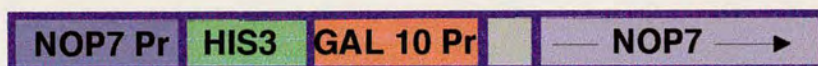
The different functions pescadillo and its yeast homologue, Nop7, appeared to have and the presence of Pes1 in human nucleoli led to the question if Nop7p could also be involved in ribosome biogenesis (Andersen et al., 2002). The further finding that Nop7p binds to the nuclear pore complex (NPC) directed us not only to study its possible involvement in ribosome synthesis but also in export of ribosomal subunits (Rout et al., 2000).

3.2. Nop7p-depleted cells are defective for rRNA processing of 60S precursor

3.2.1. Nop7p is required for pre-rRNA processing

To examine the *in vivo* function of Nop7p, its expression was placed under the control of a repressible galactose promoter (Figure 11A) using a one-step PCR technique in strain YDL401 (see Chapter 2, section 2.13). The host strain, YDL401, has reduced galactose permease activity leading to reduced *GAL*-induction (Lafontaine and Tollervy, 1996). This eliminates the over-expression generally seen with *GAL*-regulated constructs and allows faster appearance of phenotypes following transfer to repressive glucose medium. Growth of the *GAL::nop7* strain (YMO1) was not clearly different from the isogenic wild-type strain (YDL401) on permissive galactose medium but was progressively slowed following transfer to repressive glucose medium and depletion of Nop7p (Figure 11B and C).

A



B

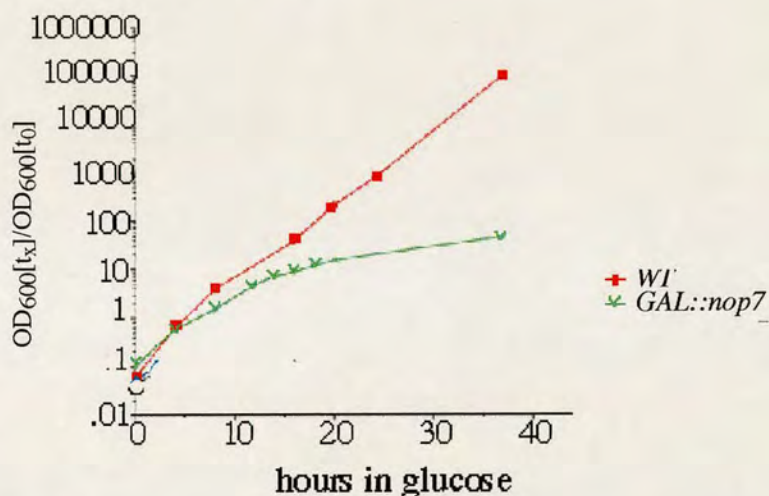


Figure 11: Construction and growth curve of a conditional *GAL::nop7* strain.

(A) Representation of the chromosomal *NOP7* gene locus (not to scale) in the *GAL10::nop7* strain (YMO1). A *HIS3-GAL10* promoter cassette was inserted 20nt upstream of the *NOP7* ATG start codon by site-specific recombination using PRC-generated targeting sequences in a *gal10-Δ* wild-type strain (YDL401).

(B) Growth curve of the *GAL10::nop7* strain (YMO1) and the otherwise isogenic wild-type strain (YDL401) after shift from permissive galactose medium to repressive glucose medium.

During synthesis of mature ribosomal RNAs, in yeast the early processing steps at sites A₀, A₁ and A₂ take place in the 5'ETS and the ITS1 yielding the 20S pre-rRNA which is transported into the cytoplasm, dimethylated and converted into mature 18S rRNA by endonucleolytic cleavage at site D (Venema and Tollervey, 1999). 18S rRNA formation requires several ATP-dependent RNA helicases (de la Cruz et al., 1999) and transport factors to export the 20S rRNA-containing pre-ribosomes to the cytoplasm (Moy and Silver, 1999; Stage-Zimmermann et al., 2000).

Processing at sites A₁ and A₂ are both endonucleolytic events (Petfalski et al., 1998). Furthermore, the cleavage at those sites is believed to be coupled since no mutation has been identified that allows processing at A₂ without prior cleavage at A₁ (Allmang and Tollervey, 1998). Mutations inhibiting A₁ or depletion of early *trans*-acting factors are found to accumulate aberrant 22S RNA, extending from A₀ to A₃, or 23S RNA, extending from 5'ETS to A₃, indicating that processing at site A₂ is also inhibited and cleavage at site A₃ occurs prior to cleavage at A₁ or A₂ (Venema et al., 1995b; Tollervey, 1996).

Cleavage at site A₂ also gives rise to the 27SA₂ precursor, which is processed further into 27SB_L and 27SB_S species by two alternative pathways in ITS1 (see Chapter 1, Figure 2). In the major pathway the pre-rRNA is cleaved at site A₃ by RNase MRP, forming the 27SA₃ pre-rRNA, and subsequent exonuclease digestion by Rat1p to site B_{1S} generates the 5' end of 27SB_S (Henry et al., 1994; Lygerou et al., 1996). The alternative, poorly understood, pathway processes the pre-rRNA at site B_{1L}, the 5' end of 27SB_L pre-rRNA. Both 27SB precursors are processed following identical pathways to generate mature 25S rRNA and 5.8S_S or 5.8S_L rRNA (see Chapter 1, Figure 2). Processing at sites C₁ and C₂ generates the 5' end of mature 25S rRNA and the 3' end of the 7S_S and 7S_L pre-rRNA, precursor to mature 5.8S rRNA. Processing of 7S pre-rRNA to the 5.8S rRNA requires a 3'→5' exonucleases complex, the exosome, and the putative RNA helicase Dop1p/Mtr4p, which has been shown to function in conjunction with the exosome (Mitchell et al., 1997; de la Cruz et al., 1999; Allmang et al., 2000). The exosome is composed of 11 components all of which, except Csl4p, have either been shown to be 3'→5' exoribonucleases *in vitro*, or are predicted to have this activity based

upon their sequence homology (van Hoof et al., 2000b). Mutations in exosome components like cause accumulation of 3' extended forms of 5.8S rRNA, showing their involvement in 5.8S rRNA 3' end processing, as well as aberrant RNAs, like 22S, 23S, extended forms of the 7S precursor and the 5'ETS-A₀ fragment all of which are degraded by the exosome in wild-type cells (Mitchell et al., 1996; Briggs et al., 1998; Allmang et al., 2000).

In cells, steady-state levels of ribosomal RNAs can be determined by Northern analysis. After transfer to a nitrocellulose membrane, specific rRNAs and pre-rRNAs can be visualized on an autoradiograph following hybridization with different radioactive labeled oligonucleotides complementary to certain sequential regions on the RNA precursors. This analysis allows the determination of different levels of rRNA precursors and mature rRNAs in mutant strains compared to wild-type and at different time points during cell growth. Some pre-rRNAs like 27SB_L and 27SB_S or cytoplasmic, dimethylated 20S pre-rRNA compared to nuclear 20S pre-rRNA are difficult to distinguish by Northern analysis. In these cases it is possible to use analysis by primer extension. An oligonucleotide is hybridized to the RNA precursor and extended by reverse transcription along the 5' end. According to the position of the oligonucleotide the analysis can visualize different precursors, all extending to the same 5' end. This method also makes it is possible to establish inhibition of cleavages at specific sites since precursors accumulated due to a block at a certain cleavage site can be detected by primer extension. Furthermore, primer extension analysis is much less affected by RNA degradation compared to analysis by Northern hybridization and can detect even low levels of RNA. Both of these methods were used to assess the effects of Nop7p depletion in yeast cells.

A problem of these methods, however, is to deduce flux of RNA precursors through the pathway from steady-state levels of RNA. During ribosome synthesis RNA intermediates are processed rapidly. Consequently, elevated steady-state levels of a certain pre-RNA species can either denote slow processing or a drop in synthesis and fast degradation of the non-processed precursor. Hence, it is necessary to interpret data obtained from steady-state level RNA carefully and in conjunction with other results.

Depletion of Nop7p resulted in mild accumulation of the 35S primary transcript and the appearance of low levels of the 23S RNA, but had little impact on levels of the 27SA₂ or 20S pre-rRNAs, or the mature 18S rRNA (Figure 12A). In contrast, the level of the 27SB pre-rRNAs was clearly reduced by 8 hours after transfer to glucose medium and the mature 25S rRNA was depleted over time.

Analysis of low molecular weight RNAs showed progressive reduction in the levels of the 7S and 6S pre-rRNAs following transfer of the *GAL::nop7* strain to glucose medium (Figure 12B, panel b). The level of the mature 5.8S was also reduced (Figure 12B, panel c) and the reduction in 5.8S_S appeared slightly greater than for 5.8S_L. Mature 5.8S_S rRNA is processed from the 27SB_S pre-rRNA, while 5.8S_L is processed from 27SB_L (see Chapter 1, Figure 2). To determine the levels of the 27SB species they were analysed by primer extension using an oligonucleotide hybridising within the 3' region of ITS2 (Figure 13A). Following growth of the *GAL::nop7* strain on glucose medium, the level of 27SB_S was clearly reduced relative to 27SB_L, as shown by the primer extension stops at B_{1S} and B_{1L}, respectively (Figure 13A, lane 4). Consistent with the Northern analysis, little alteration was seen in the level of 27SA₂, as shown by the primer extension stop at site A₂. In contrast, the level of 27SA₃, shown by the stop at site A₃, was substantially elevated. Using a primer hybridising within the mature 25S rRNA slight accumulation was seen for the primer extension stop at site C₂, the 5' end of the 26S pre-rRNA (Figure 13B). However, this effect was weak. The pre-rRNA that extends from A₂ to C₂ was not accumulated during Nop7p depletion (Figure 12B, panel a). This together with the depletion of 27SB and 7S pre-rRNAs indicate that the inhibition occurs prior to the cleavage at site C₂.

3.2.2. Synthesis of mature 5.8S rRNA is delayed in *NOP7*-mutants.

To determine if lack of Nop7p causes a delay in the synthesis of mature rRNAs a pulse-chase analysis with [³H]-uracil was performed 16 hours after transfer to glucose minimal medium (Figure 14). Pulse-chase experiments enable us to observe the fate of newly synthesized RNAs (Tollervey et al., 1993). Using radioactive labelled uracil in a

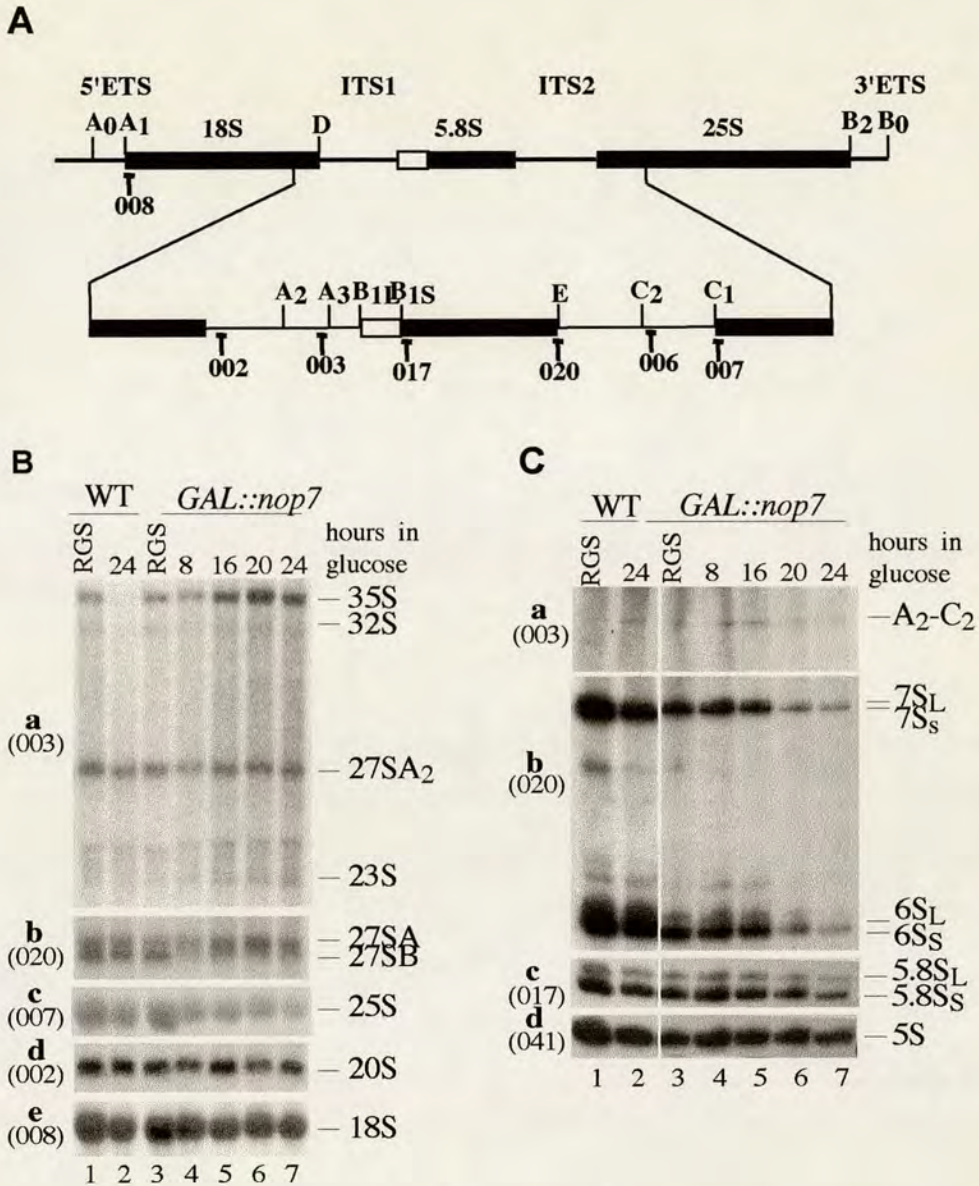


Figure 12: Northern analysis on the effects of Nop7p depletion on pre-rRNA processing.

Lanes 1 and 2: wild-type strain (YDL401) in permissive galactose medium and 24 hours after transfer to repressive glucose medium. Lanes 3 to 7: *GAL::nop7* strain (YMO1) in galactose and after transfer to glucose medium for the times indicated.

(A) Structure and processing site of the 35S pre-rRNA. This precursor contains the mature 18S, 5.8S and 25S rRNAs, which are separated by two internal transcribed spacers, ITS1 and ITS2, and flanked by two external transcribed spacers, 5'ETS and 3'ETS. The positions of oligonucleotide probes utilized in northern hybridisation and primer extension analyses are indicated.

(B) Northern analysis of high molecular weight RNAs. Probes used are indicated in parentheses on the left.

(C) Northern analysis of low molecular weight RNAs. Probes used are indicated in parentheses on the left.

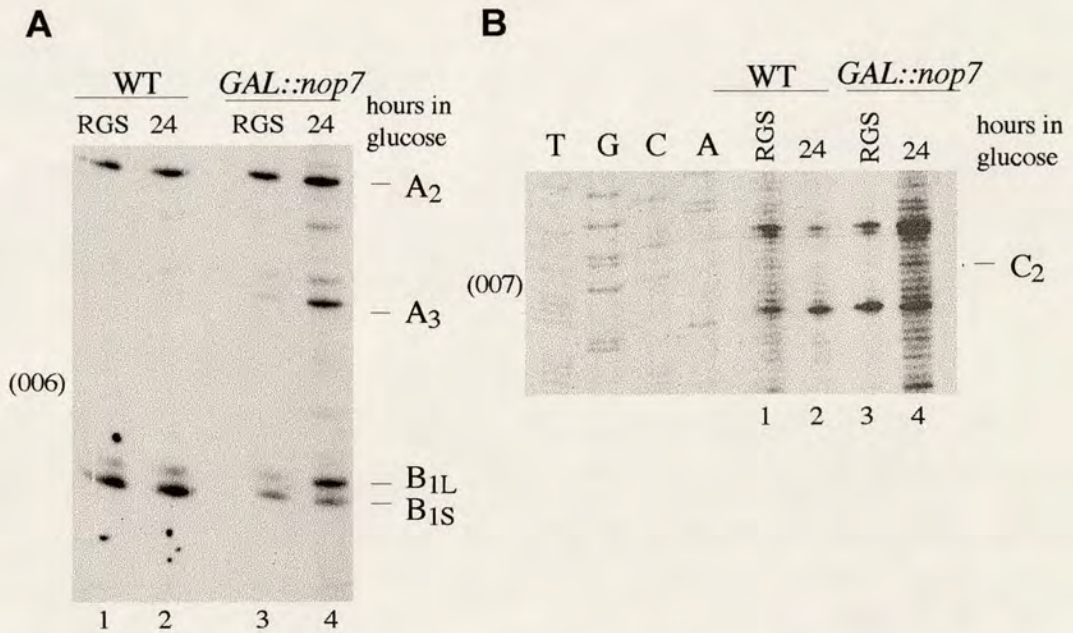


Figure 13: Levels of rRNA precursor in Nop7p depleted cells.

Lanes 1 and 2: wild-type strain (YDL401) in permissive galactose and 24 hours after transfer to repressive glucose medium. Lanes 3 and 4: *GAL::nop7* strain (YMO1) in galactose medium and 24 hours after transfer to glucose medium.

(A) Primer extension using oligo 006 (see Chapter 2, Table 2.2). Primerextension stops at sites A₂, A₃, B_{1S}, and B_{1L} show levels of the 27SA₂, 27SA₃, 27SB_S and 27SB_L pre-rRNAs, respectively.

(B) Primer extension using oligo 007 (see Chapter 2, Table 2.2). The primer extension stop at site C₂ shows the level of 26S pre-rRNA.

URA3⁺ yeast strain, the pulse creates a pool of radioactive labelled RNAs. Since transcription of rDNA takes up to 5 minutes, a longer pulse is preferable to find radioactive uracil incorporated sufficiently. Using an excess of unlabelled uracil during the chase, more unlabelled than labelled RNA molecules are synthesised depleting the pool of labelled RNAs. Ribosomal RNAs and pre-rRNAs are very abundant and, except for some precursors which are processed very quickly like 35S or 32S pre-rRNA, easily detectable in wild-type cells. The short-lived precursors can only be observed in mutant strains with delays during early processing steps. However, it should be mentioned that incorporation of labelled nucleotides into RNA is less efficient for some yeast strains compared to others, making it difficult to obtain a strong signal.

Comparison of the wild-type (YMO5) and *GAL::nop7* (YMO6) strains showed that although processing of the 27SB precursor was slowed down in the *GAL::nop7* strain over the wild-type, synthesis of 25S rRNA was not delayed (Figure 14A). However, analysis of low molecular weight RNAs showed that synthesis of the 5.8S rRNA was mildly delayed (Figure 14B).

Together these results show that depletion of Nop7p resulted in reduced exonuclease digestion from site A₃ to site B_{1S}. In consequence, the level of the 27SA₃ pre-rRNA was substantially increased while the 27SB_S pre-rRNA was depleted, together with the 7S_S and 6S_S pre-rRNAs, leading to reduced accumulation of the mature 5.8S_S rRNA. The 5' end of the 25S rRNA is also generated by exonuclease digestion from site C₂ (Geerlings et al., 2000). However, this did not appear to be strongly affected since only a small increase was seen in the primer extension stop at site C₂ and synthesis of 25S rRNA was not delayed. The mild effects on 35S processing are likely to be indirect. Accumulation of 35S next to 23S pre-rRNA indicates that this is the consequence of delayed processing at sites A₀ to A₂ rather than premature cleavage at site A₃. Many mutations that inhibit synthesis of 60S subunits were shown to result in partial inhibition of the early pre-rRNA processing steps, however, the basis of this delay is unclear (Venema and Tollervey, 1999).

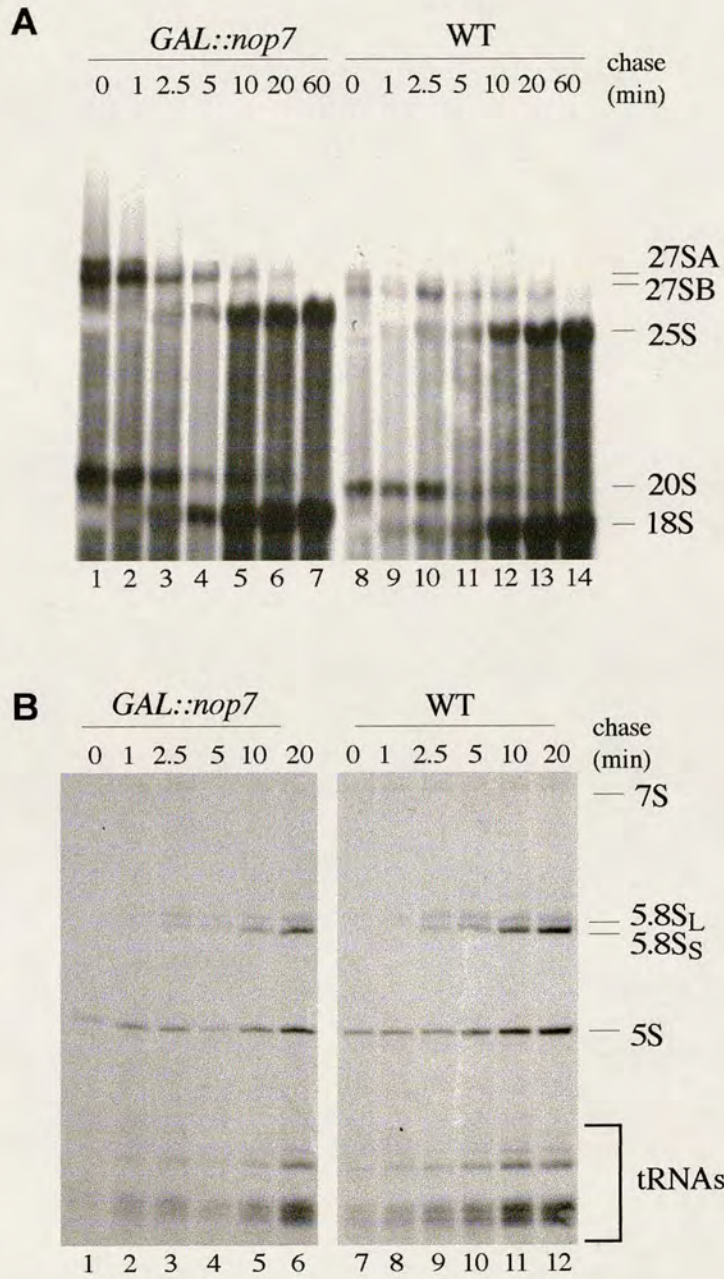


Figure 14: Synthesis of 5.8S rRNA is delayed in Nop7p depleted cells.

Pre-rRNA was pulse labelled with [³H]uracil for 2 minutes at 30°C and chased with a large excess of unlabelled uracil for the times indicated. Labelling was performed for the *GAL::nop7* (YMO6) strain (lanes 1 to 6) and a wild-type strain (YMO5) (lanes 7 to 12) 16 hours after transfer to repressive glucose medium.

(A) Analysis of high molecular weight RNAs.

(B) Analysis of low molecular weight RNAs.

3.3. Association of 60S pre-rRNAs with affinity-purified Nop7p

Nop7p was epitope-tagged by insertion of a tandem-affinity purification (TAP) tag in frame with the *NOP7* open reading frame in the chromosome, under the control of the *GAL10* promoter (Rigaut et al., 1999) (see Chapter 2, section 2.13). Correctly tagged strains were identified by western blotting, using an anti-protein A antibody bound to horseradish peroxidase (Figure 15). These exhibited no detectable growth defect on permissive galactose medium, showing the fusion construct to be fully functional (YMO2).

To determine which pre-60S precursor would associate with Nop7p, RNAs that co-purified with Nop7p-TAP in lysates from strain YMO2 were analyzed following immunoprecipitation (see Chapter 2, section 2.15). Recovered RNAs were analyzed by Northern analysis and primer extension and compared to RNA recovered in parallel in a mock precipitation from a non-tagged control strain (YDL401) and 5 μ g of total RNA. Northern hybridization showed that the 27SB and 7S pre-rRNAs co-precipitated with Nop7p-TAP, but were not detectably recovered in the mock precipitation from the non-tagged wild-type strain (Figure 16A and B). In contrast, the 27SA₂ and 6S pre-rRNAs were not detectably co-precipitated. The 25S rRNA gave a similar background signal in both, the wild-type and Nop7-TAP precipitates.

3.3.1. *Nop7p is associated with pre-rRNAs from both processing pathways*

The inhibition of pre-rRNA processing in cells depleted for Nop7p appeared to be more significant for mature 5.8S_s rRNA compared to 5.8S_L rRNA, suggesting that Nop7p might associate specifically with the pre-rRNAs on the 27SB_s branch of the processing pathway. 27SB_L and 27SB_s precursor cannot be resolved by Northern hybridisation, but primer extension using an oligonucleotide hybridising within ITS2 showed that both the 27SB_s and 27SB_L pre-rRNAs were co-precipitated with Nop7-TAP, although the recovery of 27SB_L appeared to be less efficient (Figure 16C). Moreover,

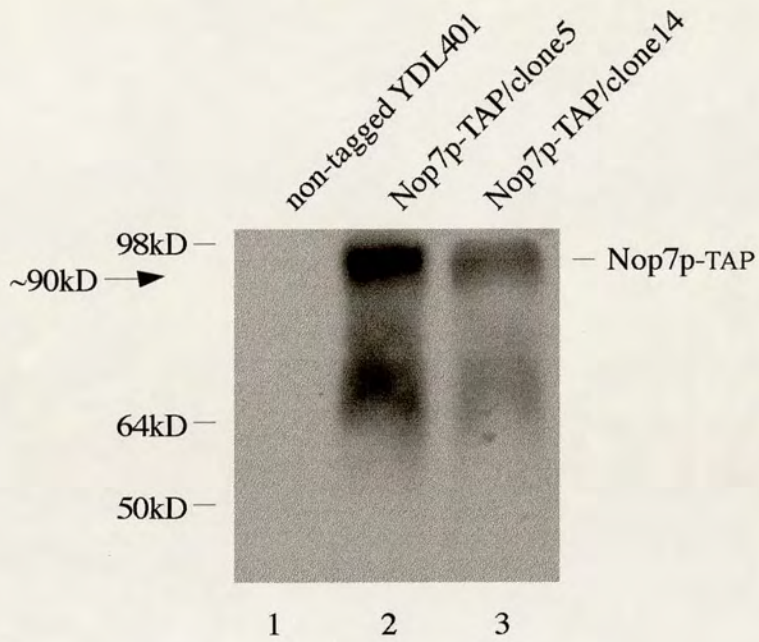


Figure 15: Western blot showing Nop7p-TAP constructs. Immuno blot analysis of strains expressing a Nop7p-TAP (affinity purification tag) fusion under the control of the *GAL 10* promoter in the YMO1 strain. The blot has been decorated with anti-ProteinA antibodies bound to horseradish peroxidase to visualise the TAP-tag . Nop7p is a 70kD protein; the tag contributes ~20kD to the size of the fusion protein.

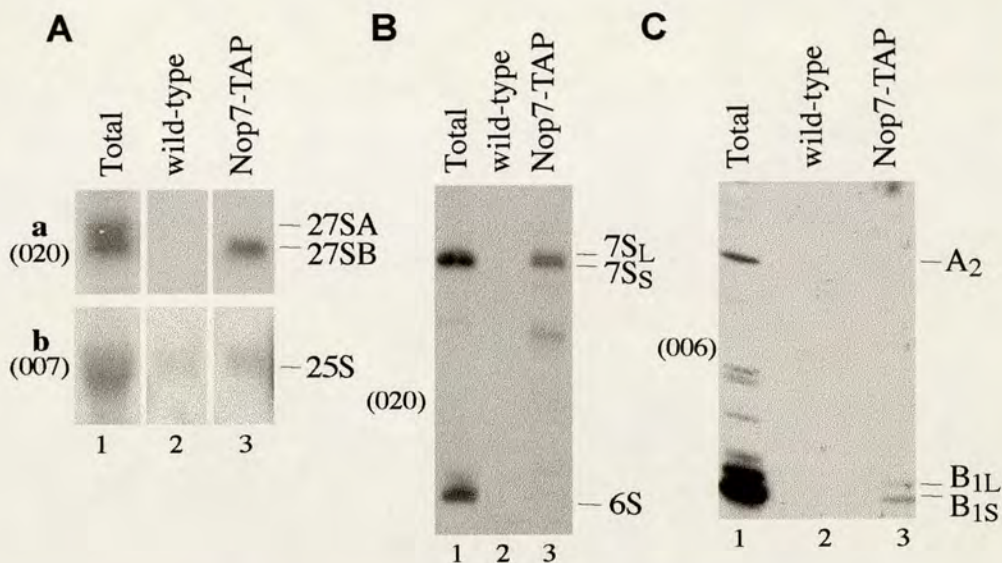


Figure 16: Nop7p co-precipitates precursors to the 25S and 5.8S rRNA.

Lane1: Total RNA control (5 μ g). Lane2: Precipitate from a wild-type control strain (YDL401). Lane3: Precipitate from a strain expressing Nop7-TAP (YMO2). The preparation used for **B** and **C** is different from that for **A** and gave lower recovery efficiency. Oligonucleotides used are indicated in parantheses on the left (see Chapter 2, Table 2.2).

(**A**) Northern hybridisation of high molecular weight RNA.

(**B**) Northern hybridisation of low molecular weight RNA.

(**C**) Primer extension analysis using oligo 006 (see Chapter 2, Table 2.2). Primerextension stops at sites A₂, B_{1S} and B_{1L} show levels of co-precipitated 27SA₂, 27SB_S and 27SB_L pre-rRNAs, respectively.

close inspection of the original autoradiograph of figure 16 showed that both the 7S_L and 7S_S pre-rRNAs were co-precipitated. This indicates that Nop7p has a specific role in formation of the 27SB_S pre-rRNA but is associated with pre-rRNAs in both processing pathways.

3.4. Nop7p is localised to the nucleolus

To determine the sub-cellular location of Nop7p, cells expressing Nop7p-TAP were examined by indirect immunofluorescence using a rabbit anti-protein A and a secondary FITC-coupled goat anti-rabbit antibody to detect the protein A region of the TAP-tag (Rigaut et al., 1999). As a marker for the nucleolus, a DsRed-fusion with the nucleolar protein Nop1p, the yeast homologue of human fibrillarin, was co-expressed from a plasmid introduced into the Nop7p-TAP fusion strain (YMO3) (Gadal et al., 2001b). The nucleoplasm was identified by DAPI staining. Anti-protein A preferentially decorated the nucleolus, with a weaker signal over the nucleoplasm (Figure 17). No cytoplasmic signal was detected. This suggests that Nop7p is localised to the nucleus with nucleolar enrichment. The significant nucleoplasmic staining would be consistent with association with late pre-ribosomes that have been released from the nucleolus (Milkereit et al., 2001).

In HeLa cells eYFP-Pescadillo was shown to predominantly localise to the nucleoli with low level of nucleoplasmic staining (Oeffinger et al., 2002). Comparison of subnucleolar distribution of eYFP-Pescadillo to fibrillarin, a component of the box C+D snoRNAs that function early in ribosome synthesis, and B23, a putative late-acting assembly factor and nuclease (Biggiogera et al., 1990; Savkur and Olson, 1998) revealed that while fibrillarin is concentrated in the dense fibrillar component (DFC) of the nucleolus, Pescadillo and B23 were largely excluded from the DFCs and concentrated in the surrounding area, which corresponds to the granular component (GC). This is consistent with the later role of B23 in nucleolar ribosome maturation, which has been reported to cleave a pre-rRNA reporter within ITS2 *in vitro* (Savkur and Olson, 1998), at

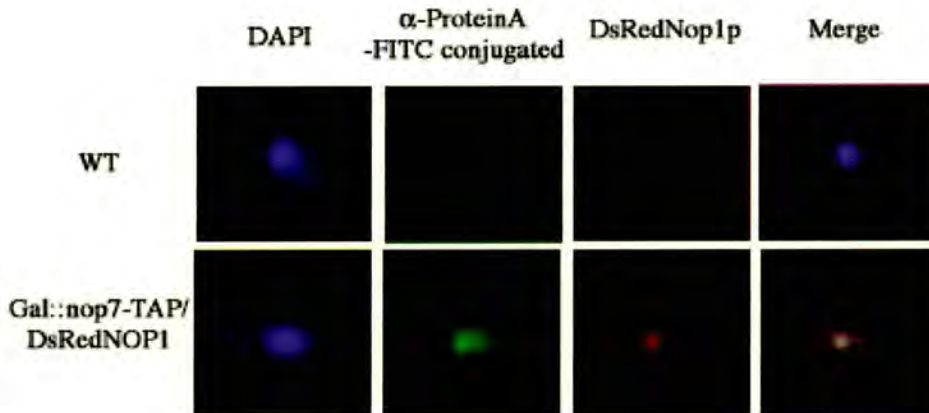


Figure 17: Nop7p is predominantly localised to the nucleolus.

The GAL::*nop7*-TAP strain (YMO3) co-expressing the nucleolar marker DsRedNop1p was examined by indirect immunofluorescence using an anti-proteinA antibody coupled to FITC. Also shown is the position of the nucleus visualised by DAPI staining and a wild-type control strain (YDL401).

a site potentially equivalent to C₂ in the yeast pre-rRNA. The distribution of Pescadillo was shown to resemble that of B23 but was distinct from that of fibrillarin. This appears to be consistent with a late role for Nop7p in nucleolar ribosome synthesis.

3.5. Nop7p-depleted cells are defective for 60S subunit export

The association of Nop7p with the purified NPC and its co-purification with ribosomal export factors suggested the possible involvement of Nop7p in ribosomal subunit export (Rout et al., 2000; Bassler et al., 2001). To follow the export of pre-60S ribosomal subunits from the nucleus to the cytoplasm several recent studies have made use of fusions between ribosomal proteins and GFP (Stage-Zimmermann et al., 2000; Bassler et al., 2001; Milkereit et al., 2001; Gadal et al., 2001a; Gadal et al., 2001b; Fatica et al., 2002). To test for potential 60S subunit export defects, Rpl25p-eGFP (Gadal et al., 2001b) was expressed from a plasmid in the wild-type (YDL401) and *GAL::nop7* (YMO4) strains. The distribution of Rpl25p-eGFP was followed during depletion of Nop7p.

During growth of the *GAL::nop7* strain on galactose medium Rpl25p-eGFP showed the normal, predominantly cytoplasmic distribution (Figure 18A, panels a to c). After transfer to glucose medium for 2 hours (Figure 18A, panels d to f), increased nuclear staining of Rpl25p-eGFP was already visible, and accumulation was strong after 8 hours (Figure 18A, panels g to i). The distribution of Rpl25p-eGFP fluorescence matched that of DAPI staining, indicating that it was not restricted to the nucleolus. This shows that Nop7p is required to allow the export of precursors to the 60S ribosomal subunit from the nucleoplasm to the cytoplasm.

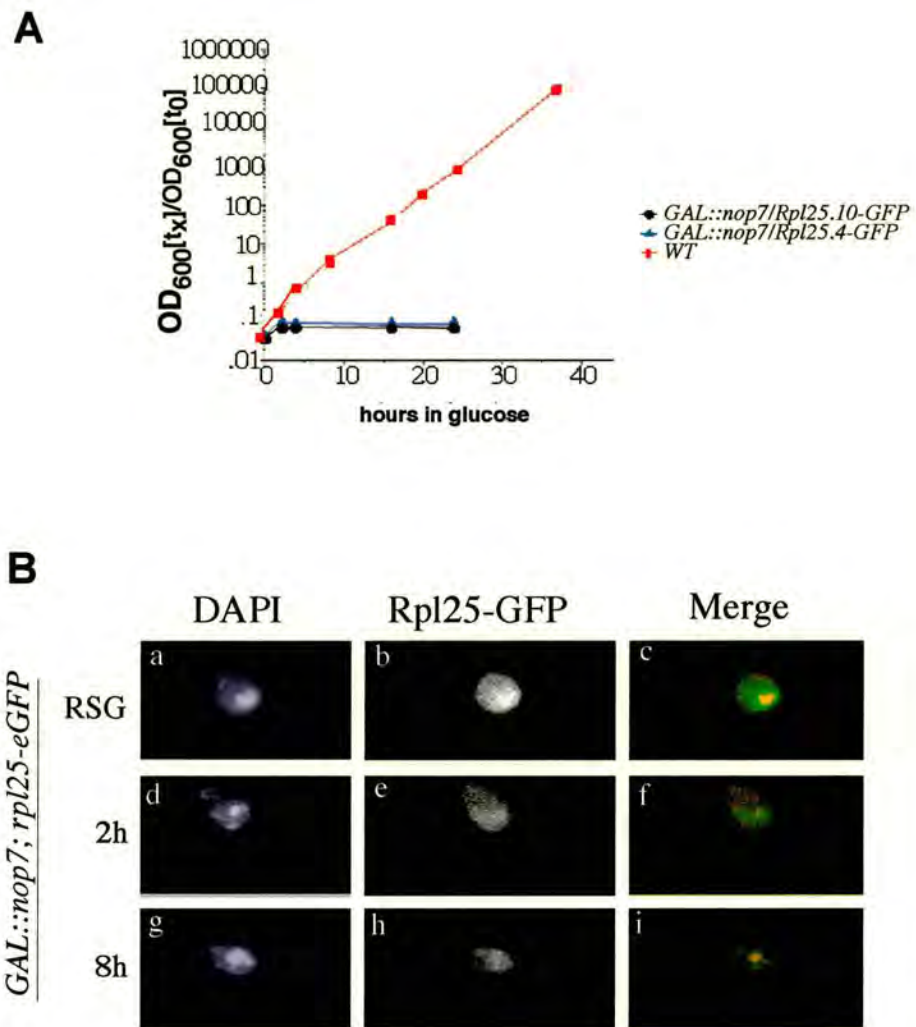


Figure 18: Depletion of Nop7p inhibits 60S subunit export and growth in Rpl25-eGFP expressing strains.

(A) Growth curve of *GAL::nop7* strain co-expressing Rpl25-(YMO4) eGFP (* and ●) and an isogenic wild-type strain (▲) (YDL401). Strains were pre-grown in permissive galactose medium and transferred to repressive glucose medium for the times indicated.

(B) Subcellular distribution of Rpl25-eGFP in a *GAL::nop7* strain (YMO4). Rpl25-eGFP was examined by fluorescence microscopy during growth in permissive galactose medium and following transfer to repressive glucose medium for 2 and 8 hours. The position of the nucleus was visualised by DAPI staining. In the merged image, DAPI staining is shown in red and Rpl25-eGFP in

3.6. Nop7p interacts genetically with GFP-tagged Rpl25p

As previously reported, expression of the Rpl25p-eGFP construct had little effect on the growth of the wild-type strain (Gadal et al., 2001b) or the *GAL::nop7* strain on galactose medium. However, growth of the *GAL::nop7/Rpl25p-eGFP* strain was very rapidly inhibited following transfer to glucose medium (Figure 18B). This strain also expresses the wild-type Rpl25p, showing that the Rpl25p-eGFP fusion construct has a dominant negative effect for growth in strains with a reduced level of Nop7p. Nevertheless, the growth inhibition is much more rapid than would have been expected for a strain that is simply unable to synthesize new ribosomes and suggests that Nop7p has an additional role in 60S ribosomal subunit assembly.

3.7. Discussion

The results show that Nop7p has multiple roles in the synthesis of the 60S ribosomal subunit. Cells depleted of Nop7p show defects in pre-rRNA processing, export of the pre-60S subunits from the nucleus to the cytoplasm and subunit assembly. Nop7p is required for the 5' to 3' exonuclease digestion that generates the 5' end of the major, short form of the 5.8S rRNA. Nuclear accumulation of Rpl25p-eGFP, which had been used as a marker for a defect in nuclear export of pre-60S ribosomal particles (ribosome export or *rix* phenotype) (Gadal et al., 2001b), was also observed following Nop7p depletion. Depletion of Nop7p also resulted in strong synergistic inhibition of growth in the presence of a GFP-tagged form of ribosomal protein Rpl25p, indicating a role in 60S ribosome assembly.

3.7.1. *Nop7p* has multiple functions during 60S subunit synthesis

Pescadillo was isolated as a mutation affecting embryonic development (Allende et al., 1996) and a mutant allele of the yeast gene resulted in growth arrest in G2 (Kinoshita et al., 2001), consistent with a specific defect in cell-cycle progression. Yeast Yhp1p/Nop7p has been reported to interact with Yvh1p (Sakumoto et al., 2001), a protein-tyrosine phosphatase with a postulated role in the regulation of sporulation and meiosis. Human pescadillo (PES1) was isolated during a proteomic analysis of human nucleoli (Andersen et al., 2002) and Ygr103p/Nop7p was identified as its homologue in yeast.

While this work was in progress, Nop7p was also shown to be a component of at least three different pre-ribosomal complexes with substantially different protein composition, as well as differences in pre-rRNA components (Bassler et al., 2001; Harnpicharnchai et al., 2001; Fatica et al., 2002). Comparison of the results from these analyses with the data presented here allows the proposal of a correlation between the pre-ribosomal particles with which Nop7p is associated and the distinct defects in ribosome synthesis that are seen on its depletion (Figure 19).

The earliest pre-60S particle with which Nop7p is known to be associated is pre-60S E₁. This complex is also associated with the 27SA₂, 27SA₃ and 27SB pre-rRNAs (Fatica et al., 2002) and it is therefore very likely that Nop7p is required for processing from 27SA₃ to 27SB₅ within the pre-60S E₁ particle (Figure 19).

A fast acting, dominant negative phenotype is associated with the expression of a GFP-tagged form of the ribosomal protein Rpl25p in strains depleted of Nop7p. The fact that expression of Rpl25-GFP is dominant indicates that in its presence the wild-type Rpl25p is no longer able to support growth. Notably, the inhibition of growth was much more rapid and complete than would be expected for a strain that was simply unable to synthesize new ribosomes due to pre-rRNA processing defects. Many such mutants have been analyzed (Venema and Tollervey, 1999) and predominantly show a gradual increase in doubling time, as preformed ribosomes are depleted by growth. The very rapid onset of growth inhibition, seen in the Nop7p-depleted strain expressing Rlp25p-GFP, indicates that this does not require substantial depletion of the pre-existing

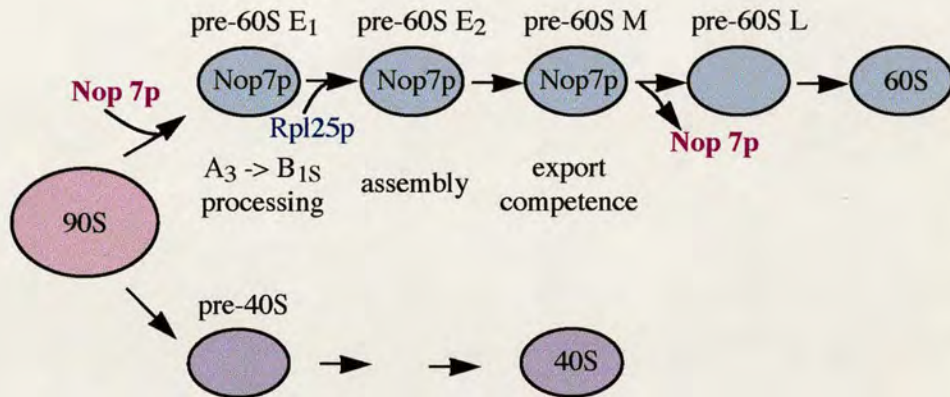


Figure 19: Model for the roles of Nop7p in 60S subunit biogenesis.

Outline pathway of biogenesis of 60S and 40S ribosomal subunits. This model indicates the presence of Nop7p in three different pre-60S complexes designated E₁, E₂ and M, which can be correlated with the different functions deduced for Nop7p. Pre-60SE₁ contains the 27SA₃ pre-rRNA, the processing of which is defective in strains lacking Nop7p. Rpl25p is not present in pre-60SE₁ but joins the pre-60SE₂ particle, and the defect in Rpl25p assembly is therefore predicted to occur at this step. The pre-60SM complex contains numerous factors required for 60S subunit export as judged by the nuclear retention of a Rpl25p reporter construct, and Nop7p is likely to be required during the acquisition of export competence within this complex.

ribosome pool. It could be speculated that production of defective subunits prevents the remaining, otherwise functional, ribosomes from carrying out efficient translation. The pre-60S E₁ complex lacks Rpl25p, which is added only to the pre-60 E₂ particle (Harnpicharnchai et al., 2001; Fatica et al., 2002). Therefore, genetic interactions between *GAL::nop7* and Rpl25p-GFP appears to reflect a requirement for Nop7p in the correct assembly of Rpl25p, and perhaps other factors, with the pre-60S E₂ complex (Figure 19).

Several recent studies have made use of the fusion between Rpl25p and GFP to follow the export of 60S ribosomal subunits from the nucleus to the cytoplasm (Bassler et al., 2001; Milkereit et al., 2001; Gadai et al., 2001a; Gadai et al., 2001b; Fatica et al., 2002). This approach relies on the observation showing that free r-proteins do not accumulate in the absence of ribosome synthesis. The accumulation of Rpl25p-GFP has therefore been taken as evidence for the accumulation of pre-ribosomal particles in the nucleoplasm, indicating a defect in their export. This assay has defined a late pre-ribosomal particle (pre-60S M in Figure 19), all tested components of which are required for 60S subunit export. These include Nug1p, Nug2p, Noc2p, Noc3p and Rix1p as well as Nop7p itself (Bassler et al., 2001; Milkereit et al., 2001; Gadai et al., 2001a; Gadai et al., 2001b). Mutations in any of these proteins leads to defects in export suggesting a requirement for the intact structure of this pre-ribosomal particle. Since multiple components of this complex are required for subunit export, we predict that export-competence is established within this particle, and that this activity requires Nop7p.

Mutations in Nug1p, Nug2p, Noc2p, Noc3p or Rix1p did not result in pre-rRNA processing defects similar to Nop7p depletion or synergistic interactions with Rpl25p-GFP (Bassler et al., 2001; Milkereit et al., 2001) indicating that these are distinct activities. Moreover, Nug1-TAP did not coprecipitate 27SA₂ or 27SA₃ (Bassler et al., 2001 and unpublished observations) indicating that it associates with the pre-rRNA particle only after processing at these sites is complete. Depletion of a specific component of the pre-60S E₁ complex, Ssf1p, also did not interact genetically with Rpl25p-GFP and did not inhibit subunit export as judged by nuclear accumulation of Rpl25p-GFP (Fatica et al., 2002). Consideration of these data leads to the proposal that

Nop7p is independently required for pre-rRNA processing, assembly and export and performs these functions within different pre-ribosomal particles (Figure 19).

At the same time as the data presented in this work was in print, another independent study describing the functions of Nop7p during ribosome biogenesis, which had been carried out by Adams et al. was also published. This work showed the synthetic lethality of combined mutations within the DEAD-box protein Dsr1p, an ATP-dependent RNA helicase that is required for maturation of 25S rRNA from 27SB rRNA precursor, and Nop7p (Adams et al., 2002). The accumulation of halfmer polyribosomes and the deficiency of processing of 27S pre-rRNA to 25S rRNA in a temperature sensitive *nop7-1* mutant strain supports the results presented for Nop7p in this thesis suggesting a role during processing of the 27SA₃ precursor. Adams and colleagues furthermore proposed that the synthetic lethal effect seen upon combination of *DSR1* and *NOP7* ts alleles is the result of miss-assembly within pre-60S subunits due to the lack of Nop7p in these cells and hence that the protein may be required to facilitate the association of Dsr1p with the pre-rRNA during processing of the 27SB precursor. This supports the conclusion drawn from the nuclear retention of Rpl25p-GFP in cells depleted of Nop7p and the synergetic growth defect observed in those cells, which suggested that Nop7p may be required for assembly of the pre-60S particle. A requirement of Nop7p for the function of Dsr1p might also explain the slightly increased primer extension stop at site C₂ in Nop7p-depleted cells, which accounts for a loss of processing at this site and could be due to an inefficient interaction between Dsr1p and the pre-rRNA in the absence of Nop7p.

3.7.2. *Pescadillo* is a multifunctional protein

There are clear precedents for proteins that function both in cell-cycle progression and ribosome synthesis. Exit from mitosis in budding yeast requires a group of proteins, including the phosphatase Cdc14p, which down regulate cyclin-dependent kinase activity. Cdc14p is sequestered in the nucleolus in the RENT (regulator of nucleolar silencing and telophase) complex with Sir2p and Net1p, which serves to anchor the

complex (Shou et al., 1999). In addition, Net1p is required for the maintenance of normal nucleolar structure and its binding stimulates RNA polymerase I (Shou et al., 1999; Shou et al., 2001). These nucleolus-specific functions of Net1p can be separated genetically from its cell-cycle functions in the RENT complex. In human cells the nucleolar p19^{ARF} protein binds and sequesters the negative regulator of p53 activity, Mdm2 (Tao and Levine, 1999; Weber et al., 1999; Zhang and Xiong, 1999a). Free Mdm2 ubiquitinates p53 and transports it to the cytoplasm where it is degraded by the proteasome (Fuchs et al., 1998; Geyer et al., 2000), and the nucleolar sequestration of Mdm2 contributes to the inhibition of this activity by p19^{ARF}. Mouse Pescadillo was identified by its up-regulation in cells lacking p53 (Kinoshita et al., 2001), but other interactions with the p53 system have not been reported.

The available data suggest that yeast Nop7p may function both in ribosome synthesis and in cell-cycle regulation. Whether its role in the cell cycle involves other protein components of the pre-ribosomal particles or a different set of interactions remains to be determined.

Chapter Four

Rrp12p functions in the maturation of both 40S and 60S ribosomal subunits

4.1. Introduction

More than 80 nonribosomal factors that are required for ribosome synthesis but only one, Rrp5p, was previously shown to function separately in both 18S and 5.8S/25S rRNA formation (Venema and Tollervey, 1996; Torchet et al., 1998; Eppens et al., 1999). Mutations in the *RRP5* gene affected the early cleavages at sites A₀, A₁ and A₂, as well as cleavage at site A₃. No protein had previously been identified that is involved in late steps of both 18S and 5.8S or 25S processing.

An uncharacterised human ORF, KIAA0690, identified during the proteomic analysis of human nucleoli (Andersen et al., 2002), was found to be homologous to yeast, YPL012w (Altschul et al., 1997). Moreover, this gene was shown to be essential in yeast, which, together with its presence within human nucleoli, initiated our investigation of the gene and its product (Winzeler et al., 1999). *YPL012w*, or *RRP12*, encodes a protein of 1228 amino acids with a predicted molecular weight of 137.5 kDa. Sequence alignment with the human homologue, Nnp86p (hRrp12p) (Andersen et al., 2002), revealed 25% identity and 44% similarity between the two proteins (Figure 20). In this work I investigated if Rrp12p is required for ribosome synthesis and if its karyopherin-like structure could relate to a function in nucleo-cytoplasmic transport.

4.2. Rrp12p-depleted cells are defective for rRNA processing

4.2.1. Aberrant and truncated RNAs accumulate cells lacking Rrp12p

To examine the *in vivo* function of Rrp12p, the *RRP12* gene was placed under the control of a repressible *GAL* promoter using a one-step PCR technique in strain YDL401 (see Chapter 2, section 2.13). This allows expression in medium containing galactose but is repressed in glucose-based medium. The host strain, YDL401, has reduced galactose permease activity leading to reduced *GAL*-induction (Lafontaine and Tollervey

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169 IRRRAQHGVCSVIGKSEFMFEKAF--AHHPAAITSTAKFCIQEIE-----KSGGSKIA--TTTLHMILLKDLLPC-- 238 ScRrp12p
RRK A V VI AF A H AA+ A FC +++ K+ +KF ++ L L+ ++
175 VRRRALDAVHAVLNNPPV----AHTAEHVAVFVADRCQKLAGILNLDLSNLSNKQLKAKTKFDINASVMRSRLITSVVSTGQ 255 hRrp12p

239 FPEGLVKSCSETLIRVMTLSHVLVTACAMQAFHSTLHARPLG---SITSAELNAQIITALLYDVPSENDLQPLLAWLKVMEKAHI 319 ScRrp12p
+P +++ + LI V S + + + + + E S + F S L + + + + + + + P S D + W + V + K
256 WESSQIEPLCDVITGVTKSSEQYLVSASFEDFESMKTMAETTISGVAENKYLRVLDTIFALKPSNVITLLTKSRLIAVIRGMS 340 hRrp12p

320 NLVRLQWDLGIGHIFRFEGTAVICLLSPHSQVLTAFQSTKEITKPCVAPHMADIGSVISSASGPA-----QSVAKMFRAVEEGL 397 ScRrp12p
O L + P F T L S + V AA+O L II E V D+ T S + + + + + + + L
341 TYATHCPKARWKLFGVSHIMCQYASQETPEVYQASQOQISITISVSKD---DILLYGPEVDEKVFKNVDEIISQIAKTFIDF 422 hRrp12p

398 TYKFAAANSSVLIQICVFEACGRQAEFVMRKCIQSLCDIRLSPH--FPHTAALDQAVGAAVTSMGPIVVVQAVLETIDGSEETL 481 ScRrp12p
+ + + + + I + + L F + + + P K L + + R + + + + GA + + + MGPI + + L P I + D
423 STRYSHCSDRELDKIVAAANKFRYRSNHFVKSFKIVDTWRVNEEQFMDLRNETELVIGASISAMGPIMLAEALNLDNPSSER 507 hRrp12p

482 DFRRSWILPVRIRDHVQETRGFTTYFLPLANTLKSAMDLAQAGSTVESKIYDILQVQWLLFGFCITRPFIDVAISHKG-IART 463 ScRrp12p
R+WILP+IRD+ + L F P + +SK + + + + + + + + O+W+ L F C P D+ SE A
508 P-GRAMMDELIRDTYKNNATQNELADYIKSFOKFDKVPK--ESIQLRVFOIVDIDSTLRECELMDLRESEPTDEFSE 590 hRrp12p

564 LGMATSERPDLRVVCOAIRLLITKGC-----QAEADRAEVSRFAKNLEPILFNLYGQVPAAGDTEAHR 628 ScRrp12p
L + +L R + C AI+ L + + + + + + S + N L + LFN+ Y O T E R
591 SSSLYSEVEARTVICHAKVIAESNVSYAESSSHNVLLQRFPISEQKNIEYLSKTSNLLAVLNNVDTOT-----TNNRS 674 hRrp12p

629 AVLEHIRTITLTDIQ-----LVNSLLEKASEKVLDPASSDFTRLVLDLWVALAPCADEAAISKLYSTIRPYLESKA 699 ScRrp12p
+LETTI YL IT + L NS+ E++S V + +LDL+ + + + S L S + S
675 YLLEHIDQIKVSKEDLEKTFNNVCGLLKNSMNPESGNVNKEKKKQLTATLDDILICMITYLPVSSYALRSMFLTVNSAD 753 hRrp12p

700 HGVOKAYRVLEEVCASPCPGALFVQSHLEDLKKTLIDSLRSTSSPAKRPRKCLLEIVRKLSAEHKEFTALIEVIECTKRV 786 ScRrp12p
+QK+AYR+ + + G A V + D+ + +DS S + AK RL + IV L +H +FI + EVIL TK+V
754 ALLQFRVRIITKLSSELKSGST--VAQFTSDIENVMVDSASVQTSAAARITALKTVELPLDLELDEWRTVAEVLISKXDM 837 hRrp12p

787 SVGARKNAFALVEMGHAFRLRFGS-----NOEEALQCYLVITYFGVGC-AVIMVSCSLALHTLIDFEFGLMGTS 856 ScRrp12p
+ +R+ AF L+ MG + O + + + + I L G I + G + MVS ST L + FEFK + +
838 NEKSEIARFDITICGRKMNEPNGIIKLFQIPGYDPTTPDOSSSISEFFKDSAGLIGESQEMVSSITGYACVVEFEKNELD 922 hRrp12p

857 TVEQLLENVCLLRSRTDVKVSAIGFKVAVTVMDVAHLAKHVQVMEATGKISDDMRHFRMKLRNLFTKFKRFGPELVKRL 940 ScRrp12p
+ + + + L L S +R+VKSA+GF KV V + + V + + + + S + HF+ K+ + + + + IR+FG+ + +
923 ILMIDYDTIEHYLTSNSREIVKSAIGFKVQVGLPEELMRPKVPELLKLLLRVSEHTGHEKAVKHIIERIIRFGYDYLEAN 1007 hRrp12p

941 IPPEEYHRYLVNIRKAEAKRHRRLSQAAVEPEEEEEEEFQCKG----DSIFHILADSDEEDN--EHEERSRQKQKRLAR 1022 ScRrp12p
PEE R+L NIRK R KR + +EE + A KG + + E + S + + E DN + +EE G + + A+
1008 EPEEDRRLITNIRKMRNENKR-----KDEPVTGVSVDVATAGSRFMSAFDFAVYGDDEPNDNGSDQENVAQCKMKNGAK 1083 hRrp12p

1023 QRSRAWLKEGGDEPLNFDLPKVAQRVLAQF-----GPGRGKKTSHSEKVSADGRLLIR 1074 ScRrp12p
O + + E GD PL+ LD + + + + P R R D + F + + C + L + + +
1084 Q----FIVE--SGDPLDLDQSLAHLISSRQKFKFNQNRARFNDAENFDSECKVVK 1138 hRrp12p

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Figure 20: Alignment of homologous sequences of *S. cerevisiae* Rrp12p and human Rrp12p.

Amino acid positions are indicated at the left and the right of each strand. Purple boxes indicate sequence identities (25%) while violet boxes indicate conservative substitutions (similarities, 44%). Alignment retrieved by BLAST search.

1996). This eliminates the over-expression generally seen with *GAL*-regulated constructs and allows faster appearance of phenotypes following transfer to glucose medium. Growth of the *GAL::rrp12* strain was not clearly different from the isogenic wild-type strain on permissive galactose medium but progressively slowed following transfer to repressive glucose medium and depletion of Rrp12p (Figure 21).

Analysis of steady state levels of precursor rRNA by Northern hybridisation showed that depletion of Rrp12p resulted in accumulation of 35S precursor RNA as well as 32S pre-rRNA (Figure 22, panel a). Mature 18S and 25S rRNA were both reduced after 12 hours in glucose medium (Figure 22, panel d and g). While 27SA₂ pre-rRNA appeared to be increased, 27SA₃ and 27SB₅ RNA precursors were reduced, suggesting a slight inhibition of cleavage at site A₃ and a processing delay during synthesis of 25S and 5.8S rRNA (Figure 22, panel a, b and c). Furthermore, there was an appearance of the aberrant 23S precursor RNA, extending from the 5'ETS to the A₃ cleavage site, together with 22S and 21S pre-rRNA, extending from site A₀ and A₁, respectively, to site A₃ (Figure 22, panel a and e). These precursors result from a cleavage at site A₃ before processing at sites A₀, A₁ and A₂ has occurred, therefore suggesting a kinetic delay of early processing steps in strains depleted of Rrp12p. Hybridisation with probe 005 also detected truncated versions of the 23S and 21S species, designated 23S* and 21S* (Figure 22, panel c). These give rise to a stronger signal with probe 005 than with probe 003. While probe 005 is located directly downstream of site A₂, probe 003 is 53 nucleotides further 3', immediately upstream of site A₃, indicating that 23S* and 21S* represent short 3' truncations of the 23S and 21S species.

Previously it has been shown that in exosome mutants short truncations of 23S and 21S RNAs are stabilized, indicating that the exosome is responsible for the degradation of these intermediates (Allmang et al., 2000). However, these truncated RNAs have not been found in mutants affecting early cleavages since in those strains the 23S intermediates are degraded by the functional exosome and inhibition of processing at sites A₀, A₁ and A₂ in exosome mutants is likely to be indirect (Mitchell et al., 1997; Venema and Tollervy, 1999). This suggests that the exosome is less active in degrading the aberrant 21S and 23s pre-rRNAs in the absence of Rrp12p.

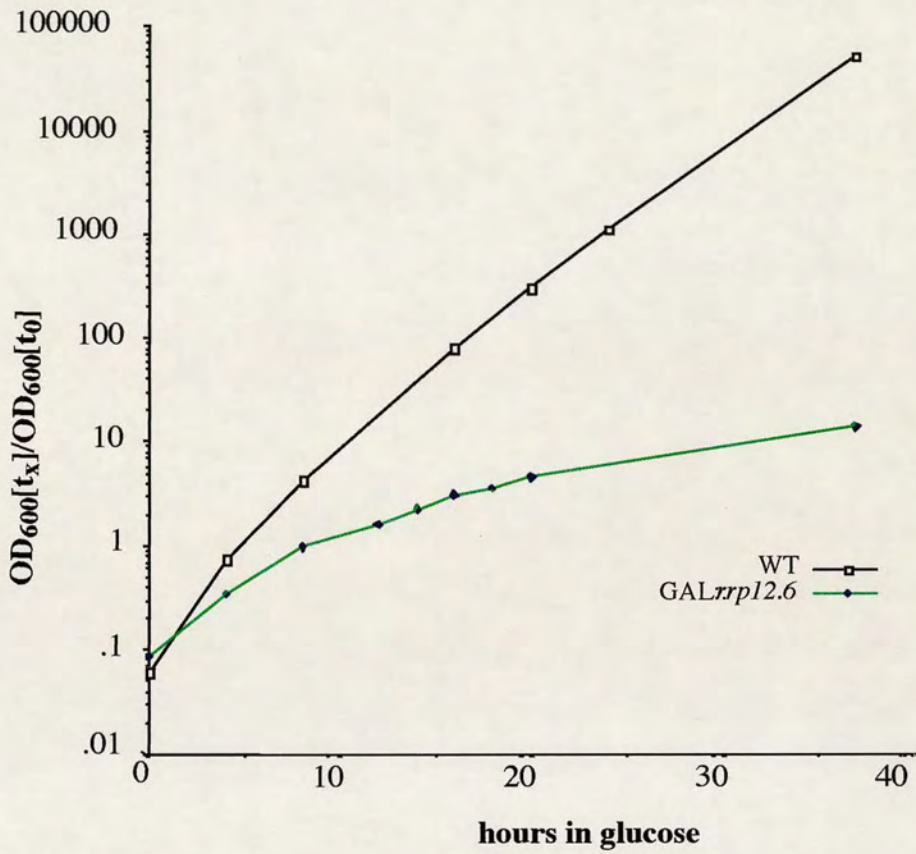


Figure 21: Growth curve of a conditional *GAL::rrp12* strain.

Growth curve of the *GAL10::rrp12* strain (YMO7) and the otherwise isogenic wild-type strain (YDL401) after shift from permissive galactose medium to repressive glucose medium.

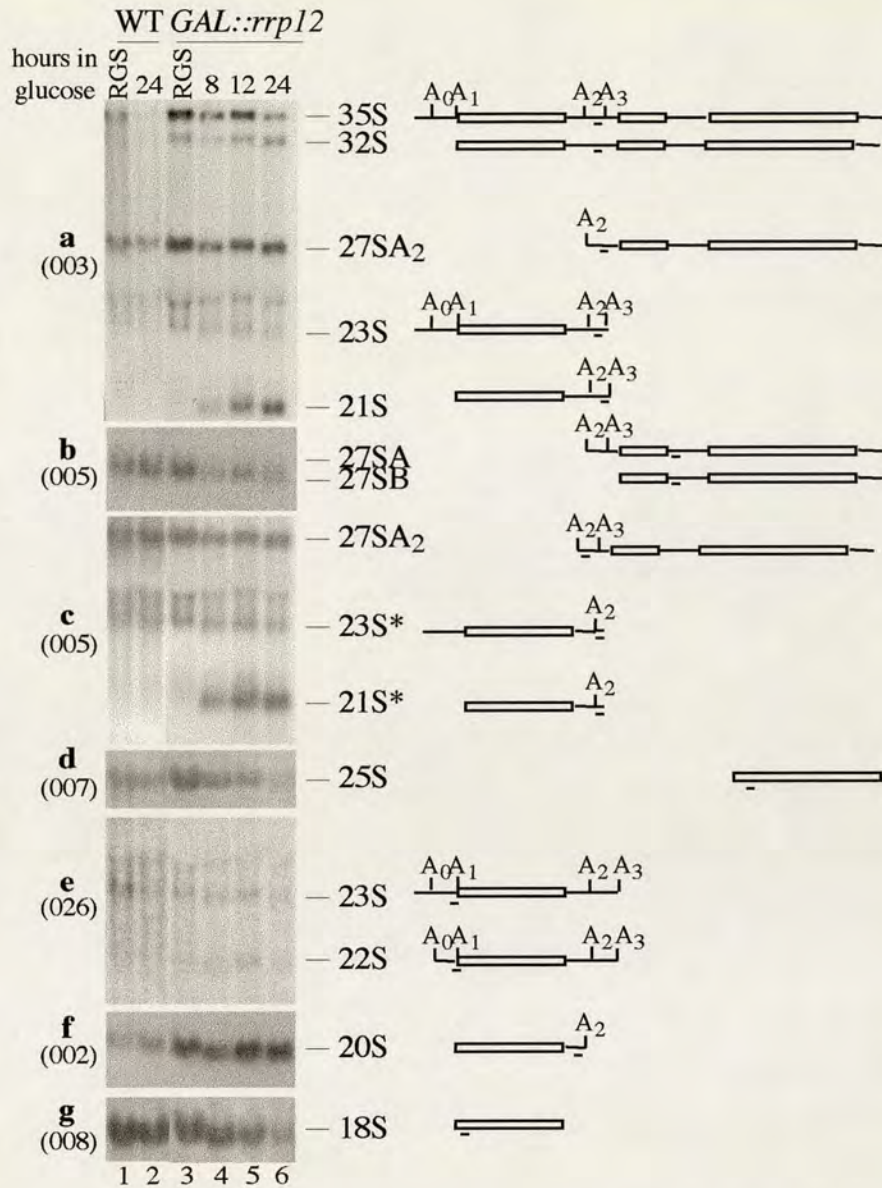


Figure 22: Northern analysis on the effects of Rrp12p depletion on pre-rRNA processing.

Lanes 1 and 2: wild-type strain (YDL401) in permissive galactose medium and 24 hours after transfer to repressive glucose medium. Lanes 3 to 6: *GAL::rrp12* strain (YMO7) in galactose and after transfer to glucose medium for the times indicated.

Probes used are shown in parentheses on the left and indicated below the schematics on the right (see Chapter 2, Table 2.2.). The pre-rRNA and rRNA species are schematically represented on the right; rectangles represent mature rRNA and thin lines the transcribed spacers. The bands labelled 23S* and 21S* are a mixture of full-length 23S, 21S pre-rRNA and the truncated * species.

4.2.1. Depletion of *Rrp12p* causes an accumulation of 3'-extended 5.8S rRNA

Analysis of low molecular weight RNAs did not show any significant changes in the level of mature 5.8S rRNA (Figure 23A). However, following depletion of Rrp12p, the Rrp12p-depleted strain exhibited an accumulation of 3' extended forms of 5.8S rRNA that extended in a ladder up to the position of 7S pre-rRNA (Figure 23A). This characteristic pattern has so far only been detected in strains mutant for exosome components or the exosome-associated helicase Dop1p, (Mitchell et al., 1996; Briggs et al., 1998; de la Cruz et al., 1998; Allmang et al., 2000) and is the result of the inhibition of 3' to 5' exonucleolytic digestion from site C₂ to the 3' end of mature 5.8S rRNA. Also noted was the appearance of fragments longer than the 7S precursor. Detected by hybridisation with a probe 3' to site A₂, these fragments extend from sites A₂ to C₂ while shorter RNAs (A₂-C₂*) extend from A₂ to sites between the 3' end of 5.8S rRNA and site C₂ (Figure 23A and B), most likely terminating at the same sites as the 3' extended forms of 5.8S rRNA (Allmang et al., 2000). Comparison of the *GAL::rrp12* strain with *GAL::dob1*, *GAL::rrp41/rrp6-1Δ* (YCA31) and *GAL::csl4* (P170) strains 24 hours after transfer to glucose medium showed A₂-C₂ and A₂-C₂* species only in the *RRP12* and *CSL4* mutants, while the 3' extended forms of 5.8S rRNA were present in all four mutant strains (Figure 23B). So far forms of 5.8S rRNA that are 5' extended to site A₂ have been observed in strains defective in cleavage at site A₃ due to mutations in components of RNase MRP. In RNase MRP mutant strains, these fragments are usually processed to site E by the exosome. However, in exosome mutants these fragments as well as the A₂-C₂ fragment accumulate, since they would normally be a substrate for the exosome (Lygerou et al., 1994; Chu et al., 1997; Dichtl and Tollervey, 1997; Allmang et al., 2000).

Primer extension analysis using an oligonucleotide hybridising within the 3' region of ITS2 (006) showed a clearly increased primer extension stop at A₂ (Figure 24A). This probably arises from the A₂-C₂ and A₂-C₂* species detected in the Northern analyses. Increased primer extension stops were also observed at sites B_{1L} and B_{1S} (Figure 24A). Primer extension using oligonucleotide 006 detects both the 27SB pre-

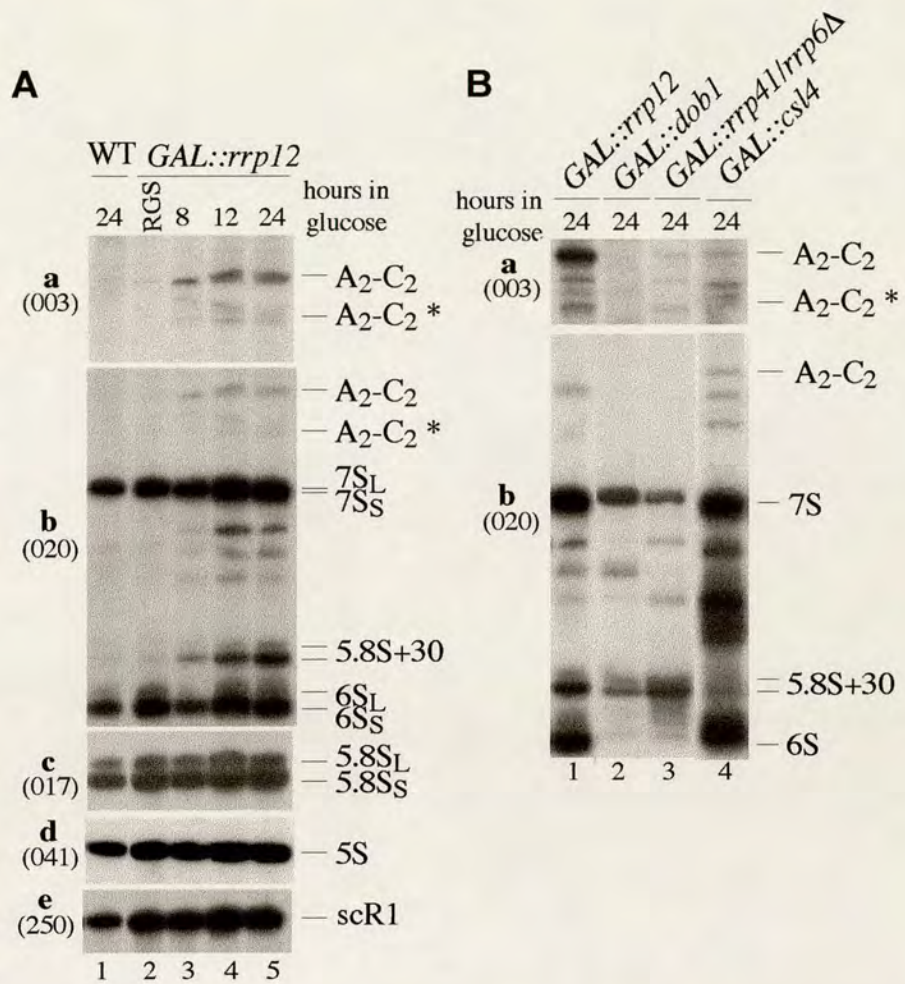


Figure 23: Appearance of aberrant pre-rRNAs in Rrp12p depleted cells.

(A) Lane1: wild-type strain (YDL401) 24 hours after transfer to repressive glucose medium. Lanes 2 to 5: *GAL::rrp12* strain (YMO7) in galactose medium (RGS) and after transfer to glucose medium for the times indicated. SCR1 was used as a loading control .

(B) Lane1, *GAL::rrp12* (YMO7), Lane2, *GAL::dob1*, Lane3, *GAL::rrp41/rrp6Δ* (YCA31), Lane4, *GAL::csl4* (P170). All strains shown 24 hours after transfer to repressive glucose medium.

Probes used are indicated in parantheses on the left (see Chapter 2, Table 2.2.).

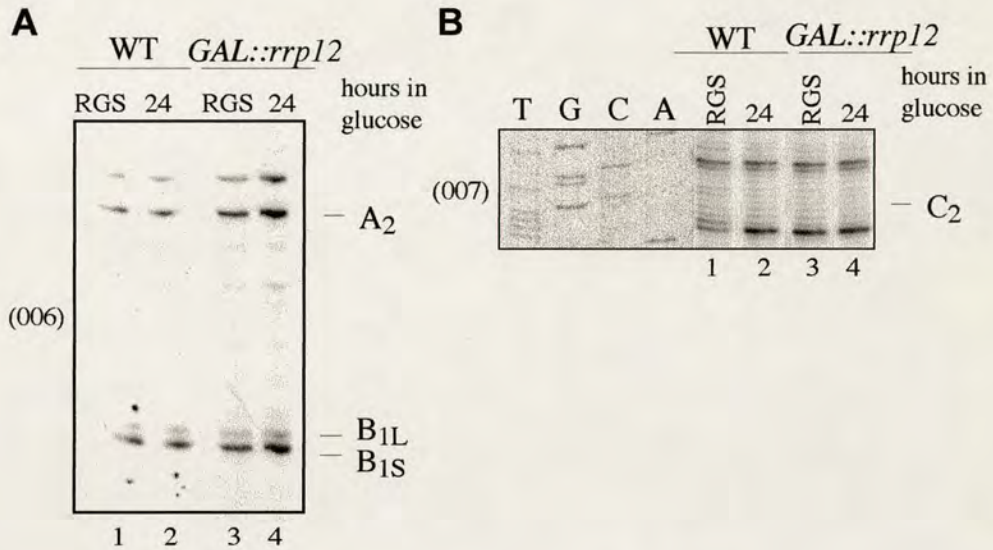


Figure 24: Primer-extension analysis of levels of rRNA precursors in *Rrp12p* depleted cells.

Lanes 1 and 2: wild-type strain (YDL401) in permissive, galactose medium and 24h after transfer to repressive glucose medium. Lanes 3 and 4: *GAL::rrp12* strain (YMO7) in galactose medium and 24h after transfer to glucose medium.

(A) Primer extension using oligo 006 (see Chapter 2, Table 2.2.). Primerextension stops at sites A₂, B_{1S}, and B_{1L} show levels of the 27SA₂, 27SB_S and 27SB_L pre-rRNAs, respectively.

(B) Primer extension using oligo 007, which hybridises within 25S rRNA. The primer extension stop at site C₂ shows the level of 26S pre-rRNA.

rRNAs and the 3' extended forms of 5.8S rRNA, since these have the same 5' ends. The observed alterations presumably reflect the accumulated 3' extended forms of 5.8S rRNA, since no elevation of 27SB pre-rRNA compared to wild-type was detected by Northern hybridisation (Figure 22). Primer extension through site C₂ using an oligonucleotide hybridising at the 3' end of ITS2 (007) did not show any significant alterations in levels of 26S pre-rRNA, which is the other product of C₂ cleavage (Geerlings et al., 2000) (Figure 24B). Considering the depletion of 27SA₃ and 27SB pre-rRNAs and the accumulation of A₂-C₂ fragment in strains depleted of Rrp12p, it appears that processing at site A₃ is slightly delayed. The remnant 27SA₂ pre-rRNA is cleaved at site C₂ in ITS2, generating the A₂-C₂ fragment. This phenotype has also been observed in strains depleted of exosome components, like GAL::*csl4* and GAL::*rrp41/rrp6-1Δ*.

Together these data suggest a possible function for Rrp12p in promoting the association of the exosome with the pre-60S particle or in the stimulation of exosome activities.

4.3. Rrp12 has a separate function during late maturation of the 40S ribosomal subunit

The 20S precursor is a late intermediate in 18S rRNA synthesis that is generated from the 35S pre-rRNA by cleavage at sites A₀, A₁ and A₂. The 20S pre-rRNA is transported into the cytoplasm as part of the 43S pre-ribosomal particle, where it is cleaved at site D to yield mature 18S rRNA and a mature 40S subunit (Udem and Warner, 1973; Venema and Tollervey, 1999).

In strains depleted of Rrp12p, following transfer to repressive glucose medium, accumulation of 20S pre-rRNA and depletion of mature 18S rRNA could be observed (Figure 22, panel f and g). The accumulation of 20S precursor is seen despite a delay of the early processing steps at sites A₀ to A₂ that lead to the production of 20S pre-rRNA. Therefore, the inhibition of 20S to 18S rRNA maturation might be more severe than can be judged from the Northern analyses. These results suggest a function for Rrp12p

following the early processing steps at sites A₀ to A₂ that lead to the production of 20S pre-rRNA, but before maturation of 20S to 18S rRNA in the cytoplasm.

To determine if lack of Rrp12p causes a delay in the synthesis of mature rRNA, a pulse-chase analysis with [³H]-uracil was performed 16 hours after transfer to glucose minimal medium. Comparison of the wild-type (YMO5) and *GAL::rrp12* (YMO9) strains showed that in wild-type cells 27SA, 27SB and 20S precursors could be detected after 1 minute, and after 5 minutes the majority of label was found in mature 25S and 18S rRNA (Figure 25A, lanes 9-12). In contrast, pre-rRNA processing was delayed in *GAL::rrp12* cells (Figure 25A, lanes 1-6). The 27S species persisted until 10 minutes into the chase and a lower yield of 25S rRNA was detected. 20S pre-rRNA could still be detected after 20 minutes, whereas mature 18S rRNA was not detected until 10 minutes of the chase and at lower levels than in the wild-type (Figure 25A lanes 5 and 6). Analysis of low molecular weight rRNAs showed that synthesis of mature 5.8S rRNA was substantially in the *GAL::rrp12* strain compared to wild-type strain (Figure 25B). However, synthesis of 5S rRNA and pre-tRNAs appeared delayed in a similar fashion to that of 5.8S rRNA, suggesting an over-all slowdown of the biosynthesis pathways in Rrp12p depleted cells at this time point. Therefore, it is possible that the delay of 5.8S rRNA synthesis is an indirect effect caused by the slowed metabolism of the cells.

Many mutant strains that are defective in the synthesis of 60S ribosomal subunit, including strains depleted for exosome components, show a delay in the early pre-rRNA cleavages (Venema and Tollervey, 1995). However, with very few exceptions, these do not alter the levels of the 20S pre-rRNA or 18S rRNA. With the exception of strains depleted for Rrp43p, depletion of other exosome components or associated proteins does not cause such a phenotype, leaving levels of 20S and 18S rRNA unaffected (Mitchell et al., 1996; Briggs et al., 1998; de la Cruz et al., 1998; Zanchin and Goldfarb, 1999a; Allmang et al., 2000). The accumulation of 20S pre-rRNA and loss of 18S rRNA indicates that Rrp12p does not only function in the maturation of the large 60S ribosomal subunit but has another, separate role in the late steps during small subunit formation.

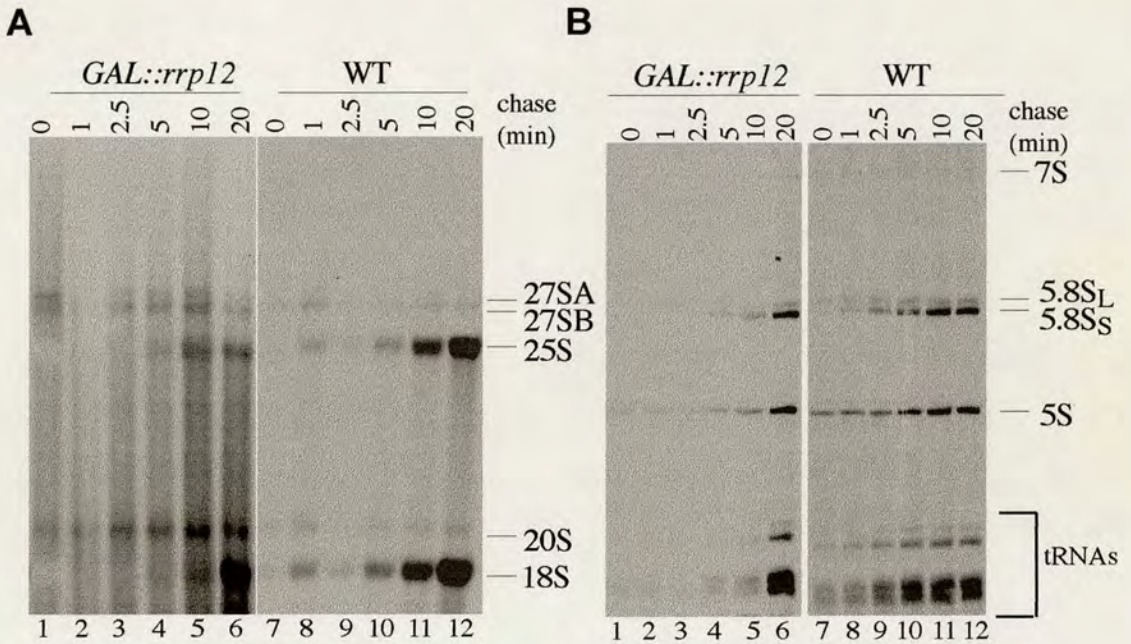


Figure 25: Ribosomal RNA synthesis is delayed in Rrp12p depleted cells.

Pre-rRNA was pulse labelled with [³H]uracil for 2 minutes at 30°C and chased with a large excess of unlabelled uracil for the times indicated. Labelling was performed for the *GAL::rrp12* (YMO9) strain (lanes 1 to 6) and a wild-type strain (YMO5) (lanes 7 to 12) 16 hours after transfer to repressive glucose medium.

(A) High molecular weight RNA separated on a 1.2% agarose/formaldehyde gel.

(B) Low molecular weight RNA separated on an 8% polyacrylamide/urea gel.

4.4. Rrp12p is associated with both ribosomal subunits

To better understand the role of Rrp12p, its association with pre-ribosomal particles and pre-rRNA precursors was analyzed. Rrp12p was epitope-tagged by insertion of a tandem-affinity (TAP) tag in frame with the *RRP12* open reading frame in the chromosome under the control of the *GAL10* promoter (Rigaut et al., 1999) (see Chapter 2, section 2.13). Correct expression of the fusion protein was confirmed by western blotting using an anti-Protein A antibody bound to horseradish peroxidase (Figure 26). The strain exhibited no detectable growth defect on permissive galactose medium, showing the fusion construct to be fully functional (YMO8).

Association with pre-ribosomal particles was assessed by sucrose gradient centrifugation, and sedimentation of Rrp12p-TAP was compared to the rRNA species and pre-rRNAs (Figure 27). Comparison to the positions of mature rRNAs detected by Northern hybridisation showed that Rrp12-TAP is enriched in the 40S and 60S regions of the gradient (Figure 27, panel a, c and e). The majority of protein was detected in the 40S region, with a smaller amount in the 60S region. The Rrp12p-TAP is clearly present in the samples that contain 20S pre-rRNA although the peak of Rrp12p is slightly lower on the gradient (Figure 27, panel a and d). The minor peak detected in the 60S regions is close to the peak of 27SB pre-rRNA (Figure 27, panel a and b). This suggests that Rrp12p associates with both, the pre-40S particle and the pre-60S particle. Under these conditions, the interaction between the exosome and the pre-60S particle is not maintained, and the Rrp12p-pre-60S association may also not accurately reflect the degree of *in vivo* association.

4.4.1. Rrp12p accompanies the small subunit precursor to the cytoplasm

RNAs associated with Rrp12p-TAP (YMO8) were analyzed following immunoprecipitation (see Chapter 2, section 2.15) by Northern analysis and primer extension and were compared to RNA recovered in parallel from a non-tagged control

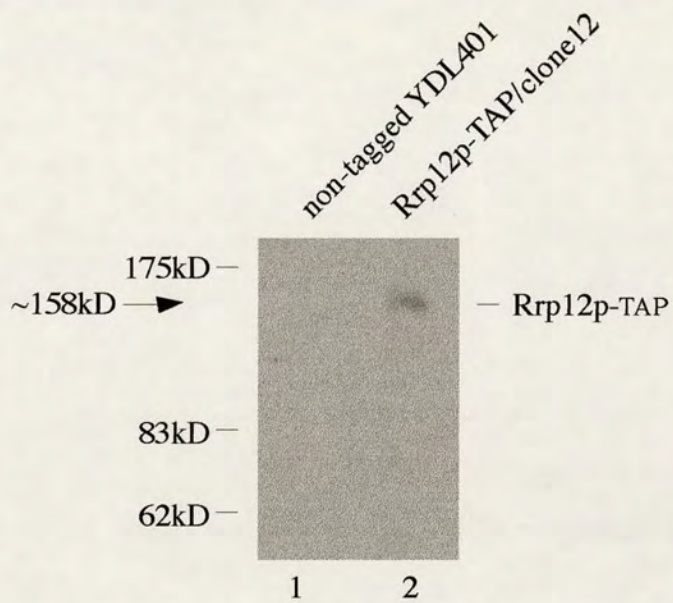


Figure 26: Western blot showing Rrp12p-TAP construct.
 Immuno blot analysis of strains expressing a Rrp12p-TAP (affinity purification tag) fusion under the control of the *GAL 10* promoter in the YMO8 strain. Blot has been probed with anti-ProteinA antibodies coupled to horseradish peroxidase to visualise the TAP-tag . The tag contributes 20kD to the size of the fusion protein.

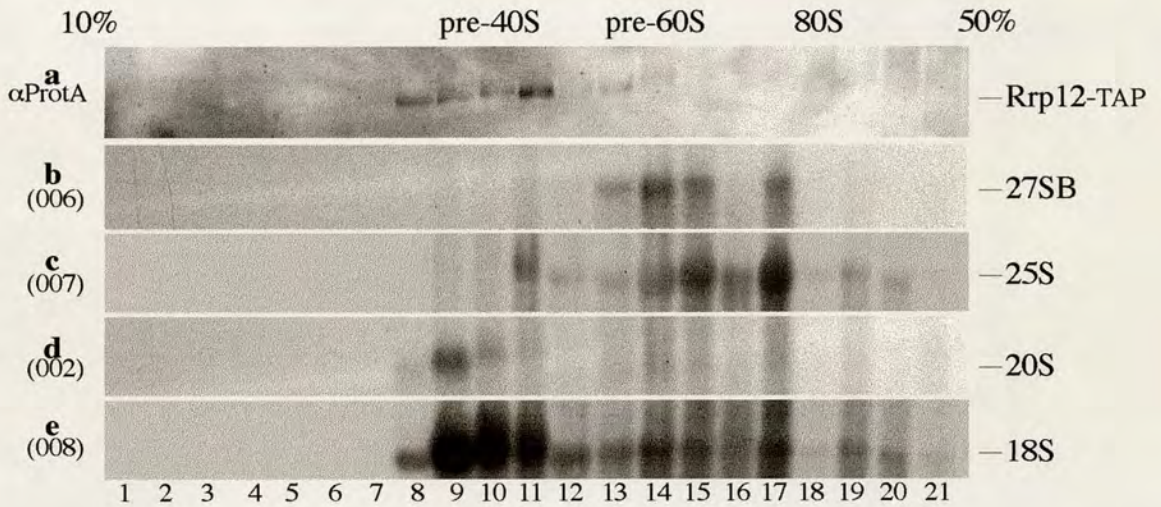


Figure 27: Rrp12p is associated with the pre-40S and pre-60S complexes.

(a) Behavior of TAP-tagged Rrp12p on a 10-50% sucrose gradient. Rrp12p-TAP was detected by Western blot analysis using PAP antibodies (see Chapter 2, section).

(b-e) Northern analysis of RNA extracted from each gradient fraction. Probes used are indicated in parentheses on the left (see Chapter 2, Table 2.2.).

Position of 40 and 60S ribosomal subunits and 80S ribosomes are indicated.

strain (YDL401) and 5µg of total RNA. Co-precipitation was seen for 35S, 27SA₂, 27SB and 7S pre-rRNAs, as well as 20S pre-rRNA (Figure 28A panels a, c; 28B and 28C, panel a). The mature 25S and 5.8S rRNAs were also precipitated (Figure 28A panel b and e), but no precipitation was seen for 6S pre-rRNA or 18S rRNA (Figure 28A, panel d and f). Significantly, Rrp12p-TAP also precipitated the cytoplasmic form of the 20S precursor (Figure 29) as shown by a primer extension stop at the 2'-O-methylated residues m⁶₂A₁₇₇₉ and m⁶₂A₁₇₈₀ within the 18S rRNA sequence. This dimethylation occurs after the precursor has been transported to the cytoplasm as was shown by cell fractionation, and is carried out by the dimethylase Dim1p (Klootwijk et al., 1972; Udem and Warner, 1973; Brand et al., 1977; Lafontaine et al., 1998a).

This supports the results from Northern analyses, primer extension and sucrose gradients concluding that Rrp12p is associated with both pre-40S and pre-60S particles (precursors of both ribosomal subunits) as well as the 90S pre-ribosome, and has distinct roles in the maturation of both subunits. Association with late precursors and, in the case of 25S and 5.8S, mature rRNAs points to a function during the late maturation steps. Furthermore, co-precipitation of the methylated 20S pre-rRNA suggests shuttling of Rrp12p to the cytoplasm.

4.4.2. Proteomic analysis

To identify non-ribosomal and ribosomal proteins associated with Rrp12p-TAP, a two-step affinity purification was performed (Rigaut et al., 1999), and purified proteins were separated by SDS-PAGE using different gel conditions (see chapter 2, section 2.14). Rrp12p is a 138kDa protein and to visualise the protein by SDS-PAGE it is necessary to use a low-percentage gel. However, to separate small sized proteins that might co-purify with Rrp12p-TAP, a high-percentage gel was used. Since it was not possible to detect any proteins by Coomassie staining, bands were visualised by silver staining. A strong band was observed at around 143kDa that most likely corresponds to Rrp12p, carrying the calmodulin-binding domain.

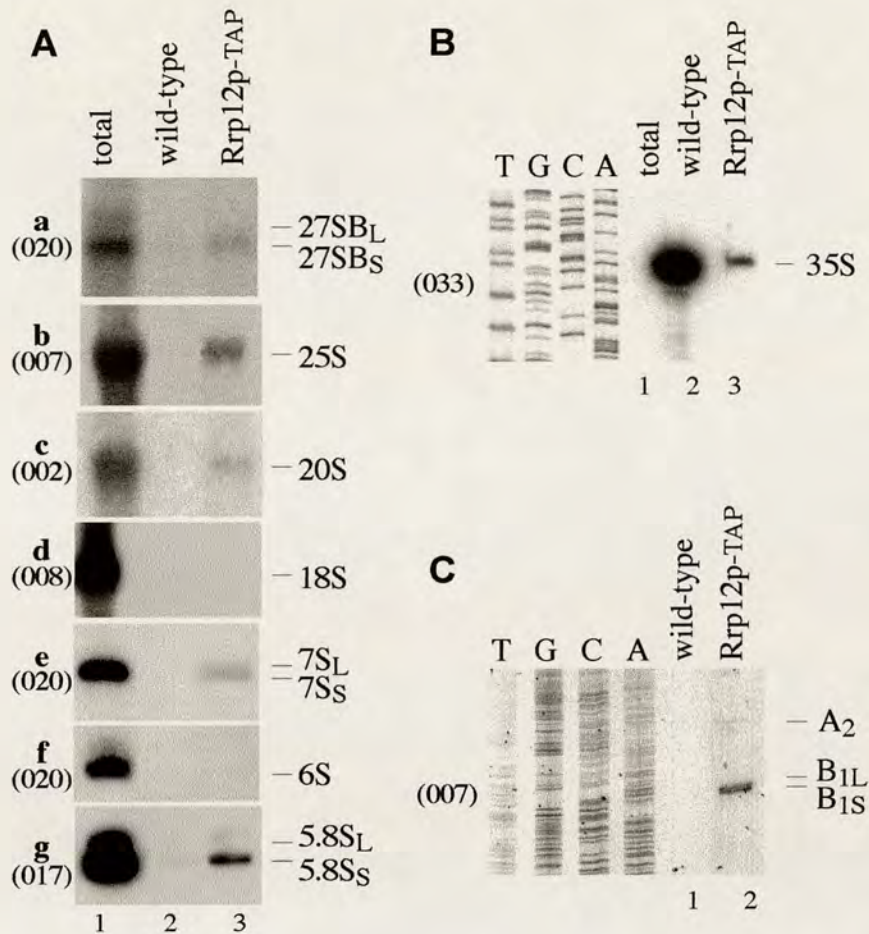


Figure 28: Rrp12 co-precipitates RNAs from both ribosomal subunits.

Nop7p-TAP was immunoprecipitated from cell lysates using IgG agarose, with release of bound RNA-protein complexes by cleavage of the protein A linker by TEV protease. RNA was recovered from released material and from a mock-treated isogenic wild-type control strain (YDL401).

(A) Northern hybridisation of pre-rRNA and rRNA co-precipitated with Rrp12p-TAP. Probes used are indicated in parentheses on the left (see Chapter 2, Table 2.2.). Lane1: total RNA control (5µg). Lane2: precipitate from a wild-type control strain (YDL401). Lane3: precipitate from a strain expressing RRP12-TAP (YMO8).

(B) Primer extension analysis using oligo 033 (see Chapter 2, Table 2.2.). Primerextension stops show levels of co-precipitated 35S pre-rRNA. Lanes 1 to 3 loaded as described in (A).

(C) Primer extension analysis using oligo 007 (see Chapter 2, Table 2.2.). Primerextension stops at sites B_{1S} and B_{1L} show levels of co-precipitated 27S_{B_S} and 27S_{B_L} pre-rRNAs, respectively. Lane1: precipitate from a wild-type control strain (YDL401). Lane2: precipitate from a strain expressing RRP12-TAP (YMO8).

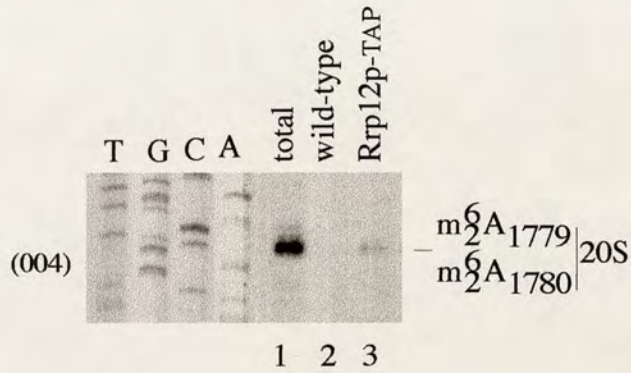


Figure 29: Rrp12 co-precipitates cytoplasmic, methylated 20S pre-rRNA.

Lane1: Total RNA control (5 μ g). Lane2: Precipitate from a wild-type control strain (YDL401). Lane3: Precipitate from a strain expressing RRP12-TAP (YMO8). Nop7p-TAP was immunoprecipitated from cell lysates using IgG agarose, with release of bound RNA-protein complexes by cleavage of the protein A linker by TEV protease. RNA was recovered from released material and from a mock-treated isogenic wild-type control strain (YDL401).

Primer extension analysis using oligo 004 (see Chapter 2, Table 2.2). Primer extension stops show levels of co-precipitated cytoplasmic, dimethylated 20S pre-rRNA.

Silver staining is a very sensitive method to detect small amounts of protein, but it was not possible to identify the protein by mass spectrometry (Figure 30A and B). Since Rrp12p is a very large protein, it is possible that a TAP-tag on either of its termini might not be fully accessible due to structural arrangements, and the purification therefore not efficient. If Rrp12p associates with a large number of proteins steric hindrance or weak binding could also interfere with a complete purification of the complex. Previously it had been shown that the efficiency of TAP purification is influenced by the properties of the bait protein. The TAP-tag on the bait could weaken the interaction with associated proteins (Gavin et al., 2002). Comparison of the protein bands obtained with Rrp12p-TAP to previous protein purifications showed that the pattern resembled that seen with Ssf1p-TAP, Nog1p-TAP or Nop7p-TAP (Bassler et al., 2001; Harnpicharnchai et al., 2001; Fatica et al., 2002). In other proteomic studies, Rrp12p was copurified with tagged proteins of the 90S complex, the pre-40S particle and different pre-60S particle (Table 4.1.) (Gavin et al., 2002; Grandi et al., 2002). This proteomic data supports the association of Rrp12p with the 90S pre-ribosome, the pre-40S and pre-60S particles as seen from Northern analysis, primer extension and RNA precipitation experiments. Rrp12p is the only protein identified so far to show this behaviour.

4.4.3. Rrp12p is localised to the nucleolus

To determine the sub-cellular location of Rrp12p, cells expressing Rrp12p-TAP were examined by indirect immunofluorescence (Figure 31) using a rabbit anti-protein A and a secondary FITC-conjugated goat anti-rabbit antibody to detect the protein A region of the TAP-tag (Rigaut et al., 1999). The anti-protein A signal was mostly found in the nucleolus, with a weaker signal over the nucleoplasm. No cytoplasmic signal could be detected. This indicates that Rrp12p is localised to the nucleus with nucleolar enrichment. The absence of staining in the cytoplasm cannot, however, be taken as a demonstration that Rrp12p does not shuttle to the cytoplasm. Nuclear import might be very rapid, leading to only a small fraction of protein being cytoplasmic at steady-state.

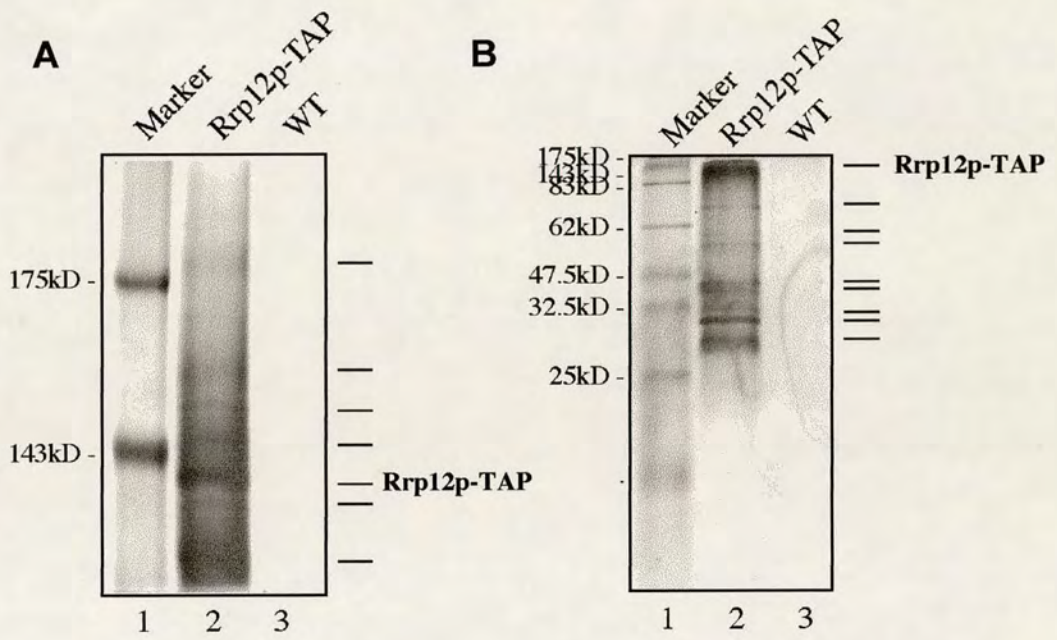


Figure 30: Proteomic analysis of Rrp12-TAP.

Purified proteins obtained from Immunoprecipitation with Rrp12p-TAP. The proteins were resolved on different SDS-PAGE gels according to size. (A) 6% Polyacrylamide gel. (B) 12% polyacrylamide gel. Bands were visualised by silver staining.

Protein	Pre-ribosomal Particle	Cellular Localisation	Function	
Pwp2p	90S	No	cell separation	Gavin et al., 2002; Gardi et al., 2002
Cbf5p	90S	No	rRNA modification	Gavin et al., 2002; Gardi et al., 2002
Gar1p	90S	No	rRNA modification	Gavin et al., 2002; Gardi et al., 2002
Enp1p	90S, pre-40S	No	unknown	Gavin et al., 2002; Gardi et al., 2002
Krr1p	90S, pre-40S	No	40S biogenesis	Gardi et al., 2002
Noc4p	90S	No	unknown	Gardi et al., 2002
Yhr196p	90S	No	unknown	Gardi et al., 2002
Nop14p	90S	No	unknown	Gavin et al., 2002; Gardi et al., 2002
Noc1p	90S, pre-60SE _{1&2}	No	60S biogenesis/export	Gavin et al., 2002
Nop4p	pre-60SE ₁	No	60S biogenesis	Gavin et al., 2002
Nop15p	pre-60SE _{1&2}	No	60S biogenesis	Gavin et al., 2002
Tsr1p	90S, pre-40S	No	40S biogenesis	Gavin et al., 2002
Rio2p	pre-40S	Cyt	40S biogenesis	Gavin et al., 2002
Pse1p(Kap121p)	-	Cyt	Importin	Gavin et al., 2002

Table 4.1. TAP-tagged proteins reported to co-purify Rrp12p.

Rrp12p was found to co-purify with the proteins listed in the table above. The TAP-tagged proteins were used as bait in studies indicated in the right-hand column.

No, nucleolar; Cyt, cytoplasmic.

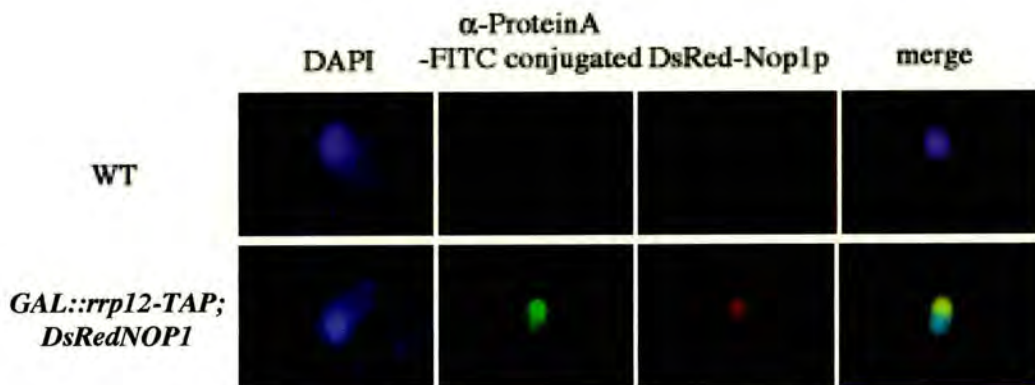


Figure 31: Rrp12p is localised to the nucleolus.

The *GAL::rrp12-TAP* strain (YMO9) co-expressing the nucleolar marker DsRedNop1p was examined by indirect immunofluorescence using an anti protein A antibody coupled to FITC. Also shown is the position of the nucleus visualised by DAPI staining and a wild-type control strain (YDL401).

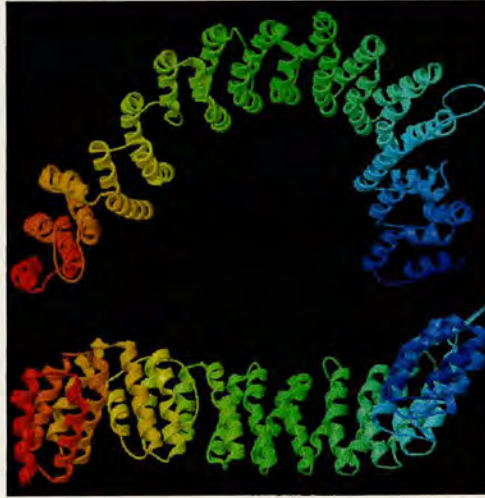
Moreover, a diffuse cytoplasmic signal would be less readily detected than the nuclear signal. This has also been demonstrated for the nucleolar protein nucleolin in vertebrate cells. Nucleolin was found to shuttle between nucleus and cytoplasm in heterokaryon interspecies, however, at steady-state it is exclusively localised to the nucleolus (Lischwe et al., 1981; Borer et al., 1989; Schmidt-Zachmann and Nigg, 1993).

4.5. Is Rrp12p involved in export of ribosomal subunits?

Rrp12p is a large 137kDa protein, but the database annotation and initial BLAST searches did not identify any structural motifs in the protein (Altschul et al., 1997). It seems unlikely that a large protein would really lack homology to known structures and we therefore initiated a collaboration with a bio-information, Dr. M. Dlakic at the University of Michigan. Using a sequence analysis program, it was possible to predict a 3-dimensional structure, which revealed that the protein consists of 15 tandemly repeated modules known as HEAT-repeats (Figure 32A). HEAT-repeats are sequences of approximately 40 residues of a loose consensus pattern of conserved hydrophobic amino acids (Figure 32B) folded into a bent helix and a straight helix (Andrade et al., 2001; Conti and Izaurralde, 2001). The multiple HEAT-repeats pack side by side in a parallel fashion, forming elongated molecules with a superhelical twist (Figure 21A) (Chook and Blobel, 1999; Cingolani et al., 1999). A number of HEAT-motif containing proteins, called karyopherins, have been implicated in nucleo-cytoplasmic transport (Chook and Blobel, 1999; Cingolani et al., 1999). The structural similarity of Rrp12p to karyopherins and the association with cytoplasmic 20S pre-rRNA suggest a possible involvement in ribosomal export.

4.5.1. 40S subunit export is inhibited in cells depleted of Rrp12p

In yeast, the pre-40S ribosomal particle is assembled in the nucleolus before being transported to the cytoplasm (Udem and Warner, 1973). In the cytoplasm, the 20S RNA

A**B**

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233  KEDINASVVRSLRLITTSVV---STGQWPSSQIEP-LCDVLLGVTKSESEQYLVSASFECFESMF 291
684  KEDLEKIFNNVCGLLKNSMNEESSGNVNKERKKPQLTATLLOLIIIMITYLPVSSYSALFSMF 786

430  SREIKILVAAFNKFYRNPFFLKS LKTYD---TWRVNEEQFMDLRNETELVIGASISAMGPENTLAEAP 497
931  SREIVKSAI-GFTKVCVLELPEELMRPKVPE(6)RW--SHEHTGFKAKVKHLIERLIRRFQYDYIEANFP 1009

779  AQFISDIENVMVDSASSVQTSAKAARLT--ALKTLVEL--LPLDHLDFIVRTVAEVLIS-TKDVNEKSREI 849
1055 AVYGSDEEN---DNGSDQEEENVASGKMNNGAKQFLVESGDNPLDLLD--SQTLAHISSTRPKKENKNQNR 1125

850  AE-DTLLICMRKMNEPNGIILKLFQIPGYDPTTPDQ--SSISSEFFKITSAG 892
1126 RFNDDAFNFD---SEGKLVVKGQPKPSTNVDDPLSAVTSGINAYLEAVKSG 1168

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Figure 32: Rrp12p consists of consensus HEAT-repeats.

(A) Predicted 3-dimensional structure of Rrp12p (residue 452 to 1018). Ribbons diagram showing folding of consensus HEAT motifs from two different orientations. Image supplied by M.Dlagic, University of Michigan.

(B) Multiple sequence alignment of HEAT motif sequences within Rrp12p. A custom-built hidden Markov model (Eddy, 1998) was used to make the alignment. Amino acids are coloured according to their physico-chemical properties with an 80% threshold. The meaning of colours is as follows: gold-charged (DEHRK), green-aliphatic (ILV), red-polar (EHKNQRST), blue-hydrophobic (ACFGHILMTVWY), purple-small residues (ACDGNPSTV), grey-tiny (ACGST), light blue-aromatic (FY), red letters-negatively charged (DE), blue letters-big residues (EFIKLMQRY), blue letters-positively charged (HKR), light blue letters-hydroxy (ST); individual residues with more than 80% identity are shown in yellow. Model and alignment supplied by Dr. M. Dlagic, University of Michigan, USA.

precursor within this complex is cleaved by an unidentified endonuclease at site D (Stevens et al., 1991), which produces the mature 18S rRNA and an approximately 200-nucleotide fragment, the 5' region of ITS1. This fragment is then degraded by the 5' to 3' exonuclease Xrn1p (Stevens et al., 1991). To assay for defects in the export of the small ribosomal subunit, the localisation of the ITS1-5' fragment was determined by fluorescent in situ hybridisation (Moy and Silver, 1999). This assay was initially performed in cells lacking the Xrn1p exonuclease, in which, after cleavage at site D, the ITS1 5'-fragment was accumulated in the cytoplasm (Moy and Silver, 1999). However, it had been shown that in *xrn1Δ* cells, the excess of nuclear ITS1 5' RNA signal can obscure a mild export defect and that the assay can also be carried out in *XRN1*⁺ wild-type yeast cells (Moy and Silver, 2002).

I assayed the export of the small ribosomal subunit in Rrp12p-depleted cells in a *XRN1*⁺ wild-type background using a fluorescent labelled probe against the ITS1 5' region (see Chapter 2, Table 2.2). The nucleoplasm was identified by DAPI staining. In wild-type (BMA64) cells and *GAL::rrp12* (YMO7) cells grown in permissive galactose medium, ITS1 5' RNA signal was concentrated in the nucleolus and along the nuclear rim (Figure 33, arrows indicating localisation at nuclear rim, panels c and i). In addition, the ITS1 5' probe showed a clear cytoplasmic signal, with some peri-nuclear enrichment (Figure 33, panels b and h). The localisation of the ITS1 5' RNA signal to the nucleolus was expected since this is the site of rRNA transcription, processing and assembly and the probe detects nascent rRNA transcripts as well as 35S, 33S, 32S, 20S pre-rRNAs in addition to excised ITS1 5'-fragments. After 24 hours growth in repressive glucose medium, wild-type cells exhibited the same localisation pattern for ITS1 5' RNA as in galactose medium (Figure 33, panels d to f). In cells lacking Rrp12p, however, the 5'ITS1 RNA signal was only detected in the nucleus and nucleolus and along the nuclear rim (Figure 33, panels j to k; arrows indicating localisation at nuclear rim) with no detectable cytoplasmic signal. This nuclear/nucleolar restriction of the pre-rRNA in the Rrp12p depleted strain demonstrates that Rrp12p is required for the export of the small ribosomal subunit to the cytoplasm. The exact role of Rrp12p in 40S export, if it

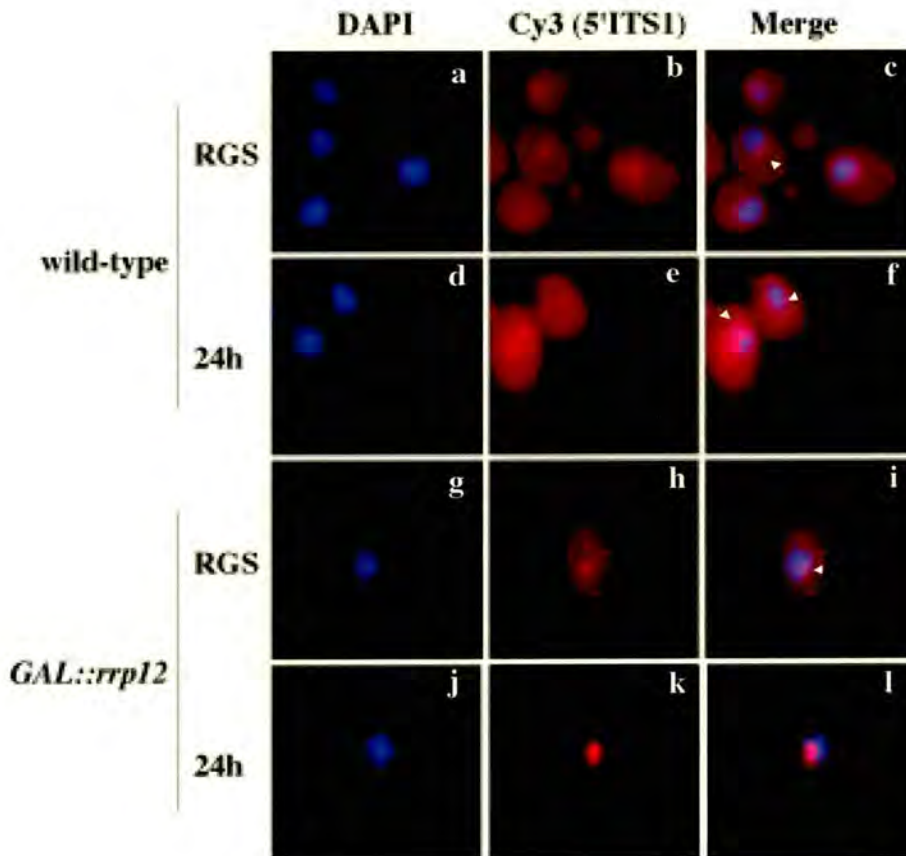


Figure 33: Depletion of Rrp12p impairs export of pre-40S particles to the cytoplasm.

Localisation of the ITS1 5' region in wild-type (YDL401) and *GAL::rrp12* (YMO7) cells grown in permissive galactose medium and after 24 hours in repressive glucose medium. Chromosomal DNA was visualised with DAPI to identify the position of the nucleus and is shown in blue (a, d, g, j). 5' ITS1 RNA was hybridised and labeled with a Cy3-conjugated 50bp probe and is shown in red (b, e, h, k). The DAPI and 5' ITS1 signals were overlaid in c, f, i and l and produce a pink staining. Arrows indicate localisation in the nucleolus and the nuclear rim.

can shuttle through the nuclear pore by itself or if it needs an adapter protein, like Xpo1p, and whether it can bind to the small GTPase Gsp1p (Yeast Ran) and the nuclear pore complex, remain to be determined (Ho et al., 2000b).

4.5.2. 60S export

The association of Rrp12p with both mature 5.8S and 25S rRNA and an involvement in 40S subunit export suggested the possibility that Rrp12p could also function during export of the large ribosomal subunit. To follow the export of pre-60S ribosomal subunits from the nucleus to the cytoplasm several recent studies have made use of fusions between ribosomal proteins and GFP (Stage-Zimmermann et al., 2000; Bassler et al., 2001; Milkereit et al., 2001; Gadal et al., 2001a; Gadal et al., 2001b; Fatica et al., 2002). To look for 60S subunit export defects, Rpl25p-eGFP (Gadal et al., 2001b) was expressed from a plasmid in the wild-type (YDL401) and *GAL::rrp12* (YMO10) strains.

Expression of the Rpl25p-eGFP construct had little effect on the growth of the wild-type strain (Gadal et al., 2001b) or the *GAL::rrp12* strain on galactose medium. However, growth of the *GAL::rrp12*/Rpl25p-eGFP strain was significantly slowed following transfer to glucose medium compared to the *GAL::rrp12* strain lacking the reporter construct (Figure 34). This strain also expresses the wild-type Rpl25p, showing that the Rpl25p-eGFP fusion construct has a dominant negative effect for growth in strains with a reduced level of Rrp12p. Nevertheless, the growth inhibition appears to be less pronounced than in the *GAL::nop7* strain (YMO4) (see Chapter 3, section 3.6). The distribution of Rpl25p-eGFP was observed before and after depletion of Rrp12p.

During growth of the *GAL::rrp12* strain on galactose medium, Rpl25p-eGFP showed a normal, predominantly cytoplasmic distribution. After transfer to glucose medium for 6 hours, no GFP-signal could be detected other than a weak yellow-green auto-fluorescence. Wild-type cells, however, displayed a normal signal for Rpl25p-eGFP throughout the cytoplasm. DAPI staining could still be seen in the nucleus. The loss of GFP-signal was clear by visual inspection but it was not possible to present a

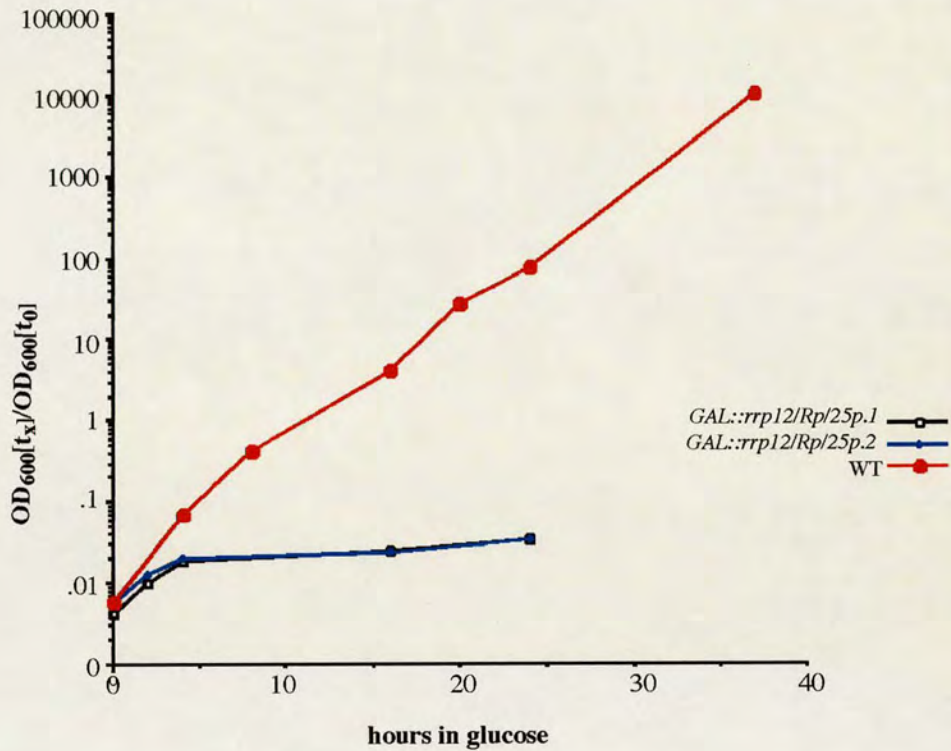


Figure 34: Depletion of Rrp12p affects assembly of Rpl25p to pre-60S particles.

Growth curve of two independent transformants of the *GAL::rrp12* strain co-expressing Rpl25p-eGFP (YMO10) and an isogenic wild-type strain (YDL401). Strains were pre-grown in permissive galactose medium and transferred to repressive glucose medium. Growth inhibition in the *GAL::rrp12* strain occurs 4 hours after transfer to glucose medium, and is both faster and more complete than in the *GAL::rrp12* strain not expressing Rpl25p-eGFP (see Fig. 21).

figure for this result since the auto-fluorescence could not be distinguished from real GFP-fluorescence in the images taken by the camera. This is due to a technical problem resulting from the fact that the camera tried to capture the maximum contrast from the images during exposure. Therefore, the weak fluorescence was enhanced in the absence of the GFP-signal. The auto-fluorescence was therefore not clearly different from the actual fluorescence in other images. This experiment will be repeated. The loss of GFP-signal in *GAL::rrp12* cells after transfer to glucose medium could account for a mild assembly defect that will be discussed in the final section of this chapter.

4.6. Discussion

The role of a novel, essential protein, Rrp12p, in ribosome biogenesis and export was the focus of this chapter. This work showed that depletion of Rrp12p results not only in accumulation of 20S pre-rRNA and depletion of 18S rRNA but also depletion of 25S rRNA and inhibition of 3' end formation of 5.8S rRNA. Localisation of the ITS1 5' RNA region has been used as a marker for a defect in nuclear export of pre-40S ribosomal particles (Moy and Silver, 1999), and was also observed following Rrp12p depletion. Depletion of Rrp12p also resulted in synergistic inhibition of growth in the presence of a GFP-tagged form of ribosomal protein Rpl25p, indicating an additional role in 60S ribosome assembly. From these results it can be concluded that Rrp12p is required for normal pre-60S assembly, efficient activity of the exosome on the 5.8S rRNA and nuclear export of the 40S subunit.

4.6.1. *Rrp12p has independent functions during biogenesis of both subunits*

Nnp86p/hRrp12p was isolated during a proteomic analysis of human nucleoli (Andersen et al., 2002) and Ypl012p/Rrp12p was identified as its homologue in yeast. Sequence analysis predicted Rrp12p to be involved in ribosomal biogenesis and structural analysis predicted Rrp12p to consist entirely of HEAT-repeats. Rrp12p was shown to be part of

different pre-ribosomal complexes (Gavin et al., 2002; Grandi et al., 2002) of the large and the small ribosomal subunit. This allows a proposal between the pre-ribosomal particles Rrp12p is associated with and the distinct defects in ribosome synthesis that are seen on its depletion.

In yeast, the early processing steps at sites A₀, A₁ and A₂ take place within the 90S pre-ribosome (Fatica and Tollervey, 2002). A kinetic delay of these early processing steps was observed in the *GAL::rrp12* strain after transfer to glucose medium. Processing at these sites is only indirectly affected by mutations in 60S synthesis factors and exosome components. However, since it was shown that Rrp12p is present in the 90S particle, this is likely to be a direct effect in *RRP12* mutant strains (Grandi et al., 2002). Truncated RNAs resulting from delays at early cleavage sites are normally degraded by the functional exosome (Mitchell et al., 1997; Venema and Tollervey, 1999). Similar to exosome mutant strains and *GAL::dobl* strains these RNAs, however, were stabilised in the *GAL::rrp12* strain (Allmang et al., 2000).

Furthermore, in the Rrp12p depleted strain 20S RNA precursor was accumulated and 18S rRNA lost. Strains with mutations in exosome components, Dob1p and many other 60S synthesis factors do not show such phenotype, leaving levels of 20S and 18S rRNA unaffected (Mitchell et al., 1996; Briggs et al., 1998; de la Cruz et al., 1998; Allmang et al., 2000). The only exception are strains depleted of Rrp43p in which synthesis of 18S rRNA was found to be inhibited (Zanchin and Goldfarb, 1999a). However, this might be a strain dependent effect, since no such result was seen in *GAL::rrp43* mutants in a different strain background (C.Allmang, unpublished data). This strongly indicates that Rrp12p has a separate function during 40S subunit synthesis.

An inhibition of processing at site A₃ and clear accumulation of 5'extended forms of 5.8S rRNA was also observed in Rrp12p depleted strains. Accumulation of truncated and aberrant RNAs as well as the appearance of a 3'end extended 5.8S rRNA that has also been observed in strains carrying mutations in exosome components and Dob1p (Allmang et al., 2000) suggests that some exosome activity is inhibited in Rrp12p depleted strains.

Recently, Rrp12p was shown to co-purify with TAP-tagged Tsr1p and Rio2p in a large-scale proteomic analysis (Gavin et al., 2002). Both proteins have been implicated in late steps of 40S maturation (Gelperin et al., 2001; Caizergues-Ferrer, personal communication). Strains depleted of Tsr1p exhibited a delay in the maturation of 20S to 18S rRNA and accumulated the 20S precursor (Gelperin et al., 2001). Rio2p is localised to the cytoplasm and its depletion resulted in loss of the 18S rRNA and strong accumulation of cytoplasmic, dimethylated 20S pre-rRNA, suggesting its involvement in cleavage at site D in the cytoplasm (Hurt, Caizergues-Ferrer, pers. comm.).

The maturation of 20S pre-rRNA to mature 18S rRNA occurs in the cytoplasm through cleavage at site D, and releases the excised ITS1 5' region. In situ hybridisation with a fluorescent-labelled probe complementary to this region was used to follow its localisation. In strains defective in small ribosomal subunit export, the 20S precursor accumulates in the nucleus without being cleaved (Moy and Silver, 1999; Moy and Silver, 2002). However, neither Tsr1p nor Rio2p has been reported to affect 40S subunit export. So far only the karyopherin Xpo1p, the yeast Ran-binding protein Yrb2p and nucleoporins have been implicated in export of the small ribosomal subunit (Wiese et al., 1997; Gleizes et al., 2001; Moy and Silver, 2002). Nuclear accumulation of ITS1 5' region was detected in strains depleted of Rrp12p and co-precipitation of cytoplasmic, dimethylated with Rrp12p-TAP shows that the protein associates with the 20S pre-rRNA in the cytoplasm. These data indicates that Rrp12p accompanies the pre-40S particle to the cytoplasm and is required for its export.

A structural analysis predicted that Rrp12p consists entirely of HEAT-repeats. This structural framework is shared by a number of proteins within the family of nuclear transport factors, the karyopherins (Conti et al., 1998; Chook and Blobel, 1999; Cingolani et al., 1999; Andrade et al., 2001). Next to fourteen importin β -like importins, in yeast, several importin β -like exportins have so far been identified, amongst them Los1p, Msn5p, Cse1p, Kap120p, Mex67p and Xpo1p (Crm1p) (Segref et al., 1997; Stade et al., 1997; Hellmuth et al., 1998; Kunzler and Hurt, 1998; DeVit and Johnston, 1999). Out of these, only Kap120p and Xpo1p have been implicated in ribosomal subunit export (Moy and Silver, 1999; Neville and Rosbash, 1999; Stage-

Zimmermann et al., 2000; Moy and Silver, 2002). Comparison of Rrp12p to members of the family of importin β -like nuclear transport factors showed that its structure is similar to that of known karyopherins. Almost all transport factors have the ability to interact with Ran, yeast Gsp1p (Nigg, 1997). Furthermore, all karyopherins were shown to interact with FG nucleoporins via their HEAT-repeat domains, however, different factors favoured different nucleoporins (Rout et al., 1997; Marelli et al., 1998; Aitchison and Rout, 2000). This might also be the case for Rrp12p, and association with Ran and nucleoporins will be tested.

A fast acting, dominant negative phenotype that is associated with the expression of a GFP-tagged form of the ribosomal protein Rpl25p has been observed in strains depleted of Nop7p (see Chapter 3, section 3.6). In strains depleted of Rrp12p, I observed a growth inhibition similar to that observed Nop7p-depleted cells in the presence of Rpl25p-GFP. The early growth inhibition in cells lacking Rrp12p could be accounted for by an additional assembly defect. The fact that expression of Rpl25p-GFP is dominant indicates that in its presence the wild-type Rpl25p is no longer able to support growth possibly because neither of the proteins, tagged and untagged, is assembled to the pre-60S particle. Loss of the plasmid can be excluded since the cells were still able to grow, if only slowly, on medium lacking uracil.

Several recent studies have made use of a fusion between Rpl25p and GFP to follow the export of 60S ribosomal subunits from the nucleus to the cytoplasm (Bassler et al., 2001; Milkereit et al., 2001; Gadai et al., 2001a; Gadai et al., 2001b; Fatica et al., 2002). There is considerable data showing that free r-proteins do not accumulate in the absence of ribosome synthesis (Woolford and Warner, 1991). The accumulation of Rpl25p-GFP has therefore been taken as evidence for the accumulation of pre-ribosomal particles in the nucleoplasm, indicating a defect in their export. The lack of accumulation of Rpl25p in the nucleus of cells depleted for Rrp12p, points against an export defect.

Inhibition of ribosome synthesis at any stage causes degradation of the pre-60S complex. Since no GFP-tagged Rpl25p is detected in Rrp12p-depleted cells, it seems likely that ribosomal proteins are degraded with the pre-60S complex. However, in

ribosomal export (*rix*) mutants degradation of pre-60S particles seems to exclude ribosomal proteins, which accumulate in the nucleus (Hurt et al., 1999). Factors required for ribosomal export of the large subunit associate with a late pre-60S complex and mutations in any of those factors causes inhibition of ribosome synthesis at a later stage (Bassler et al., 2001; Milkereit et al., 2001; Gadal et al., 2001a; Gadal et al., 2001b). It is not entirely clear which particles Rrp12p is associated with, but it is possible that if subunit synthesis is blocked at an early stage the pre-60S particles are degraded together with ribosomal proteins, which can no longer be detected. Block of ribosome synthesis at a later stage also results in degradation of the pre-60S particles, however, in this case ribosomal proteins appear to be excluded from degradation and accumulate in the nucleus. Bleomycin hydrolase, a cysteine proteinase, was shown to interact and associate with ribosomal proteins of the large subunit by two-hybrid screen (Koldamova et al., 1999). Yeast bleomycin hydrolase forms a homohexameric structure that resembles a 20S proteasome, which is believed to be the proteolytically active core of the yeast 26S proteasome, degrading ubiquitinated proteins in all eukaryotic cells (Bochtler et al., 1999). It is possible, however, that at different stages during ribosome synthesis ribosomal proteins are degraded by a different mechanism due to a change in the level of ubiquitination (Spence et al., 2000).

4.6.2. Exosome component, transport factor or assembly factor?

Rrp12p is the only known *trans*-acting factor that is required during late maturation of both ribosomal subunits. Moreover, the protein appears to fulfil very different roles in the different subunits, as assembly factor and facilitator of the exosome activity in the large subunit, and during export of the small subunit.

The rRNA processing defects seen in the *RRP12* mutant strain show some similarities to exosome mutant strains. However, Rrp12p does not appear to be a component of the exosome since it has never been co-purified together with the complex. Furthermore, the processing at site A₃ is delayed, which is carried out by the RNase MRP (Chu et al., 1994). But Rrp12p is not a component of this complex either.

However, Rrp12p might still be required to accommodate interaction between them and their target RNA precursor. Considering its predicted structure, it could wrap around the pre-ribosomal complex and conformational changes in its structure could promote interactions with other factors including the exosome or RNase MRP or even of ribosomal proteins during assembly to the particle. Importin β , a karyopherin to which Rrp12p is structural related, wraps around its cargo and binds, after conformational changes in its structure, to Ran-GTP and the nuclear pore complex to transport the cargo into the nucleus (Conti and Izaurralde, 2001). It is possible that Rrp12p functions in a similar fashion, not only during export of the 40S subunit but also to regulate or facilitate 60S subunit assembly.

Another HEAT-repeat containing protein found to be part of the 90S pre-ribosome is Ybl004p/Utp20p (Grandi et al., 2002). This 276kDa protein is predicted to consist entirely of HEAT-repeats (M.Dlakic, pers. comm.). Moreover, Ybl004p/Utp20p was found to associate with the U3 snoRNP (S.Baserga, pers.comm.). Considering the structure, it could be suggested that Ybl004p/Utp20p facilitates the interaction between the large U3 snoRNP complex and the pre-ribosomal RNA or between components of the U3 snoRNP itself. However, this possibility has not yet been investigated.

Chapter Five

Nop15p is required for processing of pre-ribosomal RNA during 60S ribosomal subunit synthesis

5.1. Introduction

The ORF for *YNL110c/NOP15* was first identified during sequencing of the *Saccharomyces cerevisiae* chromosome XIV (Philippsen et al., 1997). As part of a systematic investigation of 1000 novel genes of *S.cerevisiae* by gene disruption, it was shown that the *YNL110c/NOP15* gene product is necessary for viability (De Antoni et al., 1997; Capozzo et al., 2000). In strains carrying a *ynl110Δ* mutation, a large number of cells appeared to be arrested during the cell cycle in G1 phase, unable to pass the “Start” checkpoint control and therefore did not form buds. A small number of cells were able to form buds but these were abnormal and did not progress and the cells were arrested during G2. From these observations it was proposed that Ynl110p/Nop15p functions in cell cycle regulation (Capozzo et al., 2000).

Protein sequence analysis of the 25.4kD protein using PSORT (Nakai and Kanehisa, 1992) revealed one RNA recognition motif (RRM) from residue 92 to 168, and two nuclear localisation signals (NLS) (Figure 35A). RNA recognition motifs are known to bind single strand RNA and are found in a variety of proteins (Kenan et al., 1991; Kim and Baker, 1993). The RRM structure consists of four strands and two helices in an alpha/beta sandwich with a third helix present during RNA binding in some cases. Sequence alignment studies of Ynl110p/Nop15p with BLASTP (Altschul et al., 1997) found matches of around 28% to 30% sequence identity to the yeast proteins Nsr1p, Mrd1p, Nop4p and Nop12p, as well as a *Drosophila* RNA binding protein.

Each of these proteins contained one or more RRM and have all been implicated in ribosome biogenesis (Kondo and Inouye, 1992; Sun and Woolford, 1997; Wu et al., 2001; Jin et al., 2002). Nsr1p and Mrd1p are required during maturation of the small ribosomal subunit whereas Nop4p and Nop12p are involved in processing of large subunit precursors. The closest human homologue of Nop15p was identified in a proteomic analysis of the human nucleolus during a search for potential ribosomal synthesis factors (Andersen et al., 2002).

Together these data suggested a possible function for Nop15p during ribosome

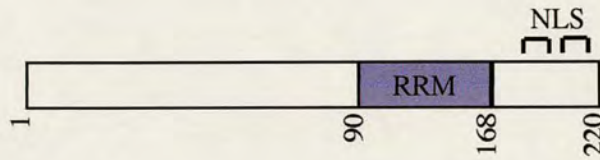
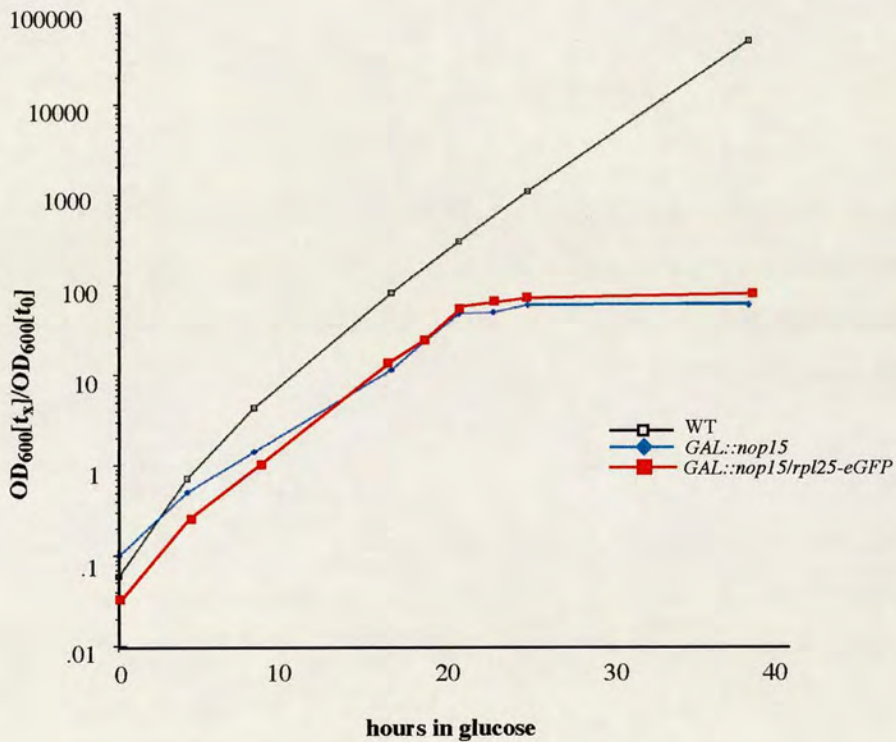
A**B**

Figure 35: Schematic diagram of Nop15p and growth curve of a conditional *GAL::nop15* strain with and without co-expression of *rpl25-eGFP*.

(A) Diagram of Nop15p. The RNA recognition motif is marked in purple.

(B) Growth curve of the *GAL10::nop15* strain with (YMO15) and without (YMO11) co-expression of *rpl25-eGFP*, and the otherwise isogenic wild-type strain (YDL401) after shift from permissive galactose medium to repressive glucose medium.

synthesis, which was investigated in this work.

5.2. Nop15p-depleted cells are defective for rRNA processing of 60S precursors

5.2.1. Nop15p is required for pre-rRNA processing

To examine the *in vivo* function of Nop15p, its expression was placed under the control of a repressible galactose promoter using a one-step PCR technique in strain YDL401 (see Chapter 2, section 2.13). Growth of the *GAL::nop15* strain (YMO11) was not clearly different from the isogenic wild-type strain (YDL401) on permissive galactose medium but slowed abruptly after 18 hours growth on repressive glucose medium and depletion of Nop15p (Figure 35B).

Depletion of Nop15p resulted in strong accumulation of the 35S primary transcript, but had little impact on levels of the 27SA₂ or 20S pre-rRNAs, or the mature 18S rRNA (Figure 36B) and led to the appearance of only very low levels of the 23S RNA. This indicates that the early cleavages are kinetically delayed but still occur with good efficiency. In contrast, the level of the 27SB pre-rRNAs was clearly reduced by 16 hours after transfer to glucose medium and the mature 25S rRNA was depleted over time. Analysis of low molecular weight RNAs (Figure 29B) showed that levels of the 7S and 6S pre-rRNAs were strongly reduced after transfer of the *GAL::nop15* strain to glucose medium (Figure 36B, panel a). The level of the mature 5.8S rRNA did not appear to be reduced (Figure 36C, panel b). It is unclear why mature 25S rRNA was depleted while levels of 5.8S rRNA did not differ from wild-type levels. This had been observed previously in pre-rRNA processing mutants, but its significance is unclear. It is possible, however, that depletion of the pool of mature 5.8S rRNA occurs at a different rate to that of 25S rRNA due to differences in stability or degradation pathways.

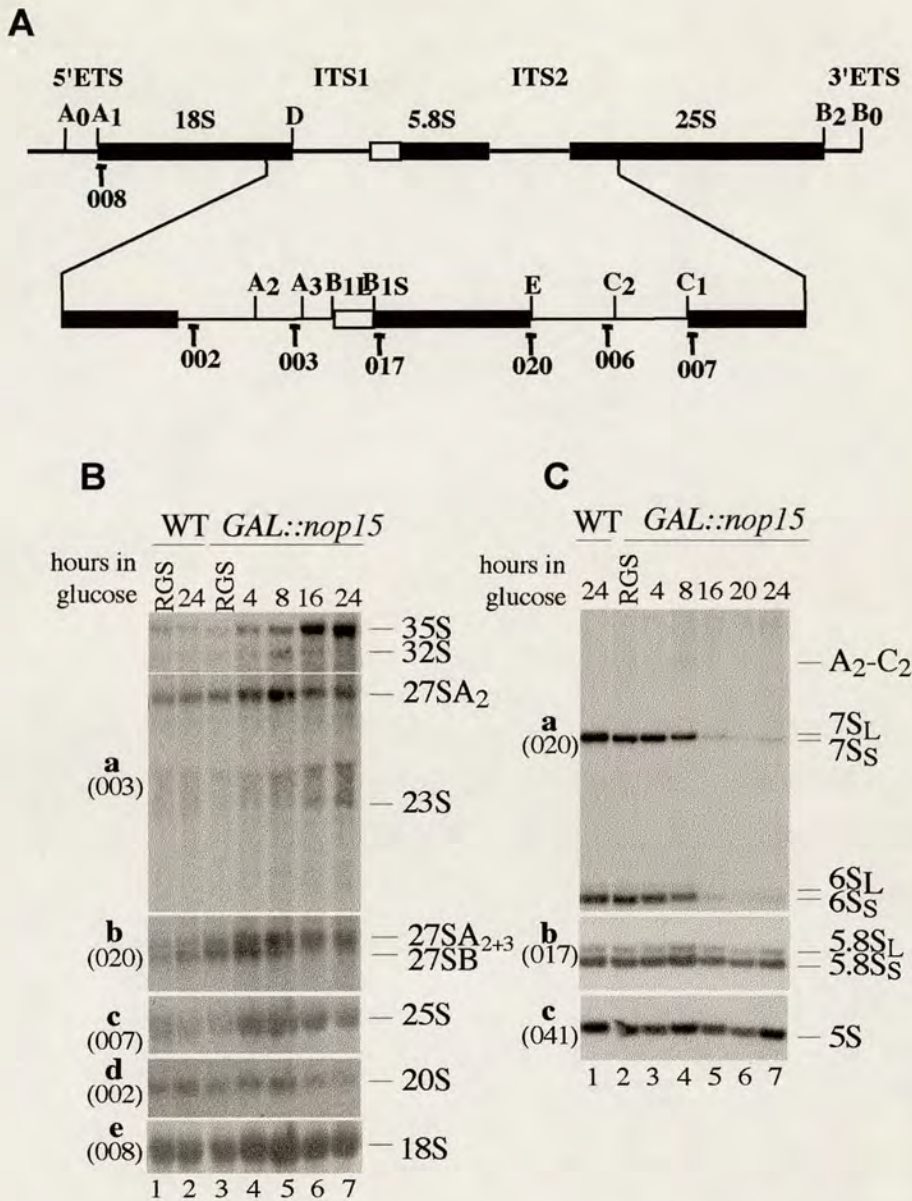


Figure 36: Northern analysis on the effects of Nop15p depletion on pre-rRNA processing.

(A) Lanes 1 and 2: wild-type strain (YDL401) in permissive galactose medium and 24 hours after transfer to repressive glucose medium. Lanes 3 to 7: *GAL::nop15* strain (YMO11) in galactose and after transfer to glucose medium for the times indicated. Northern analysis of high molecular weight RNAs. Probes used are indicated in parantheses on the left (see Chapter 2, Table 2.2.).

(B) Lane 1: wild-type strain (YDL401) 24 hours after transfer to repressive glucose medium. Lanes 2 to 7: *GAL::nop15* strain (YMO11) in galactose and after transfer to glucose medium for the times indicated. Northern analysis of low molecular weight RNAs. Probes used are indicated in parantheses on the left (see Chapter 2, Table 2.2.).

To determine the levels of the two forms of 27SB, RNA was analysed by primer extension using an oligonucleotide hybridising within the 3' region of ITS2 (Figure 37A). Following growth of the *GAL::nop15* strain on glucose medium, the level of 27SB_S was clearly reduced whereas the level of 27SB_L seemed unaffected, as shown by the primer extension stops at B_{1S} and B_{1L}, respectively (Figure 37A, lane 4). Consistent with the Northern analysis, little alteration was seen in the level of 27SA₂, as shown by the primer extension stop at site A₂. In contrast, the level of 27SA₃, shown by the stop at site A₃, was substantially elevated. Using a primer hybridising within the mature 25S rRNA a very slight increase for the primer extension stop at site C₂, the 5' end of the 26S pre-rRNA (Figure 37B) was observed. In comparison, however, the wild-type displayed a high background signal, and it is therefore difficult to draw any conclusion from this result.

The depletion of 27SB_S pre-rRNA and the loss of the subsequent 7S_S and 6S_S species indicates a strong inhibition of exonuclease processing from site A₃ to B_{1S}, which generates 27SB_S pre-rRNA in the wild-type cells. Although levels of 27SB_L are almost similar to those observed in wild-type cells, 7S_L and 6S_L are lost along with 7S_S and 6S_S pre-rRNAs (Figure 36B), suggesting an inhibition of 27SB to 7S processing. The rapid loss of 7S_L and 6S_L pre-rRNAs indicates that degradation of unprocessed 27SB_L precursor occurs at the same rate as pre-rRNA processing.

5.2.2. Synthesis of 25S and 5.8S rRNA is abolished in cells depleted of Nop15p

To determine if depletion of Nop15p causes a delay in the synthesis of mature rRNAs a pulse-chase analysis with [³H]-uracil was performed in SD-URA minimal medium 16 hours after transfer to glucose minimal medium (Figure 38).

Comparison of the wild-type (YMO5) and *GAL::nop15* (YMO14) strains showed an almost complete loss of mature 25S rRNA synthesis in the *GAL::nop15* strain over the wild-type, while synthesis of 18S rRNA remained robust (Figure 38A). Consistent with the Northern hybridisation only mild accumulation of the 35S pre-rRNA

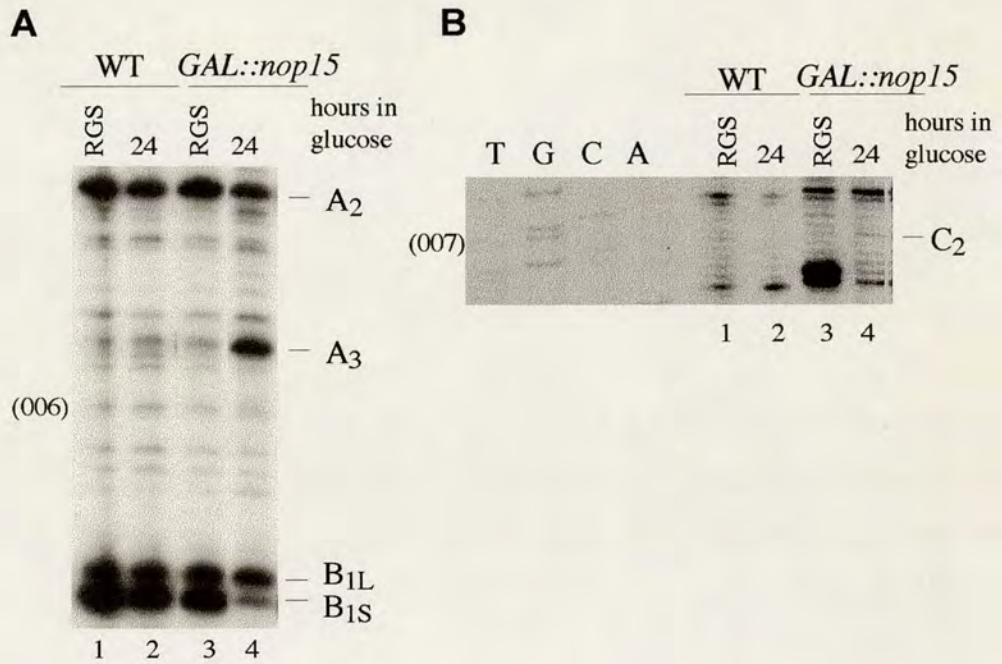


Figure 37: Levels of rRNA precursors in Nop15p depleted cells.

Lanes 1 and 2: wild-type strain (YDL401) in permissive galactose and 24 hours after transfer to repressive glucose medium. Lanes 3 and 4: *GAL::nop15* strain (YMO11) in galactose medium and 24 hours after transfer to glucose medium.

(A) Primer extension using oligo 006 (see Chapter 2, Table 2.2.). Primer extension stops at sites A₂, A₃, B_{1S}, and B_{1L} show levels of the 27SA₃, 27SA₂, 27SB_S and 27SB_L pre-rRNAs, respectively.

(B) Primer extension using oligo 007 (see Chapter 2, Table 2.2.). No primer extension stop at site C₂ was detected.

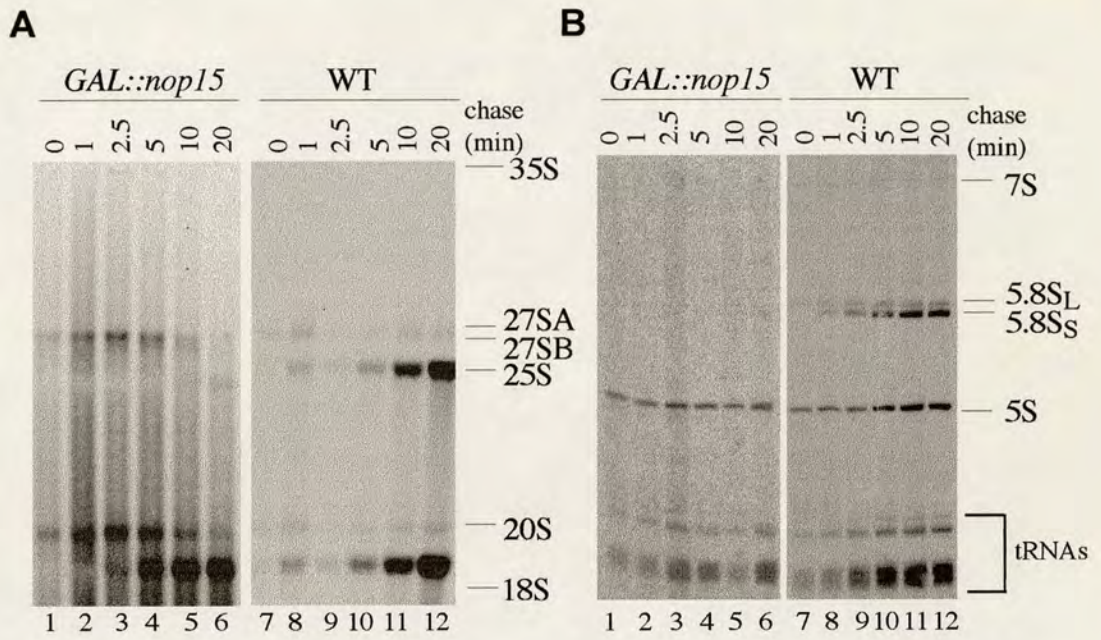


Figure 38: Ribosomal RNA synthesis is delayed in Nop15p depleted cells.

Pre-rRNA was pulse labelled with [³H]uracil for 2 minutes at 30°C and chased with a large excess of unlabelled uracil for the times indicated. Labelling was performed for the *GAL::nop15* (YMO14) strain (lanes 1 to 6) and a wild-type strain (YMO5) (lanes 7 to 12) 16 hours after transfer to repressive glucose medium.

(A) High molecular weight RNA separated on a 1.2% agarose/formaldehyde gel.

(B) Low molecular weight RNA separated on an 8% polyacrylamide/urea gel.

was observed. Analysis of low molecular weight RNAs showed that synthesis of the 5.8S rRNA was abolished while synthesis of 5S rRNA appeared normal (Figure 38B).

Together these results show that depletion of Nop15p resulted in inhibition of exonuclease digestion from site A₃ to site B_{1S}. In consequence, the level of the 27SA₃ pre-rRNA was substantially increased while the 27SB_S pre-rRNA was depleted, together with the 7S_S and 6S_S pre-rRNAs, leading to loss of the mature 25S and 5.8S rRNA. However, while levels of 27SB_L appeared normal, the loss of 7S_L and 6S_L pre-rRNAs, suggests a further inhibition of 27SB to 7S processing. The fairly mild effects on 35S processing are likely to be indirect, since many mutations that inhibit synthesis of 60S subunits result in some delay in the early pre-rRNA processing steps (Venema and Tollervey, 1999).

5.3. Nop15p is associated with precursors to the 25S and 5.8S rRNAs

Nop15p was epitope-tagged by insertion of a C-terminal tandem-affinity purification (TAP) tag in frame with the *NOP15* open reading frame in the chromosome, under the control of the *GAL10* promoter (Rigaut et al., 1999) (see Chapter 2, section 2.13). Correctly tagged strains were identified by western blotting, using an anti-Protein A antibody bound to horseradish peroxidase (Figure 39). These exhibited no detectable growth defect on permissive galactose medium, showing the fusion construct to be fully functional (YMO12).

To determine with which pre-60S precursor Nop15p is associated, RNAs that co-purified with Nop15p-TAP in lysates from strain YMO12 were analyzed following immunoprecipitation (see Chapter 2, section 2.15). Recovered RNAs were analyzed by Northern analysis (Figure 40A) and primer extension (Figure 40B) and compared to RNA recovered in parallel in a mock precipitation from a non-tagged control strain (YDL401) and 5µg of total RNA. Northern hybridization showed that the 7S pre-rRNA co-precipitated with Nop15p-TAP, but was not detectably recovered in the mock

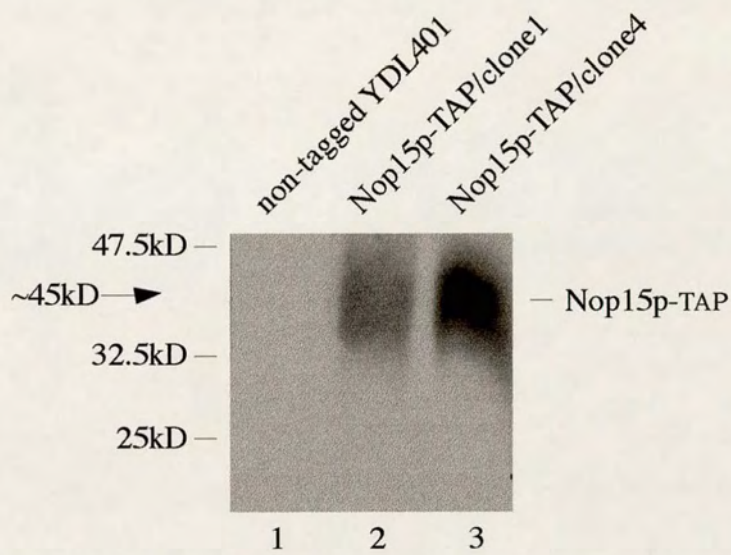


Figure 39: Western blot showing Nop15p-TAP constructs.

Immuno blot analysis of strains expressing a Nop15p-TAP (affinity purification tag) fusion under the control of the *GAL10* promoter in the YMO12 strain.

Blot has been probed with anti-ProteinA antibodies coupled to horseradish peroxidase to visualise the TAP-tag . Nop15p is a 25.4kD protein. The tag contributes 20kD to the size of the fusion protein.

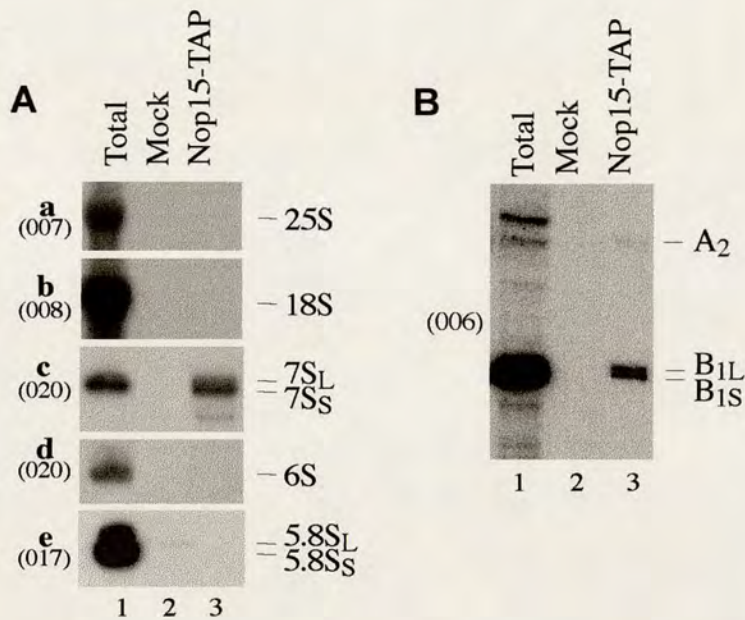


Figure 40: Nop15p is associated with precursors to mature 25S and 5.8S rRNAs.

Lane1: Total RNA control (5 μ g). Lane2: Precipitate from a wild-type control strain (YDL401). Lane3: Precipitate from a strain expressing Nop15-TAP (YMO12). Oligonucleotides used are indicated in parentheses on the left (see Chapter 2, Table 2.2.).

(A) Northern hybridization of high and low molecular weight RNA. RNA was separated on a 1.2% agarose/formaldehyde gel (a, b) or 8% polyacrylamide/urea gel (c, d and e).

(B) Primer extension analysis using oligo 006 (see Chapter 2, Table 2.2.). Primerextension stops at sites A₃, B_{1S} and B_{1L} show levels of co-precipitated 27SA₃, 27SB_S and 27SB_L pre-rRNAs, respectively.

precipitation from the non-tagged wild-type strain (Figure 40A, panel c). In contrast, the 6S pre-rRNA was not detectably co-precipitated (Figure 40A, panel d). No mature rRNAs were co-precipitated (Figure 40A, panels a, b and e).

27SB_L and 27SB_S precursor could not be resolved by Northern hybridisation, but primer extension using an oligonucleotide hybridising within ITS2 showed that both the 27SB_S and 27SB_L pre-RNAs were co-precipitated with Nop15p-TAP (Figure 40B). This indicates that Nop15p has a specific role in formation of the 27SB_S pre-rRNA but is associated with pre-rRNAs in both processing pathways and affects subsequent processing of 27SB long and short to 7S pre-rRNA. Furthermore, 27SA₂ pre-rRNA also co-precipitate with Nop15p, compared to the mock precipitation, as could be seen by the primer extension stop at A₂ (Figure 40B).

5.4. Nop15p is localised in the nucleolus

To determine the sub-cellular location of Nop15p, cells expressing Nop15p-TAP were examined by indirect immunofluorescence using a rabbit anti-protein A and a secondary FITC-coupled goat anti-rabbit antibody to detect the protein A region of the TAP-tag (Rigaut et al., 1999). As a marker for the nucleolus, a DsRed-fusion with the nucleolar protein Nop1p, the yeast homologue of human fibrillarin, was co-expressed from a plasmid introduced into the Nop15p-TAP fusion strain (YMO13) (Gadal et al., 2001b). The nucleoplasm was identified by DAPI staining. Anti-protein A decorated the nucleoplasm, with an enhanced signal over the nucleolus (Figure 41). No cytoplasmic signal was detected. This suggests that Nop15p is localised to the nucleus with nucleolar enrichment and confirms the result previously obtained with a Nop15p-GFP fusion protein (Capozzo et al., 2000).

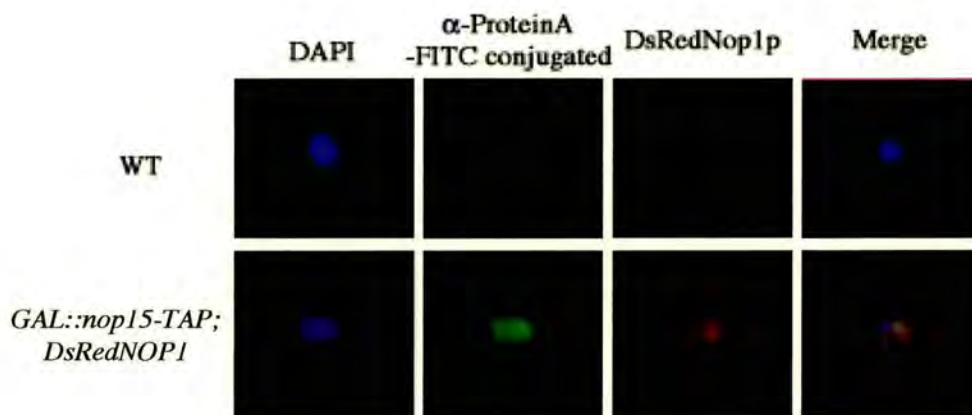


Figure 41: Nop15p is localised to the nucleolus.

The *GAL::nop15-TAP* strain (YMO13) co-expressing the nucleolar marker DsRedNop1p was examined by indirect immunofluorescence using an anti protein A antibody coupled to FITC. Also shown is the position of the nucleus visualised by DAPI staining and a wild-type control strain (YDL401).

5.5. Nop15p is not involved in the export of pre-60S particles

Analysis of pre-rRNA processing in Nop15p depleted cells revealed a similar defect to that observed in cells lacking Nop7p. Therefore, it seemed useful to look for defects in pre-60S subunit export in cells depleted of Nop15p. To test for potential 60S subunit export defects, Rpl25p-eGFP (Gadal et al., 2001b) was expressed from a plasmid in the wild-type (YDL401) and *GAL::nop15* (YMO15) strains. The distribution of Rpl25p-eGFP was followed during depletion of Nop15p.

During growth of the *GAL::nop15* strain on galactose medium and after transfer to glucose medium Rpl25p-eGFP showed the normal, predominantly cytoplasmic distribution (Figure 42, panels b, e and h). The nucleoplasm was visualized by DAPI staining (Figure 42, panels a, d and g). This shows that Nop15p does not lead to nuclear accumulation of Rpl25p-eGFP, indicating that it is not required for the export of precursors to the 60S ribosomal subunit from the nucleoplasm to the cytoplasm. Expression of the Rpl25p-eGFP construct had little effect on the growth of the wild-type strain (Gadal et al., 2001b) or the *GAL::nop15* strain on galactose medium. In *GAL::nop15* strain co-expressing *rpl25-eGFP* (YMO15) inhibition of growth following transfer to glucose medium appeared to occur at a similar rate than observed with strain YMO11 (Figure 36) indicating that assembly of 60S ribosomal subunits is not impaired in those cells.

5.6. Discussion

An investigation into the role of the essential protein Nop15p during ribosome biogenesis is reported in this chapter. The results show that Nop15p is required for synthesis of the 60S ribosomal subunit. Cells depleted of Nop15p exhibit defects in pre-rRNA processing but do not show clear defects in export of the pre-60S subunits or

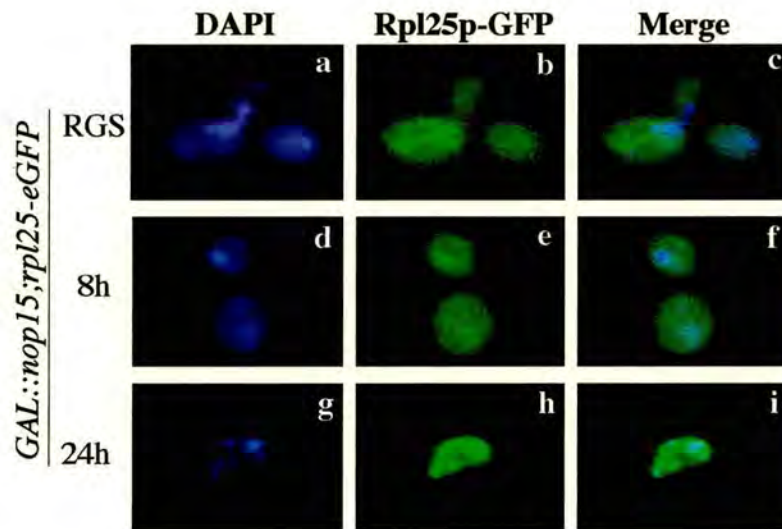


Figure 42: Depletion of Nop15p does not affect export of pre-60S ribosomal subunits .

Subcellular distribution of Rpl25p-eGFP in a *GAL::nop15* strain (YMO15). Rpl25-eGFP was examined by fluorescence microscopy during growth in permissive galactose medium and following transfer to repressive glucose medium for 2 and 8 hours. The position of the nucleoplasm was visualised by DAPI staining. In the merged image, DAPI staining is shown in blue and Rpl25p-eGFP in green.

subunit assembly. Nop15p is required for the 5' to 3' exonuclease digestion that generates the 5' end of the major, short form of the 5.8S rRNA as well as for processing of 27SB to 7S pre-rRNA. Nop15p was also found to associate with RNA precursors to the 60S subunit, namely the 27SA₂, 27SB_{L+S} and 7S pre-rRNAs.

5.6.1. *Nop15p functions specifically during processing of 27SA₃ pre-rRNA*

Nop15p was first isolated during systematic sequencing of yeast chromosome XIV and preliminary analysis of *NOP15* deleted strains suggested a function in cell cycle regulation (Philippsen et al., 1997; Capozzo et al., 2000). During a proteomic analysis of human nucleoli, the product of a previously uncharacterised human ORF was found and Nop15p was identified as its yeast homologue (Andersen et al., 2002). Sequence analysis revealed the presence of one RNA recognition motif and two potential nuclear localisation signals within the protein. Nop15p also showed significant sequence homology to Nsr1p, Mrd1p, Nop4p and Nop12p each of which is involved in ribosome synthesis (Kondo et al., 1992; Sun and Woolford, 1997; Capozzo et al., 2000; Wu et al., 2001; Jin et al., 2002).

While this work was in progress, independent studies identified Nop15p as a component of two different pre-ribosomal complexes with slightly different protein composition and differences in pre-rRNA components (Harnpicharnchai et al., 2001; Gavin et al., 2002). Comparison of the results from these analyses with the data presented here allows the proposal of a correlation between the pre-ribosomal particles with which Nop15p is associated and the distinctive defects in ribosome synthesis that are seen on its depletion.

The earliest pre-60S particle with which Nop15p appears to be associated is pre-60S E₁ in which processing of the 27SA₂, 27SA₃ and 27SB pre-rRNAs takes place (Harnpicharnchai et al., 2001; Gavin et al., 2002). In Nop15p depleted cells 27SA₃ pre-rRNA was accumulated and 27SB_S, and the subsequent 7S_S and 6S_S precursors were depleted. Although levels of 27SB_L were normal in Nop15p-depleted cells, 7S_L and 6S_L species were also lost suggesting that processing of 27SB to 7S is inhibited in both

pathways. Synthesis of 25S and 5.8S rRNA was abolished in these cells and no change was seen in 5.8S_{L,S} ratio. Furthermore, Nop15p did associate with 27SA₂ and both, 27SB_L and 27SB_S pre-rRNAs. It is therefore very likely that Nop15p is required for processing from 27SA₃ to 27SB_S within the pre-60S E₁ particle and processing of 27SB to 7S within the pre-60SE₂ particle. The exonucleolytic digestion from site A₃ to site B_{1S} is carried out by the 5'→3' exonucleases Rat1p and Xrn1p (Venema and Tollervey, 1999), which also generates the 5' end of the 25S rRNA by exonuclease digestion from site C₂ within the pre-60S E₂ particle (Geerlings et al., 2000). A very slight increase was seen in the primer extension stop at site C₂. However, this could also signify a drop in synthesis at this site and rapid degradation of the unprocessed precursor, which is consistent with the very fast disappearance of 7S_L and 6S_L pre-rRNAs. The mild effects on 35S processing are likely to be indirect, since many mutations that inhibit synthesis of 60S subunits result in partial inhibition of the early pre-rRNA processing steps (Venema and Tollervey, 1999).

Several recent studies have made use of the fusion between Rpl25p and GFP to follow the export of 60S ribosomal subunits from the nucleus to the cytoplasm (Bassler et al., 2001; Milkereit et al., 2001; Gadai et al., 2001a; Gadai et al., 2001b; Fatica et al., 2002; Chapter 3 of this work). Proteomic analysis showed that Nop15p is associated with pre-rRNAs and proteins in the pre-60S E₂ particle (Harnpicharnchai et al., 2001; Gavin et al., 2002). Since Nop15p depleted cells also revealed a similar phenotype to that observed in cells lacking Nop7p it seemed plausible to look for defects in pre-60S subunit export and assembly in cells depleted of Nop15p. However, no nuclear accumulation of Rpl25p-eGFP was detected in the absence of Nop15p concluding that Nop15p is not required for the export of pre-60S particle to the cytoplasm. Moreover, these cells did not exhibit a synergistic growth inhibition in the presence of co-expressed Rpl25p-GFP showing that Nop15p is not required for pre-60S assembly.

Reflecting on these results, it appears that Nop15p is specifically required for the processing from 27SA₃ to 27SB_S within the pre-60S E₁ particle as well as processing at site C₂ to within the pre-60S E₂ particle. The presence of a RNA recognition motif

indicates that Nop15p is likely to bind directly to the rRNA precursor. Nop15p presumably associates with the 27SA₂ pre-rRNA at or following cleavage at site A₂ and remains associated with the pre-60S complex during processing at sites A₃, B_{1S}, B_{1L} and C₂. The strong inhibition of the exonucleolytic cleavage seen in cells lacking Nop15p suggests that Nop15p might be needed to make the RNA accessible for processing at this specific site or to create an interaction between the RNA precursor and Rat1p. The same may be true for the endonucleolytic cleavage at site C₂ that generates 7S pre-rRNA, although no endonuclease has been identified for this cleavage as yet. Rat1p was not shown to co-purify with epitope-tagged Nop15p, however, none of the enzymes involved in ribosome synthesis have been found in any of the identified pre-ribosomal particles so far, and might interact only transiently with these complexes (Gavin et al., 2002). It is unlikely that Nop15p acts as a control mechanism to prevent pre-mature cleavage at C₂, as was shown for Ssf1p, since no A₂-C₂ fragment was detected in Nop15p depleted cells (Fatica et al., 2002). Interestingly, although both proteins, Nop15p and Ssf1p, are present in the same particle, the pre-60SE₁, and show similar phenotypes upon their depletion, they have not been found to co-purify with one another (Fatica et al., 2002; Gavin et al., 2002). This likely to be due to technical problems during purification of pre-ribosomal complexes or stoichiometrical differences in the presence of Nop15p and Ssf1p within the pre-60SE₁ particle.

Chapter Six

Final Discussion

The aim of this thesis was to contribute to the understanding of ribosome biogenesis and pre-ribosome assembly. For this purpose functional and biochemical analyses of three previously uncharacterised proteins were performed in *Saccharomyces cerevisiae* to investigate their function during ribosome synthesis and their association with different pre-ribosomal particles. The implication of the results presented in this work for the understanding of ribosome biogenesis and other cellular processes are discussed in this chapter.

Many features of the pre-ribosomal RNA processing pathway have become clearer over the last decade. It has recently become obvious that the number of factors involved and the complexity of their assembly into pre-ribosomal subunits has been underestimated. During the last year substantial progress has been made in the understanding of ribosomal biogenesis. Advances of proteomic techniques identified more than hundred novel protein factors implicated in ribosome synthesis and extended the capacity to analyse complete sets of proteins and RNA components associated with epitope-tagged proteins (Rigaut et al., 1999; Andersen et al., 2002). Furthermore, cryofixation and cryosubstitution in electron microscopy helped to clear the relationship between the observed subnucleolar structures and the different steps of ribosome biogenesis (Leger-Silvestre et al., 1997; Leger-Silvestre et al., 1999; Gadal et al., 2002).

6.1. The assembly of ribosomes is a highly dynamic process

The recent biochemical purification of more than a hundred novel proteins from human nucleoli indicated that ribosome biogenesis might be even more complex than previously thought (Andersen et al., 2002). A hint of this complexity was already suggested by earlier work showing that pre-rRNA from HeLa nucleoli could be isolated in the form of particles that contained not only newly formed ribosomal proteins destined to be exported with the complete ribosomal subunit, but also an equally large number of non-ribosomal proteins that recycle within the nucleolus (Warner and Soeiro, 1967; Kumar and Warner, 1972). The purification and analysis of pre-ribosomal

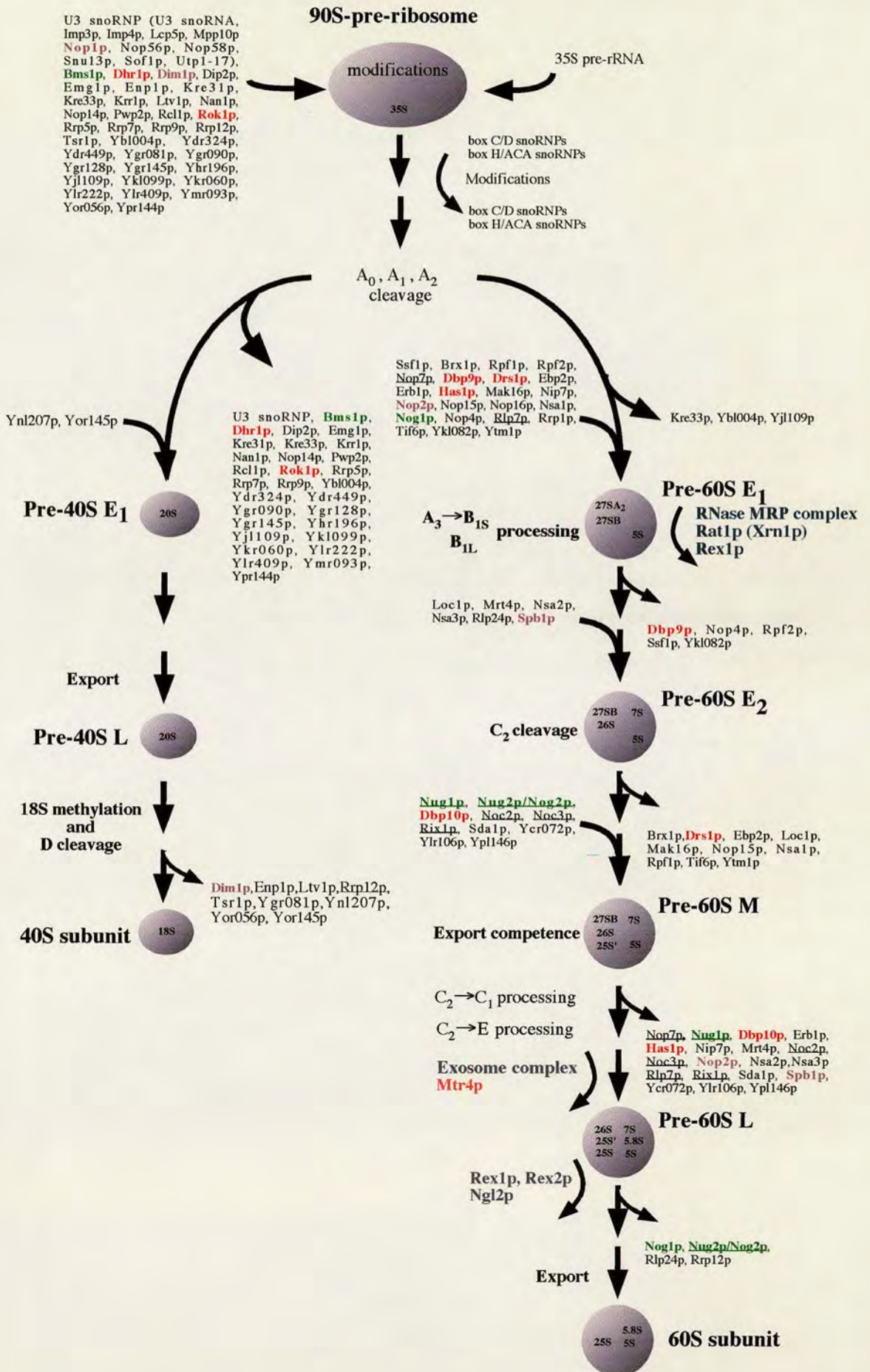
particles isolated at different stages during ribosome synthesis contributed to the latest advances made towards the understanding of ribosome assembly.

A high-throughput screen using more than eleven hundred epitope-tagged proteins as well as analyses of specific epitope-tagged nucleolar proteins, including Ssf1p, Nog1p, Nog2p and Nop7p, identified several potential 60S precursor particles with substantially different protein compositions (Bassler et al., 2001; Harnpicharnchai et al., 2001; Saveanu et al., 2001; Fatica et al., 2002; Gavin et al., 2002). Differences in pre-rRNA components defined an order between these particles and the changes in protein composition of processing factors revealed the highly dynamic nature of the 60S subunit synthesis pathway (Figure 43).

Functional studies of proteins found to co-purify with the different pre-60S complexes showed that some factors like the putative GTPase Nug1p associated with several particles, whereas others, including Ssf1p, are associated with only a single complex in which they fulfil a specific function (Bassler et al., 2001; Fatica et al., 2002). Nop7p was found to be part of three different pre-60S particles, namely the E₁, E₂ and M. This is consistent with its requirement during exonucleolytic digestion of the 27SA₃ pre-rRNA, ribosomal subunit assembly, and subunit export. Nop15p is also part of two different pre-60S particles, the E₁ and E₂, in which it is probably required for the accessibility of the pre-rRNA during exonucleolytic digestion from site A₃ to B_{1S} and endonucleolytic cleavage at site C₂, respectively. However, although Nop15p and Nop7p are both found in pre-60S E₁₊₂ and are required for processing from A₃ to B_{1S}, it is unlikely that Nop15p is required for 60S subunit assembly or export. Similarly, Nop15p and Ssf1p are both present in the pre-60SE₁ complex and show similar phenotypes when depleted in cells, however, they fulfil different functions within this particle. Ssf1p serves at least partly to prevent premature cleavage at site C₂ before the precursors are processed correctly, while Nop15p makes the precursor accessible for processing at site A₃ and perhaps C₂ (Fatica et al., 2002). This shows that the functions of different proteins are very distinct and occur only within a particular pre-ribosomal particle.

Figure 43: Outline pathway of ribosome synthesis in *S.cerevisiae*.

The 90S pre-ribosomal complex is proposed to contain the 35 S rRNA and the U3 snoRNA. The early cleavages at sites A0 to A2 lead to separation of the pre-40S and pre-60S particles. In both pathways a series of intermediates are drawn designated early (E), middle (M) and late (L) according to their position on the proposed pathway. The processing steps envisaged to be associated with each of these complexes are indicated, as is the likely time of export to the cytoplasm. Components in red are members of the DEAD/DEAH box family of putative RNA helicases. Components in green are putative GTPases. Components in purple are S-adenosyl-methionine dependent methyltransferases. Components underlined are required for subunit export. Components in blue are processing enzymes that have not yet been identified in purified complexes but are known to function in pre-rRNA processing. Figure adapted from Fatica and Tollervey, 2002.



The recent purification of a 90S pre-ribosome showed that this complex contained next to the 35S pre-rRNA, many known and unknown factors involved in modification as well as early processing of the primary transcript and factors specifically required for 40S subunit synthesis (Grandi et al., 2002). Slight variations within the particle components were observed within the particles purified with different bait proteins, which might represent an ordered assembly pathway for the 90S particle. Notably absent from all the purified 90S particles, however, were the components of previously purified pre-60S particles and most large subunit r-proteins. This and the absence of all known 40S processing factors from early pre-60S complexes identified with Ssf1p, was taken as an indication for the biphasic assembly of the processing machinery onto the pre-rRNA, with the 40S processing factors and r-proteins assembling together with the early processing machinery to the primary transcript. Association of the 60S processing factors and r-proteins with the pre-rRNA happens after the early cleavages, to the 27SA₂ pre-rRNA, when the synthesis pathways of the 40S and 60S subunit precursors separate. This unexpected separation of assembly of the 40S and 60S subunits, however, provides an explanation for the observation that many mutations inhibiting early cleavages prevent only 18S rRNA synthesis. Interestingly, most factors associated with the 35S precursor did not co-precipitate 20S pre-rRNA, indicating that they dissociate from the pre-rRNA at or immediately following cleavage at A₂.

The only processing factor that was found to be associated with precursors to both ribosomal subunits was Rrp12p. This is supported by the fact that the protein is required for synthesis of mature rRNA in both subunits. Moreover, Rrp12p is essential for export of the pre-40S particle to the cytoplasm. The structure predicted for Rrp12p is similar to that of the importin β -like proteins, or karyopherins, which contain tandemly repeated modules known as HEAT-repeats, and are mainly involved in nucleocytoplasmic transport (Aitchison and Rout, 2000). HEAT-repeats are believed to accommodate protein-protein interaction (Andrade et al., 2001) and in structural studies, Importin β was shown to wrap itself around its cargo and to facilitate transport through the nuclear pore complex by interaction of its HEAT-repeats with the hydrophobic mesh

Rrp12p could assist the pre-40S particle during its transport across the NPC by “masking” the hydrophilic 20S pre-rRNA with its hydrophobic HEAT-repeat structure against interaction with the hydrophobic nucleoporin FG-repeats. Although very recent studies have identified Rrp12p as part of a late pre-40S particle (E.Hurt, pers. comm.), its ability to shuttle between nucleus and cytoplasm as well as its interaction with nucleoporins and other factors involved in nucleocytoplasmic export needs to be established. No involvement of Rrp12p in the export of the large ribosomal subunit could be shown, however, Rrp12p is required for the optimal activity of the RNase MRP and the exosome during 60S subunit synthesis as well as assembly of the large subunit. The structure of Rrp12p is likely to assist these processes.

A role in assembly of the 60S subunit was also shown for Nop7p, which is likewise required for 60S subunit export. However, Nop7p does not contain any HEAT-motifs and is more likely involved, in conjunction with other factors including Rix1p, Rlp7p, Nug1p, Nug2p and the Noc2/Noc3 complex, in the establishment of export competence rather than directly participating in export (Figure 43). The export competence factors may accompany the pre-60S complex to the entrance of the nuclear pore, since all of them have been found to associate with the NPC, but they are less likely to be exported to the cytoplasm with the complex (Rout et al., 2000).

6.2. A correlation between ribosome synthesis and sub-nucleolar compartments

Most steps in ribosome synthesis occur in the nucleolus and subnucleolar structures have long been identified in higher eukaryotes, however, the relationship between nucleolar structures, pre-rRNA processing and ribosomal subunit assembly remained unclear. Under cryo-conditions, three compartments were clearly identified in yeast nucleoli, corresponding to the fibrillar centers (FCs), dense fibrillar component (DFC) and granular component (GC) as previously seen in higher eukaryotes (Leger-Silvestre et al., 1999; Scheer and Hock, 1999; Gadai et al., 2002). In addition, immunolocalisation on

1999; Scheer and Hock, 1999; Gadal et al., 2002). In addition, immunolocalisation on ultra thin sections obtained by these methods was used to compare different nucleolar localisation of known ribosomal processing factors to finally correlate ribosome synthesis and specific nucleolar subdomains (Gadal et al., 2001a).

Using electron tomography, a very recent study showed the FCs to be the place of rDNA transcription showing RNA polymerase I and growing nascent rRNA transcripts to be located within the fibrillar centers (Cheutin et al., 2002). During transcription the growing rRNA transcripts extend towards the DFC, which they entered rapidly after their release from the transcription machinery. Immuno-EM studies on two ribosomal processing factors, Nop1p and Rlp7p, suggested that different stages of ribosome synthesis take place in different subnucleolar compartments (Figure 44) (Gadal et al., 2002). Nop1p as part of the C/D box snoRNPs and the U3 snoRNP, is associated with the 90S pre-ribosome and involved in covalent modification and early cleavages of 35S pre-rRNA. In yeast, Nop1p localises to the DFC, and the same has been shown for fibrillarins, the human Nop1p, in HeLa cells (Pierron et al., 1989). However, Rlp7p, a processing factor present in three different pre-60S particles did not localise to the DFC but was found within the GC (Gadal et al., 2002). In mouse cells, B23 was reported to cleave ITS2 *in vitro* and was also found to localise within the GC (Biggiogera et al., 1990; Savkur and Olson, 1998). Moreover, a comparison of subnucleolar distribution of B23, fibrillarins and Pescadillo, the human Nop7p, clearly showed that while fibrillarins were localised to the DFC, B23 and Pescadillo were distributed throughout the GC but excluded from the DFC (Oeffinger et al., 2002).

Localisation of Nop1p to the DFC provides evidence of the presence of the 90S pre-ribosome in this region. Nop1p is not associated with the pre-60S particles, and it has therefore been suggested that release of the pre-ribosomal particles from the DFC coincides with formation of the pre-60S and pre-40S particles. Rlp7p and Nop7p were both shown to be part of several different pre-60S complexes and also to localise exclusively to the GC. This implies that maturation through pre-60SE₂ and pre-60SM is likely to take place within the GC. During maturation from pre-60SM to pre-60SL, the

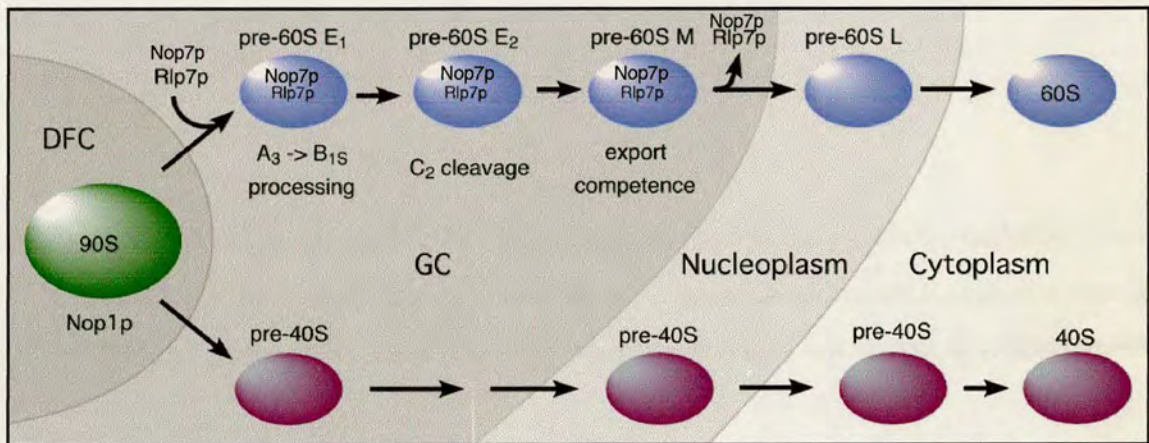


Figure 44: Correlation of ribosome synthesis and subnucleolar structure.

After transcription of rDNA in the fibrillar components (FCs), the 90S pre-ribosome is formed in the dense fibrillar component (DFC). After separation of pre-40S and pre-60S particles, the maturation of the individual subunits takes place in the granular component (GC). The pre-40S particle is released to the nucleoplasm and further on, into the cytoplasm, for final maturation. The pre-60S particle is mainly processed in the GC and only the final maturation steps take place in the nucleoplasm before the complex is transported into the cytoplasm, where both subunits form the functional 80S ribosome.

Figure adapted from Gadai et al., 2002.

complex is believed to be released into the nucleoplasm and from there, after final maturation, exported into the cytoplasm. However, the subnucleolar distribution of the factors contained within the pre-60SL has not yet been reported. The distribution of 40S processing factors and the correlation of release of pre-40S particles to the nucleoplasm and the cytoplasm with pre-40S processing and assembly also remain to be examined

It is unclear whether the intranucleolar movement of pre-ribosomes from the site of transcription to the sites of processing and assembly and, further, to the nuclear pores is an active process or is dependent on diffusion and regulated by retention and release from retention sites. The presence of specific processing factors perhaps functions as a signal for retention or release of the pre-ribosomal complex from one subnucleolar or subcellular compartment to the next.

6.3. Ribosome synthesis-part of a cellular control mechanism

6.3.1. Common factors with multiple cellular functions

Two of the proteins studied in this work, Nop7p and Nop15p, were initially identified in yeast as factors required for other cellular processes than ribosome synthesis.

Pescadillo/Nop7p is highly conserved among species and orthologs have been found in *S.pombe*, *C.elegans*, *Drosophila*, zebrafish and mouse. Nop7p was initially found to affect embryonic development in zebrafish. Two different mutant alleles of the yeast gene were isolated, one of which resulted in growth arrest in G1, consistent with defects in ribosome synthesis leading to the inability to pass the “Start” checkpoint. However, the other allele caused arrest in G2, indicating a defect in cell-cycle progression (Allende et al., 1996; Kinoshita et al., 2001). The fact that different defects are seen upon mutation of different alleles shows that these functions can be genetically separated. A recent study isolated Nop7p/Yph1p as a protein that directly interacts with the origin recognition complex (ORC) *in vitro* and *in vivo* and linking it to a large Nop7p-containing protein complex (Du and Stillman, 2002). Furthermore, two Nop7p-

checkpoint and DNA replication proteins (Du and Stillman, 2002; Fatica and Tollervey, 2002). The observation that Nop7p mutants arrested in G1 without cells entering S-phase, and moreover, that Nop7p mutants stayed viable but did not enter the cell division cycle from a G0 state led to the theory that Nop7p may be involved in sensing pathways that co-ordinate cell proliferation with other important cellular processes. Deletion of Nop15p in yeast led also to arrests during both G1 and G2 and it was therefore proposed that the protein functions at specific steps of the cell-cycle, but this has not yet been studied (Capozzo et al., 2000).

There are clear precedents for proteins that function in both cell-cycle progression or other cellular processes and ribosome synthesis. Exit from mitosis in *S.cerevisiae* requires a group of proteins which down-regulate cyclin-dependent kinase activity. These proteins are sequestered in the nucleolus in the RENT (regulator of nucleolar silencing and telophase) complex with Sir2p, Cdc14p and Net1p, which serves to anchor the complex (Shou et al., 1999b; Straight et al., 1999; Visintin et al., 1999; Cockell et al., 2000). RENT controls mitotic exit, nucleolar silencing and nucleolar localisation of Nop1p. In addition, Net1 is required for the maintenance of normal nucleolar structure and its binding stimulates RNA polymerase I and rRNA synthesis (Shou et al., 2001). These nucleolar specific functions of Net1p can be separated genetically from its cell-cycle specific functions in the RENT complex. It is also interesting to note that the interaction between the ORC and Nop7p is increased in the absence of Sir2p, a protein which is also involved in silencing of rDNA (Gotta et al., 1997) linking Nop7p and the ORC to a Sir2p control of DNA replication or ribosome biogenesis.

Transcription of rRNA was shown to be greatly affected by mutants of the Sec1 family of proteins, including Sec1, Sec7, Sec11, sec14, Sec18, Sec58, Sec68 and Sly1, all of which are involved in different stages of the secretion pathway (Mizuta and Warner, 1994). This is likely to demonstrate a requirement for the entire secretion pathway for the maintenance of ribosome synthesis. The nuclear protein Sda1p is conserved amongst *S.cerevisiae*, *S.pombe*, *Arapidopsis*, *Drosophila* as well as humans. In *S.cerevisiae* the protein is required for cell-cycle progression and actin cytoskeleton

organisation (Buscemi et al., 2000; Zimmerman and Kellogg, 2001). In temperature sensitive *sda1* mutants cells polymerisation of actin was impaired and cells arrested in G1, unable to pass through “Start” due to the absence of Cln2. However, Sda1p is also present in the late pre-60S particle and depletion of the protein blocks 60S subunit synthesis (Bassler et al., 2001; Nissan et al., 2002).

No function other than a requirement at multiple steps during ribosome biogenesis has been established for Rrp12p or most other ribosome synthesis factors. However, this might be revealed only by a genetic separation of different functions, as was shown for the proteins Nop7p and Net1p, and might therefore be determined only by analysis of multiple point mutations.

6.3.2. The regulation of cellular processes is linked

The question arises of why so many proteins function in ribosome synthesis as well as in cell-cycle regulation or secretion or actin polymerisation, rather than being specialised to only one metabolic process. Most of these proteins are well conserved among eukaryotes and some even have homologues in prokaryotic cells and archaea. During evolution, cells may have found it easier to recruit already existing proteins to novel processes within the cell instead of creating new ones, which would have involved the control of transcription, translation and degradation of additional factors.

Another, and perhaps more likely, possibility is the linking of major metabolic processes within the cell via common factors. Each cell must balance demands for resources to maintain cell viability and function with demands for resources to support growth. It would be conceivable that to balance these demands there must be cross-talk between the major synthetic pathways of the cell including the secretory pathway, the ribosome biosynthesis pathway and, of course, the cell cycle. Out of these ribosome biogenesis consumes most of the cells resources and has therefore a key role in the cells budgeting process. In a rapidly growing yeast cell, the production of new ribosomes represents an investment with the opportunity for faster growth. Synthesis of PolyA RNA as well as calculations based on abundance and stability of RNA, revealed

that in exponentially growing cells 60% of total transcription is devoted to ribosomal RNA, and 50% of RNA polymerase II transcription and 90% of mRNA splicing are devoted to ribosomal proteins (Woolford and Warner, 1991; Warner, 1999). The availability of nutrients and growth factors, the detection of changes in the source and availability of carbon and nitrogen as well as defects in other cellular processes were all shown to influence expression of rDNA and r-protein genes (Klein and Struhl, 1994; Thomas and Hall, 1997; Nierras and Warner, 1999). Moreover, the initiation of a cell cycle and subsequent cell division is dependent on the initiation of ribosome synthesis, which represents a key difference between G0 and G1 states (Spellman et al., 1998).

Co-ordinate regulation of rDNA and r-protein genes that make such prodigious use of resources is therefore essential for the economy and functionality of the cell and has to involve their regulation in conjunction with other aspects of cell growth. The suggestion that Nop7p might be sensor of pathways that co-ordinate cell proliferation with other important cellular processes, including ribosome synthesis, may also be true for other multifunctional proteins. A link between major synthetic pathways provided by such sensors would certainly be advantageous for rapid correlation of ribosome synthesis to growth rate, availability of nutrients and other aspects of cell performance.

6.4. Perspectives and conclusion

A start has been made in determining a pathway of pre-ribosome assembly but even a cursory inspection of the pathway reveals how far away any detailed understanding of ribosome assembly, and ribosome synthesis as a whole, really is. The functions of very few factors listed within the ribosome synthesis pathway (Figure 43) are known. The better-understood components, the processing enzymes, however, are not present in the pre-ribosomes analysed to date. We can guess that they associate only transiently with the pre-60S particles *in vivo* or dissociate during purification. Many steps within the synthesis of both subunits need to be investigated further and already more intermediate pre-ribosomal complexes are established to give a clearer picture of the overall process.

Many more questions arose from the recent advances, including reasons for the remarkable complexity of the pathway and the necessity for so many required factors.

Understanding of ribosome synthesis in other eukaryotes, including humans, still lags far behind yeast. This might be set to change since the techniques used to identify yeast pre-ribosomes should be applicable to human cells, and the large-scale purification of human nucleolar proteins provides much bait for purification of pre-ribosomal particles. Moreover, the large majority of factors indicated in figure 43, have clear homologues in humans and other eukaryotes, which will make functional analyses somewhat easier.

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Appendix

Yeast Pescadillo is required for multiple activities during 60S ribosomal subunit synthesis

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ABSTRACT

The Pescadillo protein was identified via a developmental defect and implicated in cell cycle progression. Here we report that human Pescadillo and its yeast homolog (Yph1p or Nop7p) are localized to the nucleolus. Depletion of Nop7p leads to nuclear accumulation of pre-60S particles, indicating a defect in subunit export, and it interacts genetically with a tagged form of the ribosomal protein Rpl25p, consistent with a role in subunit assembly. Two pre-rRNA processing pathways generate alternative forms of the 5.8S rRNA, designated 5.8S_L and 5.8S_S. In cells depleted for Nop7p, the 27SA₃ pre-rRNA accumulated, whereas later processing intermediates and the mature 5.8S_S rRNA were depleted. Less depletion was seen for the 5.8S_L pathway. TAP-tagged Nop7p coprecipitated precursors to both 5.8S_L and 5.8S_S but not the mature rRNAs. We conclude that Nop7p is required for efficient exonucleolytic processing of the 27SA₃ pre-rRNA and has additional functions in 60S subunit assembly and transport. Nop7p is a component of at least three different pre-60S particles, and we propose that it carries out distinct functions in each of these complexes.

Keywords: nucleolus; pre-rRNA; ribosome; RNA processing

INTRODUCTION

Most steps in ribosome synthesis take place within the nucleolus, a specialized subnuclear structure. During ribosome synthesis, a complex processing pathway converts a large pre-rRNA to the mature 18S, 5.8S, and 25S/28S rRNAs (see Fig. 1B). In addition, the mature rRNA sequences within the pre-rRNA undergo extensive covalent nucleotide modification and assembly with the 80 ribosomal proteins. More than 80 nonribosomal proteins that are required for ribosome synthesis have been identified by genetic and biochemical approaches in yeast (see Kressler et al., 1999; Venema & Tollervey, 1999; Warner, 2001). Biochemical analyses in human cells have identified an even larger number of nucleolar proteins (Anderson et al., 2002), although in most cases, their function in ribosome synthesis has not yet been directly addressed. Subdomains of the human nucleolus can be identified microscopically. Transcription of the rDNA is believed to occur at the boundaries of the fibrillar centers with initial processing and pre-ribosome assembly occurring in the associated dense fibrillar component (DFC) regions. Later processing and

assembly of the pre-ribosomes occurs in the surrounding granular component (GC) of the nucleolus (see, e.g., Shaw & Jordan, 1995; Scheer & Hock, 1999; Lyon & Lamond, 2000). Most analyses of subnuclear structure have been performed on vertebrates and plants, but similar structures are present in yeast (Leger-Silvestre et al., 1997, 1999).

During pre-rRNA processing, the 27SA₂ pre-rRNA can be processed by two alternative pathways (Henry et al., 1994; see Fig. 1B). In the major pathway, the pre-rRNA is cleaved at site A₃ by RNase MRP, forming the 27SA₃ pre-rRNA. Subsequent exonuclease digestion to site B_{1S} requires the two known 5' → 3' exonucleases, Xrn1p and Rat1p, and generates the 5' end of the 27SB_S pre-rRNA and mature 5.8S_S rRNA (Henry et al., 1994). An alternative, poorly understood, pathway processes the pre-rRNA at site B_{1L}, the 5' end of the 27SB_L pre-rRNA. Both 27SB pre-rRNAs are subsequently processed, by apparently identical pathways, to generate the mature 25S rRNA and either the 5.8S_S or 5.8S_L rRNAs (see Fig. 1). The ratio between the two forms of 5.8S shows some variation between strains, but around 75–80% of the population is normally made up of 5.8S_S, which is 8 nt shorter than 5.8S_L. Similar 5' heterogeneity is seen for 5.8S rRNA from many other Eukaryotes, including humans, *Xenopus*, *Drosophila*, and plants (Henry et al., 1994), sug-

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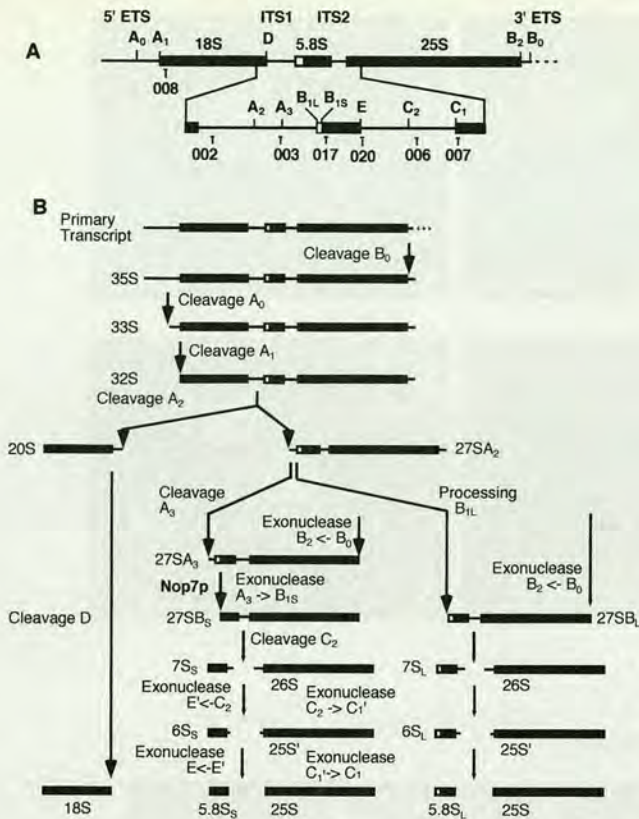


FIGURE 1. Pre-rRNA processing in *S. cerevisiae*. **A:** Structure and processing sites of the 35S pre-rRNA. This precursor contains the sequences for the mature 18S, 5.8S, and 25S, which are separated by the two internal transcribed spacers ITS1 and ITS2 and flanked by the two external transcribed spacers 5' ETS and 3' ETS. The positions of the oligonucleotide probes utilized in northern hybridization and primer extension analyses are indicated. **B:** Pre-rRNA processing pathway. The 35S pre-rRNA is generated by 3' cleavage at site B₀. 35S is then cleaved at site A₀ to produce the 33S pre-rRNA, which is rapidly cleaved at site A₁, producing the 32S pre-rRNA. 32S is cleaved at site A₂, separating the precursors to the 40S and 60S subunits, the 20S and 27SA₂ pre-rRNAs, respectively. 27SA₂ is processed via two alternative pathways. In the major pathway, cleavage at site A₃ by RNase MRP produces 27SA₃, which is then trimmed to site B_{1S} by the 5' to 3' exonucleases Rat1p and Xrn1p, producing the 27SB_S pre-rRNA. Alternatively, 27SA₂ can be processed to 27SB_L by an undetermined mechanism. 27SB_S and 27SB_L are matured to the 5.8S and 25S by identical pathways. Trimming to site B₂ generates the mature 3' end of the 25S rRNA. Cleavage at site C₂ and exonuclease digestion by Rat1p and Xrn1p generates the 5' end of mature 25S. The 3' end of the 5.8S is generated by 3' to 5' exonuclease digestion from site C₂ to E. For reviews on pre-rRNA processing and *trans*-acting factors see Kressler et al. (1999), Lafontaine and Tollervey (2001), and Venema and Tollervey (1999).

of increased nucleolar size and ribosome synthesis in such cells. *Pescadillo* was localized to the nucleolus in HeLa cells (Kinoshita et al., 2001) and the *Schizosaccharomyces pombe* homolog, SPBC19F5.05c, was also found to be nucleolar in a high throughput screen for subcellular localization of GFP fusion proteins (Ding et al., 2000). While this work was in progress, characterization of the yeast *Pescadillo* homolog Yph1p/Nop7p (YGR103w) was reported. YGR103w was originally published under the name of *YPH1* (Kinoshita et al., 2001), but has been designated as *NOP7* by the *Saccharomyces* genetic database. Nop7p is essential for viability and two temperature-sensitive (*ts*) lethal mutant alleles were reported to block growth at different steps in the cell-cycle; *yph1-24* led to arrest in G1, whereas the *yph1-45* allele caused G2 arrest (Kinoshita et al., 2001). G1 arrest is expected for mutations defective in ribosome synthesis, which are unable to pass the "Start" checkpoint control, but G2 arrest would not normally be predicted for a ribosome synthesis defect. In addition, *Pescadillo* was observed to contain a BRCT domain (Haque et al., 2000), which was originally identified in the breast and ovarian cancer gene BRCA1 and has been identified in several proteins involved in cell-cycle checkpoints and DNA repair (reviewed in Bork et al., 1997). Based on these observations, *Pescadillo* and Yph1p/Nop7p were proposed to perform some cell-cycle specific function.

A proteomic analysis of the human nucleolus identified 271 putative nucleolar proteins including *Pescadillo* (Anderson et al., 2002), the nucleolar localization of which was confirmed by YFP-tagging. A database search clearly identified YGR103w as the probable yeast homolog and we therefore analyzed its role in ribosome synthesis. While this work was in progress, the purification of a precursor to the 60S ribosomal subunit was reported that made use of a tagged form of Nop7p (Harnpicharnchai et al., 2001). This analysis did not, however, describe the effects of depletion of Yph1p/Nop7p on pre-rRNA processing or ribosome synthesis. Here we show that Nop7p is required for formation of 27SB_S, and therefore of the mature 5.8S_S rRNA, from the 27SA₃ pre-rRNA and has additional functions in subunit assembly or export.

RESULTS

Human *Pescadillo* and yeast Nop7p are localized to the nucleolus

A proteomic analysis of purified human nucleoli identified 271 proteins, one of which was *Pescadillo* (Anderson et al., 2002). To confirm this localization, an eYFP-*Pescadillo* construct was expressed in HeLa cells by transient transfection (Fig. 2A). Comparison of the localization of eYFP-*Pescadillo* (shown in green; Fig. 2A4) to a DIC image (Fig. 2A1) showed its predominant lo-

gesting that the existence of two processing pathways is both conserved and functionally significant.

The *Pescadillo* gene was initially identified in Zebrafish as the site of retrovirus insertion, which resulted in defects in embryonic development (Allende et al., 1996). *Pescadillo* mRNA showed widespread expression in developing mouse embryo brain with increased protein levels in replicating cells (Kinoshita et al., 2001). Protein levels were also increased in malignant cells (Kinoshita et al., 2001), possibly related to previous reports

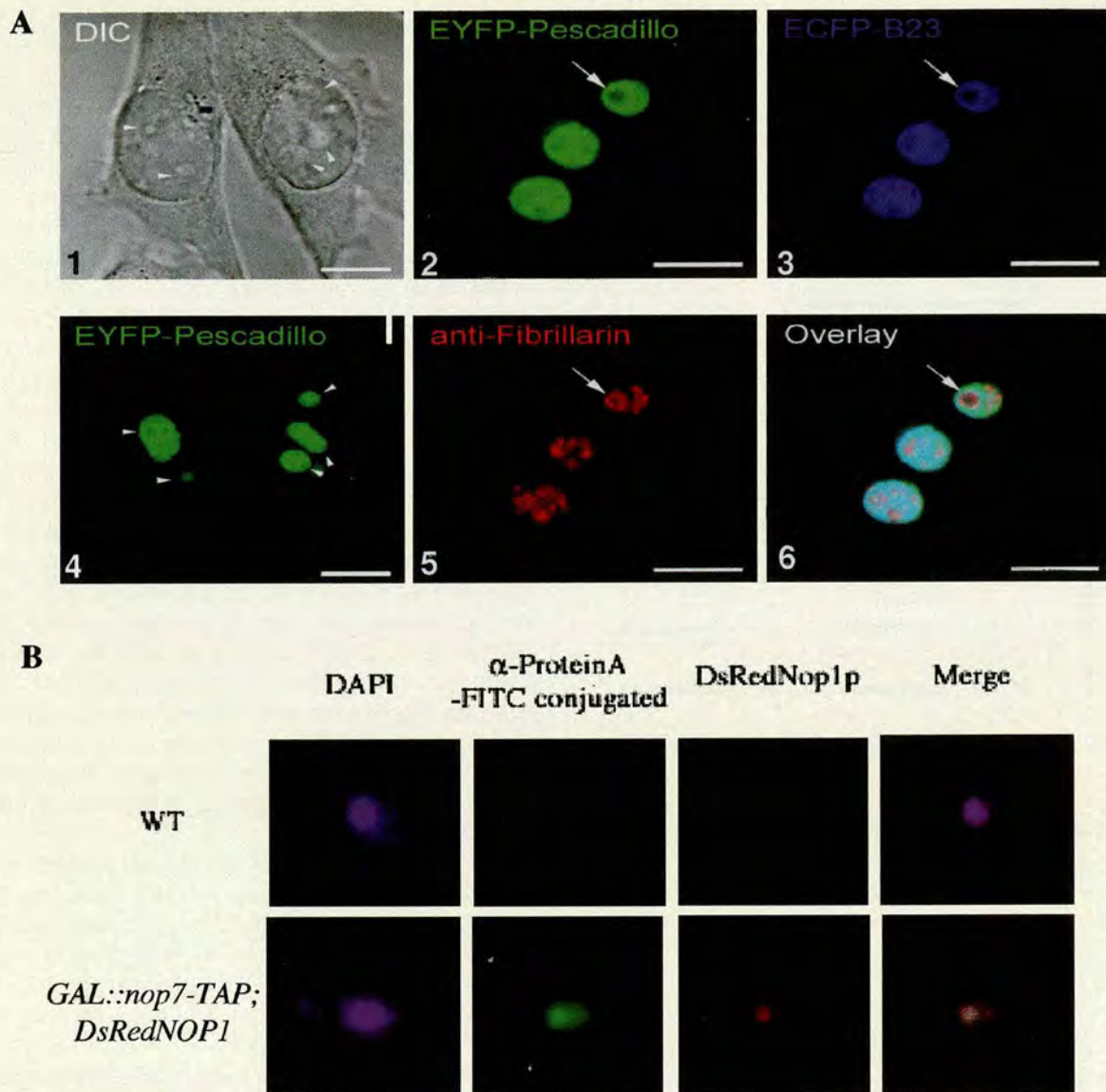


FIGURE 2. Nucleolar localization of Pescadillo and Nop7p. **A:** Localization of human Pescadillo compared with known nucleolar markers. (1, 4) HeLa cells were fixed 16 h after transfection with EYFP-Pescadillo. Comparison to the DIC image (1) shows localization of eYFP-Pescadillo (4; green) to nucleoli (arrowheads). 2, 3, 5, and 6: As markers for subnucleolar distribution, the EYFP-Pescadillo transfected cells (2; green) were cotransfected with the granular component protein ECFP-B23 (3; blue) and decorated with antibodies directed against the dense fibrillar component (DFC) protein fibrillarin (5; red). Scale bar = 5 μ m. **B:** Localization of Nop7p. The *GAL::nop7-TAP* strain also expressing the nucleolar marker DsRedNop1p was examined by indirect immunofluorescence using an anti-protein A antibody coupled to FITC. Also shown is the position of the nucleus visualized by DAPI staining and a wild-type control strain.

calization to nucleoli (indicated by arrowheads) with a low level of nucleoplasmic staining.

The subnucleolar distribution of eYFP-Pescadillo (Fig. 2A2) was compared to the GC marker eCFP-tagged B23/nucleophosmin (Npm1) (shown in blue; Fig. 2A3) and the DFC marker fibrillarin (shown in red; Fig. 2A5). Fibrillarin is a component of the box C+D snoRNAs (Schimmang et al., 1989) that function early in ribosome synthesis, whereas B23 is a putative assembly factor and nuclease that is believed to act later in ribosome synthesis (Biggiogera et al., 1990; Savkur

& Olson, 1998). In vitro, B23 is reported to cleave a pre-rRNA reporter within ITS2 (Savkur & Olson, 1998), at a site potentially equivalent to C₂ in the yeast pre-rRNA. Fibrillarin is concentrated in the DFC whereas B23 was reported to localize to the periphery of the DFC and the GC based on immuno-EM (Biggiogera et al., 1990), consistent with a later role for B23 in nucleolar ribosome maturation. The distribution of eYFP-Pescadillo resembled that of eCFP-B23, but was distinct from that of fibrillarin. eYFP-Pescadillo and eCFP-B23 were largely excluded from the DFCs (one

of which is indicated by an arrow) and concentrated in the surrounding area, which presumably corresponds to the GC. The distribution of eYFP-Pescadillo is consistent with a late role in nucleolar ribosome synthesis.

The essential yeast protein Nop7p (YGR103w) is 40% identical to human Pescadillo. To determine whether Nop7p is also nucleolar, it was epitope tagged with a tandem-affinity purification (TAP) construct (Rigaut et al., 1999) using a one-step PCR protocol (see Materials and Methods). The tagged construct was integrated at the *NOP7* locus under the control of the *GAL10* promoter and is the only source of Nop7p. The host strain, YDL401, has reduced galactose permease activity leading to reduced *GAL* induction (Lafontaine & Tollervey, 1996). This eliminates the overexpression generally seen with *GAL*-regulated constructs and allows faster appearance of phenotypes following transfer to glucose medium. The *GAL::nop7-TAP* cells exhibited no detectable growth defect on permissive RSG medium, showing the fusion construct to be fully functional (data not shown).

To determine the location of Nop7-TAP, cells were examined by indirect immunofluorescence (Fig. 2B) using a rabbit anti-protein A and a secondary FITC-coupled goat anti-rabbit antibody to detect the protein A region of the TAP tag (Rigaut et al., 1999). As a marker for the nucleolus, a DsRed fusion with the nucleolar protein Nop1p (the yeast homolog of fibrillarin) was coexpressed as previously described (Gadal et al., 2001b), and the nucleoplasm was identified by DAPI staining. Anti-protein A preferentially decorated the nu-

cleolus, with a weaker signal over the nucleoplasm. No cytoplasmic signal was detected. We conclude that Nop7p is localized to the nucleus with nucleolar enrichment. The significant nucleoplasmic staining would be consistent with association with late pre-ribosomes that have been released from the nucleolus (see Milkereit et al., 2001).

Yeast Nop7p is required for 60S subunit export and interacts genetically with GFP-tagged Rpl25p

To examine the possible functions of Nop7p in ribosome synthesis, its expression was placed under the control of a repressible *GAL10* promoter using a one-step PCR technique in strain YDL401 (see Materials and Methods). Growth of the *GAL::nop7* strain was not clearly different from the isogenic wild-type strain on RGS medium, but was progressively slowed following transfer to glucose medium, commencing around 6 h after transfer (Fig. 3A). The *ypb1-45* allele of *NOP7* is reported to lead to a G2 arrest phenotype, consistent with a specific cell-cycle defect (Kinoshita et al., 2001). However, microscopic inspection of the *GAL::nop7* strain following transfer to glucose medium showed only the accumulation of unbudded cells, even after 24 h, indicating arrest in G1 (data not shown). This is the expected phenotype for a defect in ribosome synthesis that results in the inability to pass the "Start" checkpoint.

Several recent studies have made use of fusions between ribosomal proteins and GFP to follow the ex-

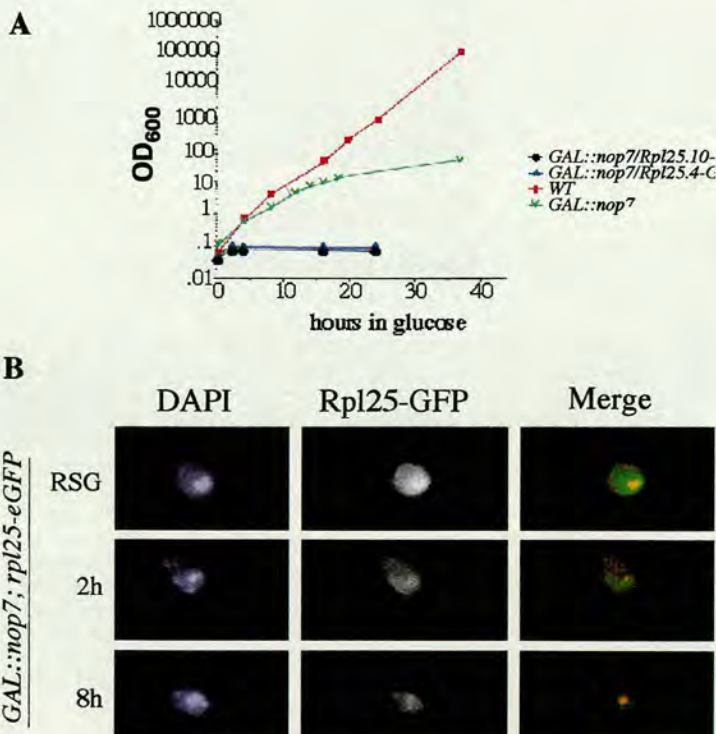


FIGURE 3. Nop7p is required for 60S subunit export and interacts genetically with GFP-tagged Rpl25p. **A:** Growth curves of *GAL::nop7* strains following transfer to glucose medium, with and without expression of Rpl25p-eGFP. Strains were pregrown in RGS medium and transferred to glucose medium for the times indicated. Strains were maintained in exponential growth by dilution with pre-warmed medium. Cell densities measured by OD_{600} are shown corrected for dilution. (■) Wild-type; (*) *GAL::nop7*; (●) *GAL::nop7; rpl25.10-GFP*; (▲) *GAL::nop7; rpl25.4-GFP*. **B:** Subcellular distribution of Rpl25-eGFP in a *GAL::nop7* strain. Rpl25-eGFP was examined by fluorescence microscopy during growth in RGS medium and following transfer to glucose medium for 2 and 8 h. The position of the nucleus was visualized by DAPI staining, which also stains the cytoplasm more weakly due to the presence of mitochondria. In the merged image, DAPI staining is shown in red and Rpl25-GFP is in green.

port of 60S ribosomal subunits from the nucleus to the cytoplasm (Stage-Zimmermann et al., 2000; Baßler et al., 2001; Gadal et al., 2001a, 2001b; Milkereit et al., 2001; Fatica et al., 2002). To look for 60S subunit export defects, Rpl25p-eGFP (Gadal et al., 2001b) was expressed from a plasmid in the wild-type and *GAL::nop7* strains. As previously reported, expression of this construct had little effect on the growth of the wild-type strain (Gadal et al., 2001b) or the *GAL::nop7* strain on galactose medium (data not shown). Unexpectedly, growth of the *GAL::nop7*/Rpl25p-eGFP strain was very rapidly inhibited following transfer to glucose medium (Fig. 3; two independent transformants are shown). These strains also express the wild-type Rpl25p, showing that the Rpl25p-eGFP fusion is dominant negative for growth in strains with a reduced level of Nop7p. The growth inhibition is much more rapid than would have been expected for a strain that is simply unable to synthesize new ribosomes (see Discussion) and we conclude that Nop7p has a role in 60S ribosomal subunit assembly.

The distribution of Rpl25p-eGFP was followed during depletion of Nop7p (Fig. 3B). During growth of the *GAL::nop7* strain on RSG medium, Rpl25-eGFP showed the normal, predominantly cytoplasmic distribution. After transfer to glucose medium for 2 h, increased nuclear staining of Rpl25-eGFP was already visible, and accumulation was strong after 8 h. The distribution of Rpl25-eGFP fluorescence matched that of DAPI staining, indicating that it was not restricted to the nucleolus. We conclude that Nop7p is required to allow the export of precursors to the 60S ribosomal subunit from the nucleoplasm to the cytoplasm.

Nop7p is required for pre-rRNA processing

The effects of depletion of Nop7p were assessed by Northern hybridization (Fig. 4), primer extension (Fig. 5), and pulse-chase labeling (Fig. 6).

Depletion of Nop7p resulted in mild accumulation of the 35S primary transcript and the appearance of low levels of the 23S RNA, but had little impact on levels of the 27SA₂ or 20S pre-rRNAs, or the mature 18S rRNA (Fig. 4A). In contrast, the level of the 27SB pre-rRNAs was clearly reduced by 8 h after transfer to glucose medium and the mature 25S rRNA was depleted over time.

Analysis of low-molecular-weight RNAs showed progressive reduction in the levels of the 7S and 6S pre-rRNAs following transfer of the *GAL::nop7* strain to glucose medium (Fig. 4Bb). The level of the mature 5.8S was also reduced (Fig. 4Bc), and the reduction in 5.8S_S appeared slightly greater than for 5.8S_L. The pre-rRNA that extends from A₂ to C₂ was not accumulated during Nop7p depletion (Fig. 4Ba), in contrast to the recently reported effects of depletion of another processing factor, Ssf1p (Fatica et al., 2002).

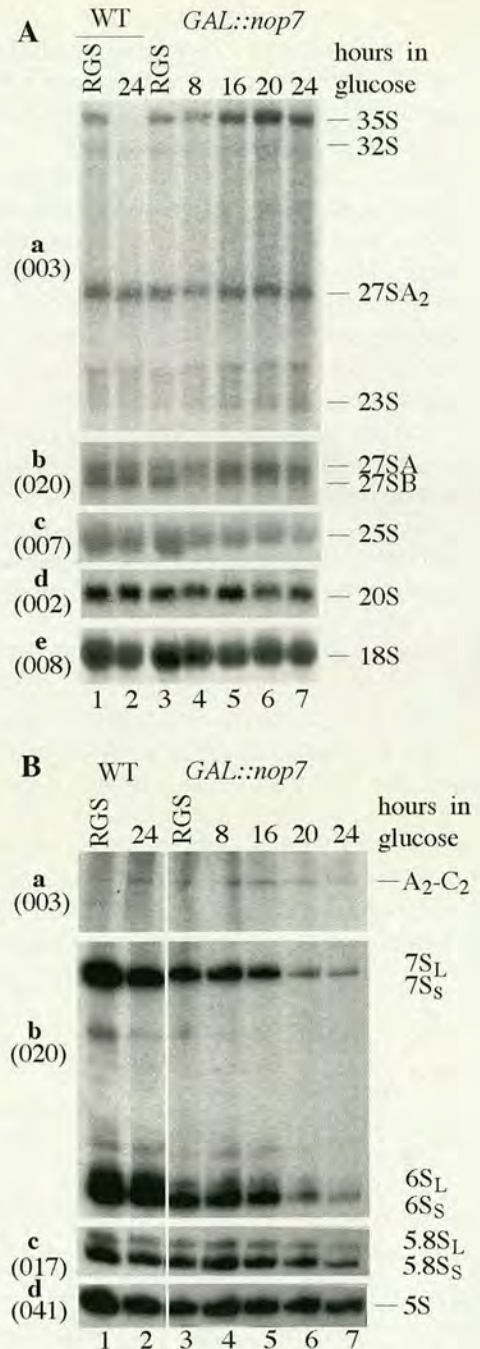


FIGURE 4. Northern analysis of the effects of Nop7p depletion on pre-rRNA processing. Lanes 1 and 2: wild-type strain in RGS medium and 24 h after transfer to glucose. Lanes 3–7: *GAL::nop7* strain in RGS medium and after transfer to glucose medium for the times indicated. **A:** (a) Hybridization of probe 003, complementary to ITS1 upstream of A₃. (b) Hybridization with probe 020, complementary to the 5.8S/ITS2 boundary. (c) Hybridization with probe 007, complementary to the 25S rRNA. (d) Hybridization with probe 002, complementary to ITS1 upstream of A₂. (e) Hybridization with probe 008, complementary to 18S rRNA. **B:** (a) Hybridization with probe 003, complementary to ITS1 upstream of A₃. (b) Hybridization with probe 020, complementary to the 5.8S/ITS2 boundary. (c) Hybridization with probe 017, complementary to 5.8S rRNA. (d) Hybridization with probe 041, complementary to 5S rRNA. RNA was separated on a 1.2% agarose/formaldehyde gel (**A**) or 8% polyacrylamide/urea gel (**B**). Probe names are indicated in parentheses on the left.

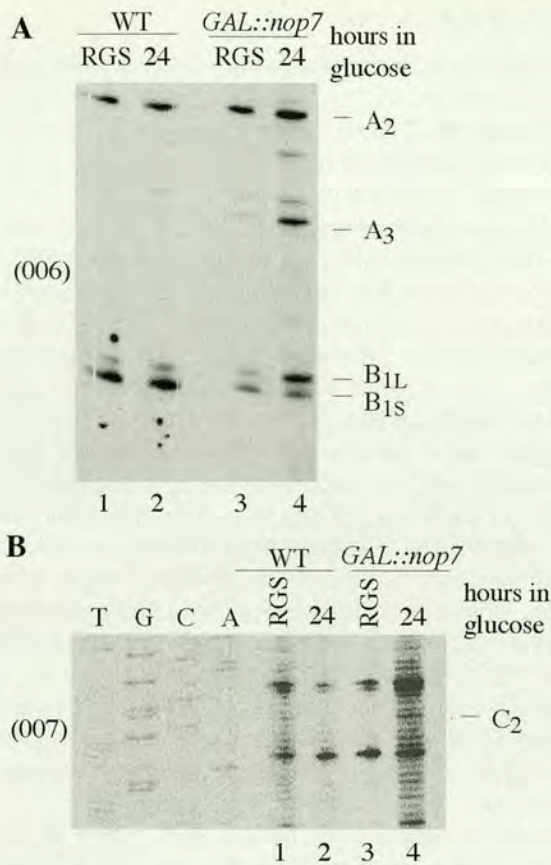


FIGURE 5. Primer extension analysis of pre-rRNA processing. Lanes 1 and 2: wild-type strain in RGS medium and 24 h after transfer to glucose medium. Lanes 3 and 4: *GAL::nop7* strain in RGS medium and 24 h after transfer to glucose medium. **A:** Primer extension using oligo 006, which hybridizes within ITS2, 3' to site C₂. Primer extension stops at sites A₂, A₃, B_{1S}, and B_{1L} show the levels of the 27SA₂, 27SA₃, 27SB_L, and 27SB_S pre-rRNAs, respectively. **B:** Primer extension using oligo 007, which hybridizes within 25S rRNA. The primer extension stop at C₂ shows the level of the 26S pre-rRNA.

5.8S_S is processed from the 27SB_S pre-rRNA, whereas 5.8S_L is processed from 27SB_L (see Fig. 1B). To determine the levels of the 27SB species, they were analyzed by primer extension (Fig. 5A) using an oligo hybridizing within the 3' region of ITS2 (oligo 006; see Fig. 1A). Following growth of the *GAL::nop7* strain on glucose medium, the level of 27SB_S was clearly reduced relative to 27SB_L, as shown by the primer extension stops at B_{1S} and B_{1L}, respectively (Fig. 5A, lane 4). Consistent with the northern analysis, little alteration was seen in the level of 27SA₂, as shown by the primer extension stop at site A₂. In contrast, the level of 27SA₃, shown by the stop at site A₃, was substantially elevated. Using a primer hybridizing within the mature 25S rRNA (oligo 007; see Fig. 1A), slight accumulation was seen for the primer extension stop at site C₂, the 5' end of the 26S pre-rRNA (Fig. 5B). This effect was weak, however, and its significance is unclear.

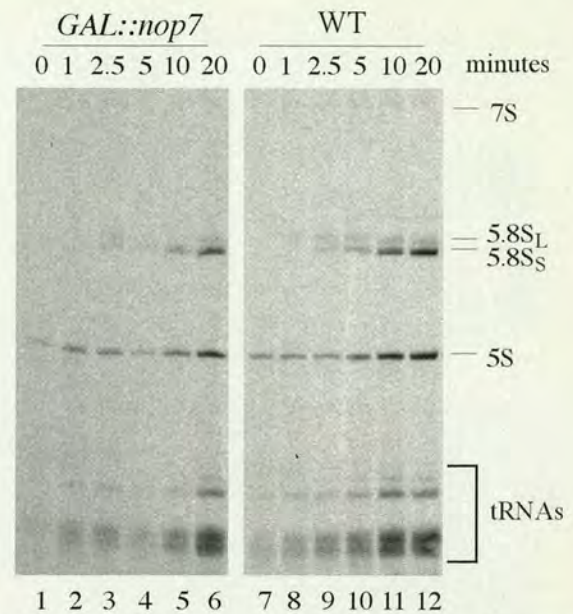


FIGURE 6. Pulse-chase analysis of rRNA synthesis. Pre-rRNA was pulse labeled with [³H]uracil for 2 min at 30°C and chased with a large excess of unlabeled uracil for the times indicated. Labeling was performed for the *GAL::nop7* strain (lanes 1–6) and a wild-type strain (lanes 7–12) 16 h after transfer to glucose medium.

Pulse-chase analysis with [³H]-uracil was performed 16 h after transfer to glucose minimal medium (Fig. 6). Comparison of the wild-type and *GAL::nop7* strains showed that accumulation of the 5.8S rRNA was mildly delayed.

Together these data show that depletion of Nop7p resulted in reduced exonuclease digestion from site A₃ to site B_{1S}. In consequence, the level of the 27SA₃ pre-rRNA was substantially increased, whereas the 27SB_S pre-rRNA was depleted together with the 7S_S and 6S_S pre-rRNAs, leading to reduced accumulation of the mature 5.8S_S rRNA. The 5' end of the 25S rRNA is also generated by exonuclease digestion (Geerlings et al., 2000; see Fig. 1B), but this did not appear to be strongly affected, as only a small increase was seen in the primer extension stop at site C₂. The mild effects on 35S processing are likely to be indirect, as many mutations that inhibit synthesis of 60S subunits result in partial inhibition of the early pre-rRNA processing steps (for further discussion see Venema & Tollervey, 1999).

Nop7p is associated with pre-rRNAs from both processing pathways

To determine whether Nop7p associated specifically with the 27SB_S branch of the processing pathway, coprecipitated RNAs were analyzed by northern analysis and primer extension. Northern hybridization (Fig. 7A,B) showed that the 27SB and 7S pre-rRNAs coprecipitated with Nop7-TAP, but were not detectably recov-

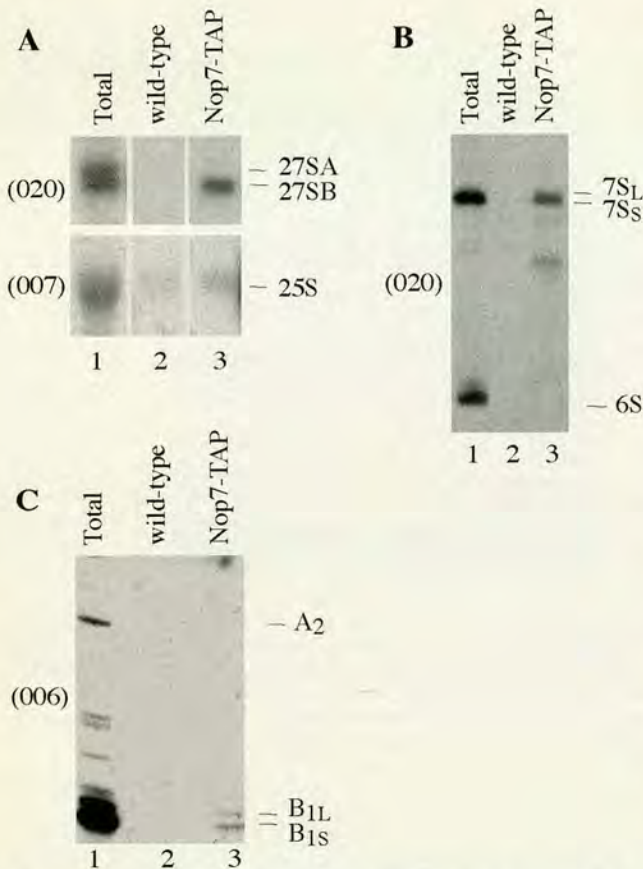


FIGURE 7. Analysis of RNAs coprecipitated with TAP-tagged Nop7p. Lane 1: Total RNA control (5 μ g). Lane 2: Precipitate from a wild-type control strain. Lane 3: Precipitate from a strain expressing Nop7-TAP. **A:** Northern hybridization of high-molecular-weight RNA separated on a 1.2% agarose/formaldehyde gel. **B:** Northern hybridization of low-molecular-weight RNA separated on an 8% polyacrylamide/urea gel. **C:** Primer extension analysis. Nop7-TAP was immunoprecipitated from cell lysates using IgG agarose, with release of bound RNA-protein complexes by cleavage of the protein A linker by TEV protease. RNA was recovered from the released material, and from a mock-treated, isogenic wild-type control strain. Oligonucleotides used are indicated in parentheses. The preparation used in **C** is different from that used for **A** and gave lower recovery efficiency.

ered in the mock precipitation from the nontagged wild-type strain. In contrast, the 27SA₂ and 6S pre-rRNAs were not detectably coprecipitated. The 25S rRNA gave the same background signal in both the wild-type and Nop7-TAP precipitates. Inspection of the original figure showed that both the 7S_L and 7S_S pre-rRNAs were coprecipitated. 27SB_L and 27SB_S cannot be resolved by northern hybridization, but primer extension from oligo 006 in ITS2 (see Fig. 1A) showed that both the 27SB_L and 27SB_S pre-rRNAs were coprecipitated with Nop7-TAP (Fig. 7C).

Nop7p has a specific role in formation of the 27SB_S pre-rRNA but is associated with pre-rRNAs in both processing pathways, consistent with the conclusion that it has additional roles in 60S subunit assembly and export.

DISCUSSION

We show here that the yeast homolog of Pescadillo is required for the 5' to 3' exonuclease digestion that generates the 5' end of the major, short form of the 5.8S rRNA. Depletion of Nop7p also resulted in strong synergistic inhibition of growth in the presence of a GFP-tagged form of ribosomal protein Rpl25p, indicating an additional role in 60S ribosome assembly. Nuclear accumulation of Rpl25-eGFP has been used as a marker for a defect in nuclear export of pre-60S ribosomal particles (*ribosome export* or *rix* phenotype; Gadgil et al., 2001b), and this was also observed following Nop7p depletion. We conclude that Nop7p is required for a specific pre-rRNA processing step as well as correct pre-60S assembly and nuclear export.

During the course of this work, Nop7p was shown to be a component of at least three different pre-ribosomal complexes with substantially different protein composition, as well as differences in pre-rRNA components (Baßler et al., 2001; Harnpicharnchai et al., 2001; Fatica et al. 2002; see Fig. 8). These analyses allow us to propose a correlation between the pre-ribosomal particles with which Nop7p is associated and the distinct defects in ribosome synthesis that are seen on its depletion.

The earliest pre-60S particle with which Nop7p is known to be associated is pre-60S E₁. This complex is also associated with the 27SA₂, 27SA₃, and 27SB pre-rRNAs (Fatica et al., 2002) and it is therefore very likely that Nop7p is required for processing from 27SA₃ to 27SB within the pre-60S E₁ particle.

A fast acting, dominant negative phenotype is associated with the expression of a GFP-tagged form of the ribosomal protein Rpl25p in strains depleted of Nop7p. The fact that expression of Rpl25-GFP is dominant in-

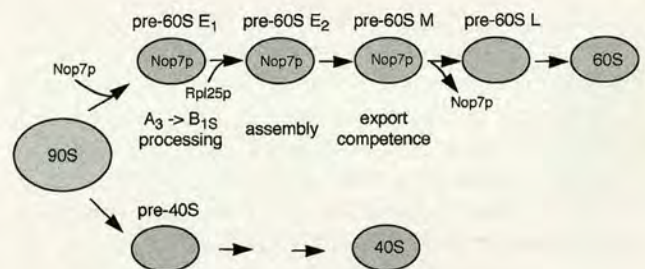


FIGURE 8. Model for the roles of Nop7p in 60S subunit biogenesis. Outline pathway of biogenesis of 60S and 40S ribosomal subunits, modified from Fatica et al. (2002). This model indicates the presence of Nop7p in three different pre-60S complexes designated E₁, E₂, and M, which can be correlated with the different functions deduced for Nop7p. Pre-60S E₁ contains the 27SA₃ pre-rRNA, the processing of which is defective in strains lacking Nop7p. Rpl25p is not present in pre-60S E₁, but joins the pre-60S E₂ particle, and the defect in Rpl25p assembly is therefore predicted to occur at this step. The pre-60S M complex contains numerous factors required for 60S subunit export as judged by the nuclear retention of a Rpl25-GFP reporter construct, and Nop7p is likely to be required during the acquisition of export competence within this complex.

dicates that, in its presence, the wild-type Rpl25p is no longer able to support growth. Notably, the inhibition of growth was much more rapid and complete than would be expected for a strain that was simply unable to synthesize new ribosomes due to pre-rRNA processing defects. Many such mutants have been analyzed (reviewed in Venema & Tollervey, 1999) and predominantly show a gradual increase in doubling time, as preformed ribosomes are depleted by growth. The very rapid onset of growth inhibition, seen in the Nop7p-depleted strain expressing Rlp25-GFP, indicates that this does not require substantial depletion of the pre-existing ribosome pool. We speculate that production of defective subunits prevents the remaining, otherwise functional, ribosomes from carrying out efficient translation. The pre-60S E₁ complex lacks Rpl25p, which is added only to the pre-60 E₂ particle (Harnpicharnchai et al., 2001; Fatica et al., 2002). We therefore predict that the genetic interaction between *GAL::nop7* and Rpl25-GFP reflects a requirement for Nop7p in the correct assembly of Rpl25p, and perhaps other factors, with the pre-60S E₂ complex.

Several recent studies have made use of a fusion between Rpl25p and GFP to follow the export of 60S ribosomal subunits from the nucleus to the cytoplasm (Baßler et al., 2001; Gadal et al., 2001a, 2001b; Ho et al., 2000; Milkereit et al., 2001; Fatica et al., 2002). There is considerable data showing that free r-proteins do not accumulate in the absence of ribosome synthesis. The accumulation of Rlp25-GFP has therefore been taken as evidence for the accumulation of pre-ribosomal particles in the nucleoplasm, indicating a defect in their export. This assay has defined a late pre-ribosomal particle (pre-60S M in Fig. 8), all tested components of which are required for 60S subunit export. These include Nug1p, Nug2p, Noc2p, Noc3p, and Rix1p as well as Nop7p itself (Baßler et al., 2001; Gadal et al., 2001a, 2001b; Milkereit et al., 2001). Mutations in any of these proteins leads to defects in export, suggesting a requirement for the intact structure of this pre-ribosomal particle. Because multiple components of this complex are required for subunit export, we predict that export competence is established within this particle, and that this activity requires Nop7p.

Mutations in Nug1p, Nug2p, Noc2p, Noc3p, or Rix1p did not result in pre-rRNA processing defects similar to Nop7p depletion or synergistic interactions with Rpl25-GFP (Baßler et al., 2001; Milkereit et al., 2001) indicating that these are distinct activities. Moreover, Nug1-TAP did not coprecipitate 27SA₂ or 27SA₃ (Baßler et al., 2001; E. Petfalski & D. Tollervey, unpubl. observations) indicating that it associates with the pre-rRNA particle only after processing at these sites is complete. Depletion of a specific component of the pre-60S E₁ complex, Ssf1p, also did not interact genetically with Rpl25-GFP and did not inhibit subunit export as judged by nuclear accumulation of Rpl25-GFP (Fatica et al.,

2002). We therefore propose that the roles of Nop7p in pre-rRNA processing, assembly, and export are distinct and performed within different pre-ribosomal particles (see Fig. 8).

Pescadillo is a multifunctional protein

Pescadillo was isolated as a mutation affecting embryonic development (Allende et al., 1996) and a mutant allele of the yeast gene resulted in growth arrest in G2 (Kinoshita et al., 2001), consistent with a specific defect in cell-cycle progression. Yeast Yhp1p/Nop7p is also reported to interact with Yvh1p (Sakumoto et al., 2001), a protein-tyrosine phosphatase with a postulated role in the regulation of sporulation and meiosis.

There are clear precedents for proteins that function both in cell-cycle progression and ribosome synthesis. Exit from mitosis in budding yeast requires a group of proteins, including the phosphatase Cdc14p, which down-regulate cyclin-dependent kinase activity. Cdc14p is sequestered in the nucleolus in the RENT (regulator of nucleolar silencing and telophase) complex with Sir2p and Net1p, which serves to anchor the complex (Shou et al., 1999). In addition, Net1p is required for the maintenance of normal nucleolar structure and its binding stimulates RNA polymerase I (Shou et al., 1999, 2001). These nucleolus-specific functions of Net1p can be separated genetically from its cell-cycle functions in the RENT complex. In human cells, the nucleolar p14/ARF protein binds and sequesters the negative regulator of p53 activity, Mdm2 (Tao & Levine, 1999; Weber et al., 1999; Zhang & Xiong, 1999). Free Mdm2 ubiquitinates p53 and transports it to the cytoplasm where it is degraded by the proteasome (Fuchs et al., 1998; Geyer et al., 2000), and the nucleolar sequestration of Mdm2 contributes to the inhibition of this activity by ARF. Mouse *Pescadillo* was identified by its up-regulation in cells lacking p53 (Kinoshita et al., 2001), but other interactions with the p53 system have not been reported.

The available data suggest that yeast Nop7p may function both in ribosome synthesis and in cell-cycle regulation. Whether its role in the cell cycle involves other protein components of the pre-ribosomal particles or a different set of interactions remains to be determined.

MATERIALS AND METHODS

Strains

Growth and handling of *Saccharomyces cerevisiae* were by standard techniques. *GAL*-regulated strains were pregrown in RGS medium, containing 2% raffinose, 2% galactose, and 2% sucrose, and harvested at intervals following a shift to medium containing 2% glucose. Strains for pulse-chase analysis were pregrown in minimal RGS medium lacking uracil and shifted to minimal glucose medium lacking uracil. Strains for

immunofluorescence studies were grown in minimal glucose medium lacking leucine.

Yeast strains used and constructed in this study are listed in Table 1. Conditional mutants under the control of the repressible *GAL10* promoter were generated by one-step PCR strategy in the strains YDL401 and BMA64 (Lafontaine & Tollervey, 1996). Transformants were selected for HIS⁺ prototrophy and screened by PCR. TAP-tagged strains were constructed by one-step PCR strategy in the GAL-mediated strain *GAL::nop7* (Rigaut et al., 1999). Transformants were screened by immunoblotting and PCR.

TAP-tagged strains were transformed with pUN100Ds RedNOP1 (kindly provided by E. Hurt and U. Heidelberg) to allow ready identification of the nucleolus, and pYEplac195-L25-eGFP to look at nuclear export of 60S ribosomal subunits.

For construction of eYFP-PES, complementary DNA of human Pescadillo gene (GI:2194202) was isolated by PCR amplification from Marathon-Ready HeLa cDNA library (Clontech) using specific primers with *Bgl*II and *Eco*RI restriction sites attached to the 5' and 3' primer, respectively. The amplified fragment was subsequently cloned to the *Bgl*II-*Eco*RI fragment of eYFP-C1 and verified by DNA sequencing.

RNA extraction, northern hybridization, and primer extension

RNA was extracted as described previously (Tollervey & Mattaj, 1987). For high-molecular-weight RNA analysis, 7 µg of total RNA were separated on a 1.2% agarose gel containing formaldehyde and transferred for northern hybridization as described previously (Tollervey, 1987). Standard 6% or 8% acrylamide-8 M urea gels were used to analyze low-molecular-weight RNA species and primer extension reactions. Primer extensions were performed as described previously (Beltrame & Tollervey, 1992) on 5 µg of total RNA using primers 007 and 006.

For pre-rRNA and rRNA analysis the following oligonucleotides were used:

002: 5'-GCTCTTTGCTCTTGCC;
 003: 5'-TGTTACCTCTGGGCC;
 006: 5'-AGATTAGCCGAGTTGG
 007: 5'-CTCCGCTTATTGATATGC;
 008: 5'-CATGGCTTAATCTTTGAGAC;
 017: 5'-GCGTTGTTTCATCGATGC;

020: 5'-TGAGAAGGAAATGACGCT;
 041: 5'-CTACTCGGTCAGGCTC.

Immunofluorescence

For localization of yeast Nop7p, cells were grown in selective medium to midexponential phase, fixed by incubation in 4% (v/v) formaldehyde for 30 min at 25 °C, and spheroplasted. Immunofluorescence was then performed as described previously (Grandi et al., 1993; Bergès et al., 1994). Protein A fusions were detected with a rabbit anti-Protein A antibody (Sigma) and a secondary goat anti-rabbit antibody coupled to FITC (Sigma) at 1:1,000 and 1:200 dilutions, respectively. To stain nuclear DNA, DAPI was included in the mounting medium (Vectashield, Vector Laboratories). Cells were viewed on a Zeiss Axioscop microscope.

Cells containing pYE195-Rpl25-eGFP were grown in SD-LEU to midexponential phase, fixed in 4% (v/v) formaldehyde for 30 min, and pelleted. Cells were resuspended in 100 mM KH₂Ac/K₂HAc/1.1 M sorbitol and mounted onto slides using moviol, containing DAPI. To detect Rpl25-eGFP in vivo in the fluorescence microscope, the GFP-signal was examined in the fluorescein channel of a Zeiss Axioscop microscope (Hurt et al., 1999). Pictures were obtained with SmartCapture VP.

The localization of eYFP-Pescadillo was determined after transient transfection into HeLa cells. EYFP-PES and eCFP-B23 were cotransfected for 6 h using Effectene (Quiagen) according to the manufacturer's protocol and fixed after 42 h using 3.7% paraformaldehyde in CSK buffer. Cells were permeabilized and decorated with antibodies against dense fibrillar component marker fibrillarin (72B9) and the granular component marker B23 (anti-B23). Cells were imaged using a Zeiss LSM410 confocal microscope or a Zeiss DeltaVision Restoration microscope (Applied Precision, Inc.). Images presented here are maximal projections of the entire nuclear fluorescence.

Immunoprecipitation of *GAL::nop7*-TAP

For immunoprecipitation of *GAL::nop7*-TAP, cells were grown in YPgal to OD₆₀₀ = 2 and lysed in buffer A (150 mM KAc, 20 mM Tris-Ac, pH 7.5, 5 mM MgAc) with 1 mM DTT, 0.5% Triton X-100, 2.5 mM vanadyl-ribonucleoside complexes (VRC), and 5 mM PMSF (phenylmethylsulphonyl fluoride) at

TABLE 1. Yeast strains used and constructed in this study.

Strain	Genotype	Reference
YDL401	MATa <i>his3Δ200 leu2Δ1 trp1 ura3-52 gal2 galΔ108</i>	Lafontaine & Tollervey, 1996
YMO1	as YDL401 but <i>GAL10::nop7-HIS3</i>	This work
YMO2	as YDL401 but <i>GAL10::nop7-TAP-TRP1</i>	This work
YMO3	as YMO2 but pUN100-DsRednop1 LEU1	This work
YMO4	as YMO1 but pRS315-Rpl25-eGFP	This work
BMA64	MATa <i>ade2-1 his3-11,-15 leu2-3,-112 trp1Δ, ura3-1</i>	F. Lacroute
YMO5	as BMA64 but pA3ura3	This work
YMO6	as BMA64 but <i>GAL10::nop7, pA3ura3</i>	This work
YCA31	as YDL401 but <i>GAL10::prot.A-RRP4, RRP6:(K)TRP1</i>	Allmang et al., 1999
GAL::DOB1	MATa <i>ura3-1 ade2-1 his3-11,-15 leu2-3,-112 trp1-1</i> Dob1::HIS3MX6 +[pAS24-DOB1]	de la Cruz et al., 1998

4°C using glass beads (Sigma). Immunoprecipitation with rabbit IgG agarose beads and subsequent TEV cleavage were performed as described (Rigaut et al., 1999). RNA was extracted with buffer AE/phenol-chloroform, ethanol precipitated (Schmitt et al., 1990), and analyzed by northern hybridization and primer extension.

Pulse-chase labeling experiments

Pulse-chase labeling of pre-rRNA was performed as previously described (Tollervey et al., 1993) using 100 μ Ci [5,6- 3 H]uracil (Amersham) for 2 min at 30°C. Unlabeled uracil was added to a final concentration of 240 μ g mL $^{-1}$. Samples (1 mL) were taken, transferred to microcentrifuge tubes at room temperature, and spun for 10 s at full speed in an Eppendorf centrifuge. Cell pellets were frozen in liquid N $_2$. Total RNA was extracted with buffer AE/phenol-chloroform and ethanol precipitated (Schmitt et al., 1990). [3 H]-labeled pre-rRNA and rRNA was resolved on 1.2% agarose gels for high-molecular-weight RNAs and 8% acrylamide-8 M urea gels for low-molecular-weight RNAs. RNA was transferred to Hybond-N $^+$ Nylon membranes (Amersham), dried, and exposed to X-ray film for 10 days at -80°C with an intensifying screen.

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