Requirements for the nuclear export of the small ribosomal subunit

Terence I. Moy[1] and Pamela A. Silver[2,*]

[1]Department of Molecular Biology, Massachusetts General Hospital, 50 Blossum Street, Boston, MA 02114, USA
[2]The Dana-Farber Cancer Institute, 1 Jimmy Fund Way, Boston, MA 02115, USA
*Author for correspondence (e-mail: pamela_silver@dfci.harvard.edu)

Accepted 7 May 2002
Journal of Cell Science 115, 2985-2995 (2002) © The Company of Biologists Ltd

Summary

Eukaryotic ribosome biogenesis requires multiple steps of nuclear transport because ribosomes are assembled in the nucleus while protein synthesis occurs in the cytoplasm. Using an in situ RNA localization assay in the yeast *Saccharomyces cerevisiae*, we determined that efficient nuclear export of the small ribosomal subunit requires Yrb2, a factor involved in Crm1-mediated export. Furthermore, in cells lacking *YRB2*, the stability and abundance of the small ribosomal subunit is decreased in comparison with the large ribosomal subunit. To identify additional factors affecting small subunit export, we performed a large-scale screen of temperature-sensitive mutants. We isolated new alleles of several nucleoporins and Ran-GTPase regulators. Together with further analysis of existing mutants, we show that nucleoporins previously shown to be defective in ribosomal assembly are also defective in export of the small ribosomal subunit.

Key words: Ribosome, Nuclear export, In situ hybridization

Introduction

Eukaryotic ribosome biogenesis requires multiple steps of nuclear import and export. In yeast, the small (40S) ribosomal subunit consists of 32 ribosomal proteins and an 18S rRNA whereas the large (60S) ribosomal subunit consists of 46 ribosomal proteins and the 25S, 5.8S, and 5S rRNAs (Verschoor et al., 1998). Following their synthesis in the cytoplasm, the nascent ribosomal proteins are imported into the nucleolus where they assemble with the rRNA to form the individual subunits. In the nucleolus, RNA pol III transcribes the 5S rRNA, and RNA pol I transcribes the 35S pre-rRNA. The 35S pre-rRNA is processed to the mature 18S, 5.8S and 25S rRNAs. Most of these pre-rRNA processing steps occur in the nucleolus (reviewed by Kressler et al., 1999; Venema and Tollervey, 1995). Once fully assembled, the ribosomal subunits must be translocated through the nuclear pore complex to enter the cytoplasm.

All macromolecular transport into or out of the nucleus is believed to occur through the nuclear pore complex (NPC). In yeast, the nuclear pore is a 60 MDa complex composed of proteins called nucleoporins (reviewed by Davis, 1995; Fabre and Hurt, 1997). The dimensions of the nuclear pore channel are such that only one ribosomal subunit could pass through at any given time. Several models have been put forth for how individual proteins pass through the NPC on their way into the nucleus (Ben-Efraim and Gerace, 2001; Ribbeck et al., 1999; Rout et al., 2000). However, the precise mechanism for nuclear exit of large ribonucleoprotein complexes such as the ribosomal subunits remains unknown.

One key determinant of the directionality of nuclear transport is the nucleotide-bound state of the small GTPase Ran (reviewed by Koepp and Silver, 1996). Directionality is determined by a GTPase-activating protein, in yeast termed Rna1, and by a guanine exchange factor, in yeast termed Prp20 (Rcc1 in mammals). Rna1 is localized in the cytoplasm while Prp20 is inside the nucleus. Consequently, the concentration of Ran-GDP is elevated in the cytoplasm while the concentration of Ran-GTP is elevated in the nucleoplasm. The nucleotide-bound state of Ran affects its interactions with nuclear transport factors containing a Ran-binding domain. One family of yeast Ran-binding proteins includes Yrb1 (RanBP1 in mammals), a protein that resides in the cytoplasm, and Yrb2 (RanBP3 in mammals), a protein that resides in the nucleus (Mueller et al., 1998; Schlenstedt et al., 1995; Taura et al., 1997). The second family of Ran-binding proteins consists of transport receptors termed importins, exportins or karyopherins (reviewed by Görlich and Kutay, 1999).

Importins/karyopherins bind cargo in the cytoplasm and enter the nucleus through the NPC. Once inside the nucleus, the protein cargo dissociates from its importer. This dissociation is driven by Ran-GTP. Conversely, proteins are exported out of the nucleus by binding to exportins in association with Ran-GTP. Once in the cytoplasm the export complex dissociates when the GTP of Ran is hydrolyzed (Görlich and Kutay, 1999).

Crm1/Xpo1 (or exportin) is the major karyopherin that exports various cargoes out of the nucleus. Crm1 is perhaps best known for exporting proteins that have the consensus leucine-rich nuclear export sequence (NES) (Fornerod et al., 1997; Fukuda et al., 1997; Neville et al., 1997; Stade et al., 1997). Crm1 also exports some proteins that do not bear the consensus leucine-rich NES. A case in point is snurportin, a protein involved in U snRNP import (Huber et al., 1998; Paraskeva et al., 1999). Additionally, Crm1 exports some of its cargo through adapter proteins. Crm1 exports U snRNAs and the HIV intron-containing RNA through the PHAX (phosphorylated adapter for RNA export) and HIV Rev adapters, respectively (Askjaer et al., 1998; Ohno et al., 2000).

Table 1. Yeast strains used in this study

Strain	Genotype	Source
PSY207	*rat3-1 ura3-52 trp1Δ63 leu2Δ1 mat**a***	Li et al., 1995
PSY209	*rat2-1 ura3-52 trp1Δ63 leu2Δ1 mat**a***	Heath et al., 1995
PSY225	*nsp1(10A) ade2-1 can1-100 leu2-3,112 lys1-1 ura3-52 mat**a***	Nehrbass et al., 1990
PSY580	*ura3-52 trp1Δ63 leu2Δ1 mat**a***	Winston et al., 1995
PSY581	*his3Δ200 ura3-52 leu2Δ1 matα*	Winston et al., 1995
PSY635	*rat7-1 his3Δ200 ura3-52 leu2Δ1 matα*	Gorsch et al., 1995
PSY637	*rat9-1 trp1Δ63 ura3-52 leu2Δ1 matα*	Goldstein et al., 1996
PSY1077	*gle2-1 ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 matα*	Murphy et al., 1999
PSY1190	*xrn1*–Bgl*::URA3 ura3-52 ade2-101 lys2-801 leu2Δ1 trp1Δ63 his3Δ200 mat**a***	Larimer and Stevens, 1990
PSY1734	*kap104::ura::HIS xrn1Δ::URA3 ura leu lys trp mat**a** +pRS314-kap104-16^{ts} (TRP1)*	Aitchison et al., 1996
PSY1740	*nmd5Δ::HIS3 xrn1Δ::URA3 leu2 trp1 ade mat**a***	Ferrigno et al., 1998
PSY1750	*xrn1Δ::URA3 ura3-52 trp1Δ63 leu2Δ1 mat**a***	Moy and Silver, 1999
PSY1762	*TRP1::nup49 xrn1Δ::URA3 ade2 ade3 his3 leu2 ura3 mata +pUN100::LEU2- nup49-313*	Doye et al., 1994
PSY1776	*nup116-5::HIS3 xrn1Δ::URA3 his3 leu2 trp1 ura3 mat**a***	Wente et al., 1992
PSY1772	*nic96::HIS3 xrn1Δ::URA3 ade2 ura3 trp1 leu2 mat**a** +pUN100-nic96-1 (LEU2)*	Zabel et al., 1996
PSY1948	*ubi3Δub-1 ura3 his3 lys2 leu2 trp1 mat**a***	Finley et al., 1989
PSY1968	*crm1Δ::kanR leu2 his3 trp1 ura3 mat**a*** +pDL-*CRM1* (*LEU2 CEN*)	Neville and Roshbash, 1999
PSY1969	*crm1Δ::kanR leu2 his3 trp1 ura3 mat**a*** +pDL-*crm1*-T539C (*LEU2 CEN*)	Neville and Roshbash, 1999
PSY2070	*yrb2Δ::HIS3 ura3-52 leu2Δ1 trp1 his3Δ200 mat**a***	This study
PSY2090	*nsp1*(L697A*) trp1Δ63 ura3-52 leu2Δ1 mat**a***	This study
PSY2092	*gle2*(N273K, D290N) *trp1Δ63 ura3-52 leu2Δ1 mat**a***	This study
PSY2095	*mtr4/dob1^{ts} trp1Δ63 ura3-52 leu2Δ1 mat**a***	This study
PSY2159	*prp20*(S297N) *ade his3 trp1Δ63 leu2Δ1 ura3 mat**a***	This study
PSY2460	*yrb1*(F191S) *his3Δ200 ura3-52 leu2Δ1 matα*	This study

Recently, Crm1 has been shown to be important for the nuclear export of the large ribosomal subunit using the NES-bearing adaptor Nmd3 (Gadal et al., 2001; Ho et al., 2000b). No such adaptor has yet been determined for the small subunit, but analysis of a *crm1/xpo1-1* mutant suggested that export of the small subunit relies on the NES/Crm1 pathway (Moy and Silver, 1999).

Yrb2 is required for the efficient nuclear export of proteins containing a leucine-rich NES (Taura et al., 1998). In vitro studies show that Yrb2 can bind to Ran-GTP but not Ran-GDP (Noguchi et al., 1997). Yrb2 contains the phenylalanine-glycine repeat motifs that are also found on many nucleoporins, but Yrb2 is not a stable component of the NPC (Taura et al., 1998). *YRB2* is not an essential gene in yeast, but cells lacking *YRB2* are slower growing at 15°C and accumulate the NES reporter protein in the nucleus (Taura et al., 1998). The mammalian Yrb2 homologue, RanBP3, has been shown to promote the interaction of Crm1 with its substrates and thus affect export (Englmeier et al., 2001; Lindsay et al., 2001).

In order to study the export of the small ribosomal subunit in yeast, we previously reported the implementation of a novel assay to monitor the distribution of nascent small ribosomal subunits by in situ analysis. The small ribosomal subunit is exported as a 43S particle containing 20S pre-rRNA. In the cytoplasm, the 20S pre-rRNA is cleaved to produce the mature 18S rRNA and a 209 base fragment, the 5′ ITS1 RNA (Stevens et al., 1991; Trapman et al., 1975; Udem and Warner, 1973). Defects in the nuclear export of the small ribosomal subunit can be detected by localizing the 5′ ITS1 RNA by fluorescent in situ hybridization; 5′ ITS1 RNA accumulates in the nucleoplasm in these cells. Furthermore, in cells defective in small ribosomal subunit export, the 20S pre-rRNA does not mature to the 18S rRNA (Moy and Silver, 1999). With this assay, we previously reported that export of the small ribosomal subunit depended on the nucleotide-bound state of Ran and certain nucleoporins. We have now further refined the assay and extended its use to a number of export factor and nucleoporin mutants. In addition, we have used the assay to screen a large collection of temperature-sensitive mutants for small subunit export defects.

Materials and Methods

Small ribosomal subunit export assay

Yeast strains used in this study are listed in Table 1. Crm1 (PSY1968, MNY7) and crm1 (T539C) (PSY1969, MNY8) were grown to a density of 2×10^7 cells/ml in YPD at 30°C (Burke et al., 2000; Neville and Rosbash, 1999) and leptomycin B was added to a final concentration of 200 nM (kind gift of M. Rosbash, Howard Hughes Medical Institute, Brandeis University, Waltham, MA). Cells were fixed with formaldehyde at time points of 5, 15 or 60 minutes as previously described (Moy and Silver, 1999). PSY580 (wild-type, FY23) (Winston et al., 1995) and PSY2070 (*yrb2Δ*) were grown a density of 1×10^7 cells/ml in met$^-$ dropout media at 30°C (Burke et al., 2000). The cultures were diluted by 1:10 with fresh media and shifted to 15°C. Cells were fixed at time points 0, 3, 6, 12, 18 or 24 hours after the temperature shift. FY23 with pPS293 (vector) or pPS1082 (GAL_{pro}-*YRB2 URA2* 2μ) was grown in ura$^-$ dropout with 2% raffinose to 1×10^7 cells/ml at 25°C. Galactose was added to 2% and cells were fixed after 1, 2 or 3 hours. For *ts*$^-$ strains, cells were grown to a density of $1\text{-}2\times10^7$ cells/ml in YPD at 25°C before the temperature shift to 37°C and treated as previously described (Moy and Silver, 1999). 5′ ITS1 rRNA was localized by fluorescence in situ hybridization as described (Amberg et al., 1992) with the following modification. An oligonucleotide complementary to the first 50 bases of ITS1 rRNA, 5′-ATGCTCTTGCAAAACAAAAAAATCA-TTTTCAAAATTATTAAATTTCTT, was synthesized with the Cy3 fluorophore at its 5′ end (IDT, Coralville, IA). Samples were hybridized with this oligonucleotide at a concentration of 50 nM.

rRNA pulse-chase experiments

In each of the following experiments, cells were grown a density of $1\text{-}2\times10^7$ cells/ml in met$^-$ dropout media. Cultures of FY23 and PSY2070 were grown at 30°C, diluted by 1:10 with fresh media, and

shifted to 15°C for 24 hours. 5×10^8 cells were concentrated to a volume of 3 ml and pulse labeled with 250 μCi of [methyl-^{3}H]methionine for 5 minutes to methylate rRNA (70-85 Ci/mmol, 1 mCi/ml; NEN, Boston, MA). 500 μl of culture was transferred to a new tube, 1 ml of ice-cold media was added, the tube was centrifuged, supernatants were removed, and the cell pellets were frozen on dry ice. Chase was initiated by adding 150 μl unlabeled methionine at concentration of 20 mg/ml to the 2.75 ml of culture. At time points of 5, 10, 20, 40 and 60 minutes of chase, 500 μl of culture were removed and processed as described above. RNA was isolated by the hot acid phenol method (Ausubel et al., 1997). 10,000 cpm of radioactivity was loaded per lane onto a 1.2% agarose-formaldehyde gel. RNA was transferred to Hybond-N$^+$ membranes by vacuum blotting (Amersham-Pharmacia), UV-crosslinked, and sprayed with En^{3}hance (NEN). The membrane was exposed to film for 4 days at –80°C.

PSY1968 and PSY1969 were grown at 30°C, and 10^8 cells were concentrated to a volume of 1 ml in met$^-$ media and treated with leptomycin B at a final concentration of 200 nM for 15 minutes. PSY580, PSY2090, PSY2092, and PSY2460 were grown at 25°C and shifted to 37°C for 1 hour. Cells were pulse labeled with 50 μCi of [methyl-^{3}H]methionine for 1 minute and chased with 45 μl unlabeled methionine at a concentration of 20 mg/ml for 2, 4 or 10 minutes. Samples were processed as described above.

In separate experiments, PSY580 and PSY635 were grown at 25°C and shifted to 37°C for 1 hour. PSY580 and PSY1772 were grown at 25°C, shifted to 37°C for 3 hours, and then shifted back to 25°C for 0.5 hours. 10^9 cells were concentrated to a volume of 3 ml in met$^-$ media, pulse labeled with 250 μCi of [methyl-^{3}H]methionine for 3 minutes, and chased with unlabeled methionine at a final concentration of 1 mg/ml for 0, 3 or 10 minutes. Samples were processed as previously described (Moy and Silver, 1999).

Polysome profiles

Polysome profiles and ribosomal subunit profiles were performed as described (Kressler et al., 1997) with the following modifications. PSY2070 was covered by pPS2098 (*YRB2 TRP1 CEN)* or pPS327 (*TRP CEN*) and grown in trp$^-$ dropout media at 30°C to a density of 2×10^7 cells/ml. The culture was diluted and shifted to 15°C for 24 hours. For the polysome profiles, lysate (4 A_{260} units) was layered onto 10 ml linear 7-49% sucrose gradients. Samples were centrifuged in a Beckman SW41Ti rotor for 2 hours at 261,000 ***g*** at 4°C (Beckman Instruments, Fullerton, CA). For the ribosomal subunit profiles, lysate (2 A_{260} units) was layered onto 10 ml linear 10-35% sucrose gradients and centrifuged in a Beckman SW41Ti rotor for 4 hours at 261,000 ***g*** at 4°C. A Beckman fraction recovery system was used to pass the gradients through a Pharmacia UV-1 monitor to measure A_{254}.

ts$^-$ mutant screening

The yeast strain FY23 was disrupted in *XRN1* (Moy and Silver, 1999). The *xrn1Δ* strain was mutagenized with ethyl methanesulfonate (EMS) as described (Ausubel et al., 1997) to produce 50% cell death. 160 temperature-sensitive mutants were isolated, essentially as described (Amberg et al., 1992). Approximately 800 *XRN1*$^+$ *ts*$^-$ mutants, previously used in the screen for mRNA trafficking mutants (*RAT*), were kindly provided by C. N. Cole (Dartmouth Medical School, Hanover, NH) (Amberg et al., 1992). Yeast strains were grown on YPD plates at 25°C for 3-4 days. The strains were transferred to 2 ml YPD at a density of 10^7 cells/ml and cultured for 1 hour at 25°C to allow cells to resume growth and stimulate ribosome biogenesis. The cultures were shifted to 37°C for 1 hour. Half of the culture was fixed with 100 μl 37% formaldehyde for 2 hours at 25°C. The other half of the culture was shifted back to 25°C for 1 hour before fixation. 5′ ITS RNA was localized as previously described (Moy and Silver, 1999). Complementation group analysis was used to determine which mutants are defective in known nuclear transport factors. Mutants that were not identified by complementation grouping were backcrossed to the FY23 or FY86 strains three times (Winston et al., 1995). Then, the mutations were cloned by complementation of their temperature-sensitivity with a genomic library (Rose et al., 1987). The mutations were verified by complementation group analysis. The mutated gene was PCR amplified, and the PCR product was subjected to DNA sequencing.

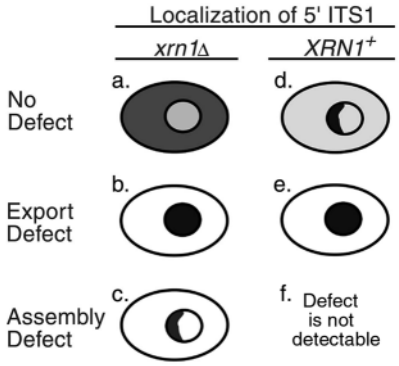

Fig. 1. The localization pattern of 5′ ITS1 from the small ribosomal subunit export assay. After the small ribosomal subunit is exported from the nucleus, the 5′ ITS1 RNA is cleaved away from the 18S rRNA in the cytoplasm and then degraded by the exonuclease Xrn1. Cells lacking *XRN1* accumulate 5′ ITS1 in the cytoplasm as long as ribosome assembly and export occurs (a). A *xrn1Δ* mutant defective in small ribosomal subunit assembly accumulates 5′ ITS1 to the nucleolus, the site of rRNA transcription and processing (c). 5′ ITS1 also localizes to the nucleolus of wild-type *XRN1*$^+$ cells (d). A mutant defective in small ribosomal subunit nuclear export accumulates 5′ ITS1 throughout the nucleoplasm in both *xrn1Δ* (b) and *XRN1*$^+$ strain backgrounds (e). Defects in ribosome assembly cannot be detected in *XRN1*$^+$ strains because 5′ ITS1 remains localized to the nucleolus.

Results

Assay for localization of the small ribosomal subunit

We previously reported the first cell-based assay for the localization of the nascent small ribosomal subunit. The assay relies on the localization of 5′ ITS1 RNA using fluorescent in situ hybridization (Moy and Silver, 1999). We originally performed this assay in strains lacking the Xrn1 exonuclease, which degrades the 5′ ITS1 fragment. In *xrn1Δ* cells, the 5′ ITS1 RNA is distributed throughout the cytoplasm because, in these cells, the small ribosomal subunit is exported, the 5′ ITS1 RNA is cleaved off, and the 5′ ITS1 fragment accumulates in the cytoplasm (Fig. 1a). We were able to assay various temperature-sensitive mutants in the *xrn1Δ* strain background and to detect both ribosomal assembly defects and nuclear export defects. In the case of a *ts*$^-$ ribosomal assembly mutant in the *xrn1Δ* strain background, the 5′ ITS1 RNA accumulates in the crescent-shaped nucleolus at the restrictive temperature (Fig. 1c) after sufficient time elapses to allow for degradation of the cytoplasmic 5′ ITS1 fragment. In the case of a *xrn1Δ* mutant blocked in export, the 5′ ITS1 accumulates in the nucleolus and the rest of the nucleoplasm after shift to the nonpermissive temperature (Fig. 1b).

The assay for the localization of 5′ ITS1 can also be carried

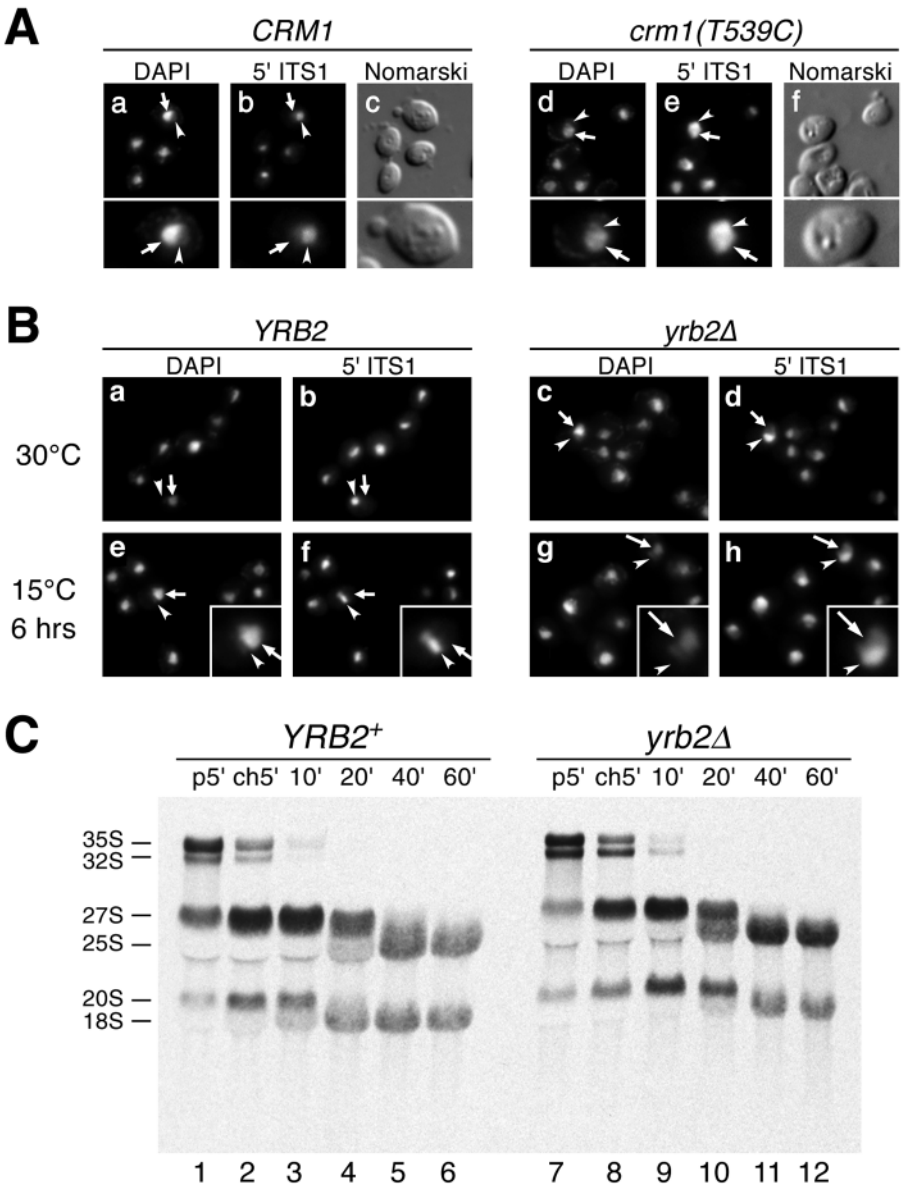

Fig. 2. *CRM1* and *YRB2* are required for nuclear export of the small ribosomal subunit. (A) 5′ ITS1 accumulates in the nucleoplasm of a *crm1* mutant. 5′ ITS1 was localized in *CRM1* (wild-type, b) and *crm1*(T539C) (e) cells after treatment with leptomycin B for 15 minutes. Chromosomal DNA was labeled with DAPI to identify the location of the nucleus (a,d), and Nomarski optics were used to visualize cell morphology (c,f). Arrows point to DAPI-stained chromatin while arrowheads point to the nucleolus. A magnified view of a cell is shown below the lettered panels. (B) 5′ ITS1 accumulates in the nucleoplasm of a *yrb2Δ* mutant. 5′ ITS1 was localized in *YRB2* (wild-type, b,f) and *yrb2Δ* (d,h) cells grown at 30°C (b,d) or shifted to the restrictive temperature of 15°C (f,h). Chromosomal DNA was labeled with DAPI to identify the location of the nucleus (a,c,e,g). Arrows point to DAPI-stained chromatin, while arrowheads point to the nucleolus. A magnified view of a cell is shown for e, f, g and h. (C) 20S pre-rRNA processing is delayed in *yrb2Δ* cells. *YRB2* (lanes 1-6) and *yrb2Δ* (lanes 7-12) cells were shifted to 15°C for 24 hours, pulse-labeled for 5 minutes, and chased for 5, 10, 20, 40 or 60 minutes. RNA was extracted and run on a formaldehyde-agarose gel. Sizes of the rRNAs are indicated.

out in *XRN1*$^+$ wild-type yeast cells. Although the loss of cytoplasmic 5′ ITS1 fluorescent signal is an easily identifiable change in *xrn1Δ* mutants, we determined that the excess 5′ ITS1 RNA in *xrn1Δ* strains can obscure a mild nuclear export defect. In addition, we wished to use the assay to screen existing mutant collections, which are *XRN1*-positive. Consequently, we have used *XRN1*$^+$ strains to examine small ribosomal subunit nuclear export. Wild-type *XRN1*$^+$ strains primarily localize 5′ ITS1 RNA to the nucleolus and a very small amount to the cytoplasm (Fig. 1d). However, in a mutant blocked in export, accumulation of 5′ ITS1 can be observed in both the nucleolus and nucleoplasm (Fig. 1e).

Crm1 and its co-factor, Yrb2, affect the export of the small ribosomal subunit

Cells bearing a leptomycin-B-sensitive (LMB) allele of *crm1* display a block in the export of the small ribosomal subunit dependent on drug treatment. Wild-type *S. cerevisiae* are insensitive to LMB because LMB does not interact with wild-type Crm1 protein. However, the *crm1*(T539C) mutant is inhibited by LMB, resulting in the nuclear export defect of leucine-rich NES proteins (Neville and Rosbash, 1999). We performed the small ribosomal subunit export assay on strains treated with LMB. LMB does not affect the localization of 5′ ITS1 RNA in wild-type (*XRN1*$^+$*)* cells (Fig. 2Aa-c); 5′ ITS1 remains localized to the nucleolus as indicated by arrowheads, and it does not significantly accumulate in the regions of the nucleus occupied by DAPI-stained chromatin as indicated by arrows. However, LMB treatment causes 5′ ITS1 to accumulate in the entire nucleoplasm in *crm1(T539C)* cells as indicated by arrows and arrowheads (Fig. 2Ad-f). Mislocalization of 5′ ITS1 in these cells occurs 5-15 minutes after addition of LMB.

Cells deleted for *YRB2*, which is involved in Crm1-mediated export, also show a defect in export of the small subunit. *yrb2Δ* cells are cold-sensitive for growth, and export of NES-containing proteins is blocked more so at the non-permissive temperature. While 5′ ITS1 RNA is confined to the nucleolus of wild-type

cells (Fig. 2Ba,b,e,f), 5′ ITS1 localizes throughout the nucleoplasm in *yrb2Δ* cells (Fig. 2Bc,d,g,h). The mislocalization of 5′ ITS1 in *yrb2Δ* cells is mildly detectable at the permissive temperature of 30°C (Fig. 2Bc,d), and the mislocalization is more evident after the shift to the restrictive temperature of 15°C (Fig. 2Bg,h). Since *YRB2* is not essential for growth in yeast, *yrb2Δ* cells cannot be completely blocked in small subunit export, but this mutant may be less efficient in this process.

In order to estimate the rate of small subunit export in *yrb2Δ* cells, we examined pre-rRNA processing. Strains were shifted to 15°C, pulse-labeled with [^{3}H-methyl]-methionine for five minutes, and then chased with excess unlabeled methionine for up to 60 minutes. In wild-type cells, 35S pre-rRNA is quickly converted to 27S and 20S pre-rRNAs, and then these pre-rRNAs are matured into the 25S and 18S rRNAs (Fig. 2C, lanes 1-6) (Kressler et al., 1999). At 15°C, wild-type cells convert 20S pre-rRNA to 18S rRNA after 10-20 minutes of chase (Fig. 2C, lanes 1-6). However, in *yrb2Δ* cells, this conversion is delayed and occurs after 20-40 minutes of chase (Fig. 2C, lanes 7-12). Furthermore, the levels of 18S rRNA in *yrb2Δ* cells at the 40-60 minute timepoints are significantly lower than the levels of 25S rRNA. In contrast, wild-type cells produce equimolar quantities of the 18S and 25S rRNAs since both rRNA species are transcribed together in the 35S pre-rRNA. Therefore, in cells lacking *YRB2*, the nascent small ribosomal subunit appears to be less stable and its nuclear export is delayed.

To determine the effect of the *yrb2Δ* mutation on ribosome activity, we examined polysome profiles. When cell lysates were subjected to sucrose gradient centrifugation, the ribosome migrates as an 80S particle and as polysome peaks, which contain multiple 80S ribosomes. In wild-type cells, approximately 10% of the ribosomal subunits are not assembled into ribosomes, and these subunits migrate as 40S or 60S peaks (Fig. 3A, left profile). In *yrb2Δ* cells, the amount of free 40S subunit is decreased while the amount of free 60S ribosomal subunit is dramatically increased (Fig. 3A, right profile). The amount of polysomes in *yrb2Δ* is marginally decreased, and these results suggest that the deficiency of the small ribosomal subunits is limiting mRNA translation. We examined the relative concentration of 60S to 40S ribosomal subunits by disassociating the ribosome and separating the subunits by sucrose gradient centrifugation. Wild-type cells contain an equal molar ratio of 60S to 40S ribosomal subunits and the ratio of 60S:40S subunits is 2.1:1 when measured by A_{254} because the 60S subunit contains twice as much RNA as the 40S subunit (Fig. 3B, left profile). In *yrb2Δ*, the ratio of 60S:40S is increased to 2.5:1 (*t*-test $P<0.05$, Fig. 3B, right profile). Therefore, the concentration of 40S ribosomal subunits is decreased by 10-20% relative to the 60S ribosomal subunit in *yrb2Δ* cells.

Overexpression of *YRB2* also causes a defect in small subunit export similar to the effect on NES-containing proteins (Taura et al., 1998). *YRB2* was expressed at high levels from a galatose-inducible promoter in wild-type yeast cells. After a 1 hour induction with galactose, 5′ ITS1 acccumulates in the nucleoplasm of cells expressing *YRB2* (Fig. 4Ac-d). Overexpression of *YRB2* also causes the accumulation of 20S pre-rRNA (data not shown). 5′ ITS1 localization and pre-rRNA processing was not affected in galactose-treated cells containing the empty vector (Fig. 4Aa-b). While expression of *YRB2* from its own promoter on a high copy plasmid is not toxic to wild-type cells, high copy expression of *YRB2* impairs the growth of *rat2^{ts}–/nup120*, a nucleoporin mutant defective in small ribosomal subunit export (see below), at the permissive temperature of 25°C (Fig. 4B). Only full-length *YRB2* had this effect as N terminal, C terminal, or FG repeat *YRB2* truncation mutants were not toxic to *rat2-1* cells (Fig. 4B) (Taura et al., 1997). The percentage of *rat2^{ts}* cells mislocalizing 5′ ITS1 at the permissive temperature was mildly increased in the presence of high copy *YRB2*. The synergistic alteration in ribosome export in these cells may explain their impaired growth rate. In any case, these data indicate that proper levels of Yrb2 are required for the efficient nuclear export of the small ribosomal subunit.

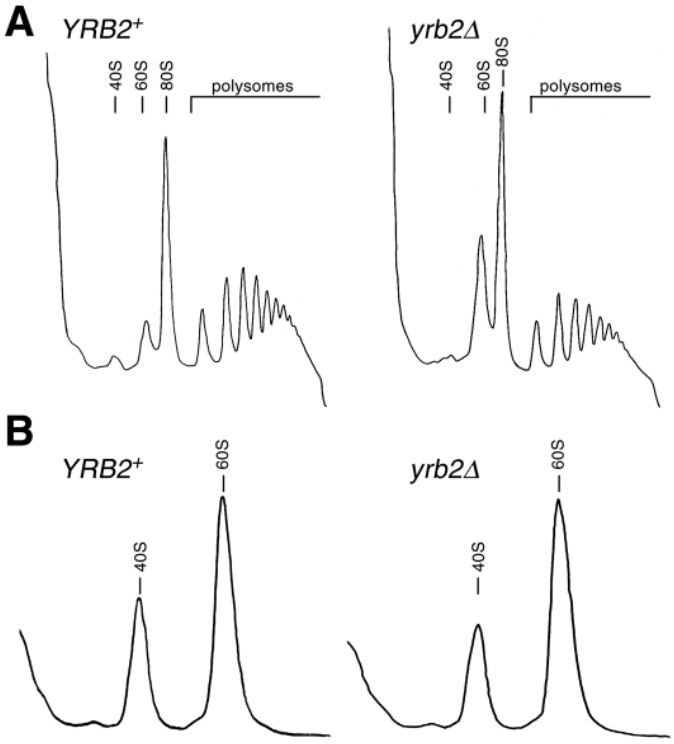

Fig. 3. *yrb2Δ* cells are deficient in the levels of the small ribosomal subunit. (A) *YRB2*$^+$ and *yrb2Δ* cells were shifted to 15°C for 24 hours, and polysome profiles were resolved in 7-49% sucrose gradients. Abs_{254nm} was monitored continuously and represents the vertical axis of the profiles. The top of the gradient is on the left-hand side of the profile. The positions of free 40S and 60S subunits, 80S ribosomes, and polysomes are indicated. (B) *YRB2*$^+$ and *yrb2Δ* cells were shifted to 15°C for 24 hours, and ribosomal subunit profiles were resolved in 10-35% sucrose gradients.

The large ribosomal subunit appears to be exported out of the nucleus via a Crm1-dependent mechanism (Gadal et al., 2001; Ho et al., 2000b; Stage-Zimmermann et al., 2000). Therefore, we expected that the decreased efficiency of NES export in *yrb2Δ* would also cause defects in 60S ribosomal subunit export. However, we do not see mislocalization of the 60S nuclear export reporter proteins Rpl11b-GFP, Rpl25-GFP, or GAL_{pro}-Rpl25-GFP in *yrb2Δ* cells (data not shown). It may be that the 60S export assays are not sensitive enough to detect subtle export defects in *yrb2Δ* cells. Alternatively, Yrb2 is not involved in 60S export.

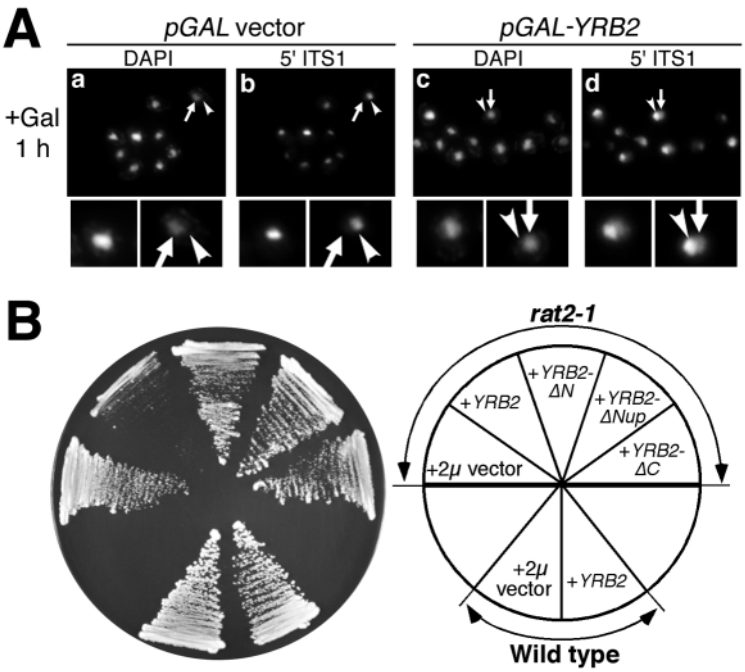

Fig. 4. Overexpression of *YRB2* is toxic. (A) Overexpression of *YRB2* causes the mislocalization of 5′ ITS1. 5′ ITS1 was localized in wild-type cells containing p*GAL* vector (b) or p*GAL-YRB2* (d) after 1 hour induction with galactose. Chromosomal DNA was labeled with DAPI (a,c). Arrows point to DAPI-stained chromatin while arrowheads point to the nucleolus. Magnified views of two cells are shown below each panel. (B) High-copy expression of *YRB2* inhibits the growth of *rat2-1/nup120* cells. *rat2-1* (PSY209) or wild-type (PSY580) cells containing 2μ vector (pPS701), full-length *YRB2* (pPS2096), N-terminal truncation (pPS2104), deletion of the Nup FG motifs (pPS2105), or the C-terminal/Ran-binding domain truncation (pPS2106) were grown on trp$^-$ dropout plates at 25°C.

Identification of nucleoporin and Ran regulator mutants that are defective in small ribosomal subunit nuclear export

In order to further characterize small ribosomal subunit export, we performed the small ribosomal subunit export assay on libraries of *ts*$^-$ mutants, and we examined the involvement of known nuclear transport factors by analyzing the corresponding mutants in greater detail. In addition to examining *ts*$^-$ mutants at the restrictive temperature, our screens also used a shift-back protocol in which cells at 37°C were transferred back to 25°C. This shift-back step is useful when examining mutants that are defective in ribosome assembly at restrictive temperatures. Ribosome assembly defects prevent the detection of ribosome export defects. The shift-back protocol allows cells to resume ribosome assembly, and ribosome export defects become detectable if the rate of ribosome assembly exceeds the rate of ribosome export (Hurt et al., 1999).

The utility of the shift-back protocol is illustrated with the nucleoporin mutant *nic96-1* that lacks the Xrn1 exonuclease (Zabel et al., 1996). At permissive temperatures, 5′ ITS1 RNA is localized throughout the cytoplasm in these cells similar to *NIC96*$^+$ *xrn1Δ* cells (data not shown). At the restrictive temperature, 5′ ITS1 accumulates in the nucleolus in the *nic96-1 xrn1Δ* strain, indicating a defect in ribosome assembly (Fig. 5Ae-f). Upon shift-back to permissive temperatures, the localization of 5′ ITS1 RNA expands to fill the entire nucleus (Fig. 5Ag,h). In contrast, the localization of 5′ ITS1 does not change in *NIC96*$^+$ *xrn1Δ* cells at any temperature (Fig. 5Aa-d). Upon shifting back to permissive temperatures, the *nic96-1 XRN1*$^+$ strain is less efficient than wildtype at converting 20S pre-rRNA to 18S rRNA (Fig. 5B, lanes 10-12). *nic96-1* cells are still able to export the small ribosomal subunit export, at a reduced rate, since 18S rRNA is produced in these cells, but these results demonstrate that mild nuclear export defects can be detected using this shift-back protocol.

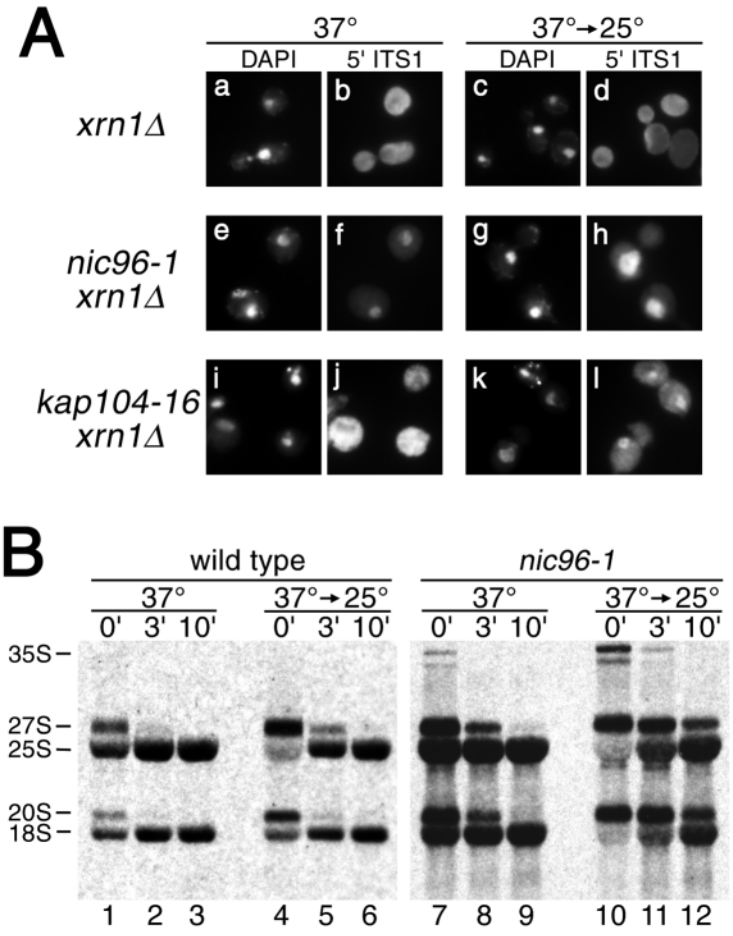

Fig. 5. A shift-back protocol facilitates the detection of ribosome biogenesis defects. (A) 5′ ITS1 was localized in *xrn1Δ*, *nic96-1 xrn1Δ*, and *kap104-16 xrn1Δ* after shifting to 37°C for 3 hours (b,f,j, respectively) or shifting back from 37°C to 25°C for 30 minutes (d,h,l). Chromosomal DNA was labeled with DAPI (a,c,e,g,i,k). (B) pre-rRNA processing in wild-type (lanes 1-6) and *nic96-1* (lanes 7-12) cells shifted to 37°C or shifted-back to 25°C. Cells were pulse labeled for 3 minutes and chased for 0, 3 or 10 minutes. RNA was extracted and run on a formaldehyde-agarose gel. Sizes of the rRNAs are indicated.

Table 2. Classification of yeast mutants affecting the assembly and nuclear export of the small ribosomal subunit

Class I. Mutations that affect the export of the small ribosomal subunit

	Small ribosomal subunit nuclear export			
Mutation	Permissive temp.	Restrictive condition	Shift back	Source
yrb2Δ	–*	–*	nd	Taura et al., 1997
crm1(T539C)	+	–	nd	Neville and Rosbash, 1999
nic96-1	+	+	–	Zabel et al., 1996
gle2/nup40 (N273K, D290N)	+	–*	–	This study
gle2-1(W334Stop)	+	–*	nd	Murphy et al., 1999
nup49-313	+	–†	–	Doye et al., 1994
nup116-5	+	–†	–	Wente et al., 1992
nup120/rat2-1	–*	–†	–	Heath et al., 1995
nup133/rat3-1	–*	–†	–	Li et al., 1995
nup159/rat7-1	–*	–*	–*	Gorsch et al., 1995
nsp1(10A)	–*	–*,†	–	Nehrbass et al., 1990
nsp1(L697P)	–*	–*	–*	This study
prp20(S297N)	+	–	nd	This study
yrb1(F191S)	+	–	nd	This study

Class II. Mutations that affect small ribosomal subunit assembly

	Assembly of the small ribosomal subunit			
Mutation	Permissive temp.	Restrictive condition	Shift back	Source
kap104-16	+	+	–†	Aitchison et al., 1996
nmd5Δ	+	+	–†	Ferrigno et al., 1998
nup82Δ108	+	–†,‡	nd	Hurwitz and Blobel, 1995
rps31Δub	–§	nd	nd	Finley et al., 1989

Class III. Mutations that do not show defects in small ribosomal subunit assembly or export

Mutation	Source
nup85/rat9-1	Goldstein et al., 1996
xrn1Δ	Larimer and Stevens, 1990

Class I mutants accumulated 5′ ITS1 RNA to the nucleoplasm. The conditions of nucleoplasmic accumulation are indicated as occurring under permissive conditions, restrictive conditions/temperatures, or upon shift back from 37°C to 25°C. + indicates wild-type export and – indicates a defect; nd, not done. Class II mutants affect the assembly of ribosomal subunits and accumulate 5′ ITS1 to the nucleolus as an *xrn1Δ* strain or to the cytoplasm as an *XRN1*$^+$ strain. Class III mutants do not mislocalize 5′ ITS1 and do not have detectable defects in ribosome assembly or export. This table partially updates Table 1 described in Moy and Silver, 1999.

*Mislocalization of 5′ ITS1 is only detectable in *XRN1*$^+$ strains.

†Mutants are defective in ribosome assembly; they accumulate 5′ ITS1 to the nucleolus as a *xrn1Δ* strain.

‡Mutant is also defective in small ribosomal subunit nuclear export.

§Mutant is defective in the cytoplasmic maturation of the small ribosomal subunit, and it accumulates 5′ ITS1 in the cytoplasm as a *XRN1*$^+$ strain.

The shift-back protocol is also useful in identifying mutants that affect ribosome assembly. We previously reported that *kap104-16 xrn1Δ* and *nmd5Δ xrn1Δ* strains do not mislocalize 5′ ITS1 RNA at 25°C or at 37°C (Moy and Silver, 1999). Using the shift-back protocol, we show that a small amount of 5′ ITS1 accumulates in the nucleolus of these cells (Fig. 5Ak,l). Mammalian orthologues of *KAP104* and *NMD5* have been shown to be importers of ribosomal proteins (Jakel and Görlich, 1998). We interpret the defect of *kap104-16* and *nmd5Δ* to be a result of decreased import of ribosomal proteins.

In an attempt to identify additional factors important for export of the small ribosomal subunit, we screened 960 *ts*$^-$ mutants with the small ribosomal subunit export assay. Twelve mutants were found to mislocalize 5′ ITS1 RNA. We determined that nine of these mutants are defective in the *RAT2/NUP120, RAT3/NUP133, RAT7/NUP159, NSP1, GLE2/NUP40, MTR4/DOB1, PRP20* and *YRB1* genes (Table 2). Two independent *rat3*$^{ts-}$ mutants were identified by this screen.

The *RAT2*, *RAT3*, and *RAT7* (mRNA trafficking) genes encode nucleoporins that are required for mRNA export (Gorsch et al., 1995; Heath et al., 1995; Li et al., 1995). Both the *rat2-1* and the *rat2-2* alleles display strong small ribosomal subunit assembly and export defects. 5′ ITS1 accumulated in the nucleolus in *rat2-1 xrn1Δ* cells shifted to the restrictive temperature (Moy and Silver, 1999), and 5′ ITS1 accumulated to the entire nucleoplasm after shifting back to the permissive temperature (data not shown). In *rat2-1 XRN1*$^+$ cells, 5′ ITS1 accumulated in the nucleoplasm at both permissive temperatures (Fig. 6Ab,i) and upon shift back. These data suggest that *RAT2/NUP120* is involved in both ribosome assembly and nuclear export.

The *rat3-1* and *rat7-1* mutants have milder defects in small ribosomal subunit nuclear export; the conditions in which these mutants mislocalize 5′ ITS1 are more limited. In *rat3-1* cells, nucleoplasmic accumulation of 5′ ITS1 is most noticeable when these cells are grown to stationary phase and then transferred to fresh media. These conditions allow cells to

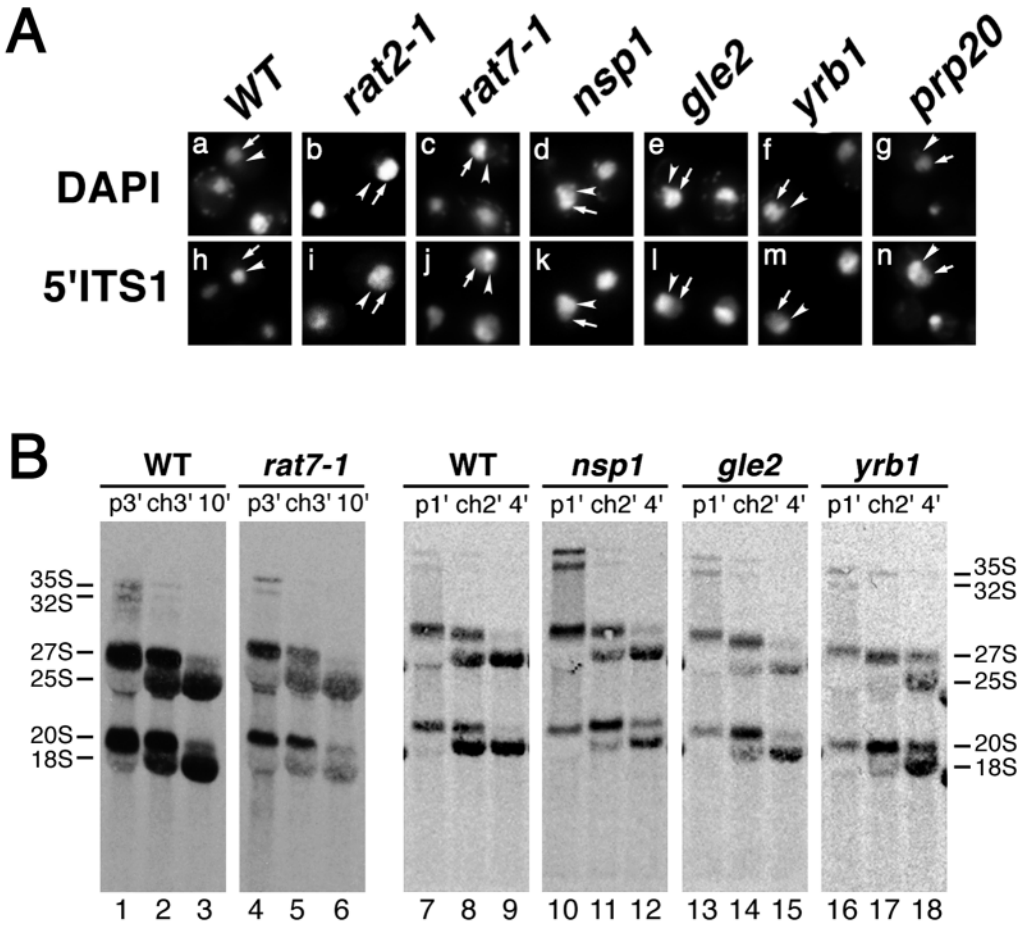

Fig. 6. Ribosome export mutants identified by screening *ts*⁻ mutant libraries. (A) 5′ ITS1 localization in *rat2-1/nup120* (i) grown at 25°C and in wild type (h), *rat7-1/nup159* (j), *nsp1*(L697P) (k), *gle2*(N273K,D290N) (l), *yrb1*(F191S) (m), and *prp20*(S297N) (n) mutants shifted to 37°C for 1 hour. Chromosomal DNA was labeled with DAPI (a-g). Arrows point to DAPI-stained chromatin while arrowheads point to the nucleolus. (B) Pre-rRNA processing in wild-type (lanes 1-3 and 7-9), *rat7-1/nup159* (lanes 4-6), *nsp1*(L697P) (lanes 10-12), *gle2*(N273K,D290N) (lanes 13-15), and *yrb1*(F191S) (lanes 16-18) mutants shifted to 37°C for 1 hour. Cells were pulse labeled for 3 minutes (lanes 1-6) or 1 minute (lanes 7-18). Pulse and chase times are indicated above each lane. Sizes of the rRNAs are indicated.

resume growth which stimulates ribosome biogenesis and facilitates the detection of ribosome export defects (Hurt et al., 1999). In *rat7-1* cells, nucleoplasmic accumulation of 5′ ITS1 was detectable only when the mutant contained the Xrn1 exonuclease; *rat7-1 xrn1Δ* cells did not mislocalize 5′ ITS1 at restrictive temperatures or upon shift-back (Moy and Silver, 1999). *rat7-1 XRN1*$^+$ cells accumulate nucleoplasmic 5′ ITS1 at all temperatures (Fig. 6Ac,j; Table 2). Furthermore, the conversion of 20S pre-rRNA to 18S rRNA was defective in *rat7-1* cells (Fig. 6B, lanes 4-6). Similar to the *yrb2Δ* mutant, the *rat7-1* mutant appears to be less efficient in the export of the small ribosomal subunit.

In our screen of the *ts*⁻ mutant libraries, we also identified a novel allele of the nucleoporin *NSP1*. Previously, we have shown that the *nsp1(10A) xrn1Δ* strain is defective in ribosome assembly in that it accumulated 5′ ITS1 to the nucleolus at the restrictive temperature (Moy and Silver, 1999). Here, we identify a *nsp1* mutant that encodes a protein with a single amino acid change in which leucine 697 is altered to a proline. The *nsp1(L697P)* mutant accumulates 5′ ITS1 to the entire nucleoplasm at all temperatures (Fig. 6Ad,k; Table 2). Addition of a plasmid containing wild-type *NSP1* rescues the mislocalization defect of the *nsp1(L697P)* mutant (data not shown). At 37°C, *nsp1*(L697P) is delayed in processing the 35S, 32S and 20S pre-rRNAs (Fig. 6B, lanes 10-12). We conclude that Nsp1 affects both assembly and nuclear export of the small ribosomal subunit.

We identified a *gle2* mutant that accumulates 5′ ITS to the nucleoplasm (Fig. 6Ae,l) and has a mild delay in 20S pre-rRNA processing at the restrictive temperature (Fig. 6B, lanes 13-15). This *gle2* mutant encodes a protein containing two amino acid changes in which asparagine 273 is altered to a lysine and aspartate 290 is altered to an asparagine. Previously, we reported that the *gle2-1 xrn1Δ* mutant does not mislocalize 5′ ITS1 at permissive or restrictive temperatures (Moy and Silver, 1999; Murphy et al., 1999). The *xrn1Δ* mutation appears to have obscured the ribosome export defect because the *gle2-1 XRN1*$^+$ mutant accumulates 5′ ITS1 at the restrictive temperature (data not shown).

In addition to the nucleoporin mutants identified by our *ts*⁻ screen, we examined previously characterized nucleoporin mutants to determine whether they affect small ribosomal subunit export. Similar to the *nic96-1 xrn1Δ* mutant, *nup49-313 xrn1Δ* and *nup116-5 xrn1Δ* mutants accumulate 5′ ITS1 to the nucleus after shifting the cells back to their permissive temperature. Furthermore, the *nup49-313* mutant has a delay in 20S pre-rRNA processing after the shift back in temperature (data not shown; Table 2).

Finally, we identified two novel alleles of genes encoding regulators of the Ran GTPase: the RanGEF, *PRP20*, and the RanGAP accessory factor, *YRB1*. The *yrb1* mutant encodes a protein in which phenyalanine 191 is mutated to a serine. This allele is similar to the previously identified *yrb1-1* allele in that the majority of cells, when shifted to the restrictive temperature, accumulate nucleoplasmic 5′ ITS1 (Fig. 6Af,m) and 20S pre-rRNA (Fig. 6B, lanes 16-18).

The novel *prp20* allele is different from the much-studied *prp20-1* allele. The *prp20* allele that we identified encodes a protein in which serine 297 is converted into an asparagine. This results in a weak allele of *PRP20* with regard to nuclear transport defects. Although this *prp20(S297N)* strain does not grow at 37°C, at this restrictive temperature only a small fraction of cells (10-20%) exhibit nucleoplasmic accumulation of 5′ ITS1 (Fig. 6Ag,n; data not shown). In contrast, greater than 95% of *prp20-1* cells accumulate 5′ ITS1 at restrictive temperatures (Moy and Silver, 1999). Furthermore, we cannot detect an mRNA export defect in *prp20(S297N)* while the *prp20-1* mutant has a strong mRNA export defect (Amberg et al., 1993). Interestingly, the *prp20(S297N)* mutant was previously identified from the same *ts*$^-$ mutant library in a screen for mutants that mislocalize Npl3 (Corbett and Silver, 1996).

Discussion

In this paper, we used a novel assay in combination with yeast mutants to further define the requirements for proper export of the small ribosomal subunit out of the nucleus. *YRB2* is required for both the stability of the nascent small ribosomal subunit and the efficient nuclear export of this subunit. In addition, a large-scale screen of temperature-sensitive mutants yielded additional evidence for the involvement of Ran and the nuclear pore complex in small ribosomal subunit export. These findings are discussed in the context of what we know about the role of these factors in nuclear export.

In previous studies, we showed that small subunit export is inhibited in the temperature-sensitive mutant *xpo1-1*. However, it remained a formal possibility that this was an indirect effect since the *xpo1-1* mutant mislocalizes the RanGAP Rna1 to the nucleus at the restrictive temperature resulting in the disruption of the Ran gradient (Feng et al., 1999). Consequently, when *xpo1-1* cells are shifted to the restrictive temperature, they immediately show a defect in mRNA export (Stade et al., 1997), which can be suppressed by overexpression of *DBP5*, an RNA helicase essential for mRNA export (Hodge et al., 1999). In contrast, upon leptomycin B addition, the *crm1*(T539C) mutant accumulates the NES reporter in the nucleus within 5 minutes, but the *crm1*(T539C) strain does not accumulate mRNA in the nucleus until 1 hour after treatment, suggesting that the primary defect is indeed in NES-dependent export (Neville and Rosbash, 1999). We now show that LMB-treated *crm1*(T539C) cells accumulate the small ribosomal subunit in the nucleus with timing similar to that of NES accumulation. Therefore, the involvement of Crm1 in small ribosomal subunit export is likely to be direct.

Yrb2 is a member of the Ran binding protein family and is required for the efficient export of Crm1-mediated cargo (Taura et al., 1998). Here, we show that the export of the small ribosomal subunit is delayed in *yrb2Δ* cells. Initially, we could not detect mislocalization of 5′ ITS1 in *yrb2Δ xrn1Δ* cells because the accumulation of cytoplasmic 5′ ITS1 fragment conceals the small ribosomal subunit export defect. However, in *yrb2Δ XRN1*$^+$ cells we could detect nuclear accumulation of 5′ ITS1 and the delay in processing 20S pre-rRNA.

The exact function of Yrb2 in Crm1-mediated export is still not clear. In vitro, Yrb2 disassociates the Crm1/NES/Ran-GTP export complex so Yrb2 may function in the terminal release step in Crm1-mediated export (Maurer et al., 2001). In contrast, the mammalian orthologue of Yrb2, RanBP3, stimulates formation of the export complex (Lindsay et al., 2001). These contrasting activities of Yrb2 and RanBP3 may stem from different experimental conditions. When RanBP3 is at sub-stoichiometric concentrations, the Crm1/NES/Ran-GTP complex formation is increased while higher concentrations of RanBP3 inhibit complex formation (Englmeier et al., 2001). Importantly, RanBP3 has variable effects on Crm1 export complex formation depending on the cargo substrate. When Snurportin and the leucine-rich NES cargos are mixed with Crm1 and RanGTP, the Crm1-Snurportin-Ran-GTP complex is favored over the Crm1-NES-Ran-GTP complex because Snurportin forms a higher affinity complex (Englmeier et al., 2001; Paraskeva et al., 1999). However, when RanBP3 is added to this mixture, the Crm1/NES/RanGTP complex is efficiently formed and the complex formation of Crm1/Snurportin/RanGTP is decreased (Englmeier et al., 2001).

The differential effects of RanBP3 on Crm1 function may help to explain why loss of Yrb2 in yeast affects the biogenesis of the small ribosomal subunit more than the biogenesis of the large ribosomal subunit. Both ribosomal subunits appear to use a Crm1-dependent export pathway (Gadal et al., 2001; Ho et al., 2000b). If Yrb2 functions similarly to RanBP3, Yrb2 may favor the nuclear export of the small ribosomal subunit over the export of the large ribosomal subunit. The decreased abundance of the small subunit in *yrb2Δ* cells could be a result of the degradation of unexported small subunits. Nuclear export of 60S subunits is required for their stability (Ho and Johnson, 1999; Ho et al., 2000a).

The question remains as to how Crm1 and Yrb2 promote export. One possibility is that Crm1 binds directly to the small subunit. However, despite extensive attempts we could not detect Crm1 bound to small subunits and could not reconstitute Crm1 binding to purified ribosomal subunits. An attractive alternative is that there is an adaptor protein that promotes binding of Crm1 to the small subunit. Such an adaptor would be analogous to the role of Nmd3 in promoting binding of Crm1 to the large subunit. Yrb2 could be such an adaptor. However, *YRB2* is not essential for normal growth while one might expect such an adaptor to be essential if it is a critical part of the small subunit export pathway. Further experiments will be required to identify such an adaptor.

In order to identify additional *trans*-acting factors involved in small ribosomal subunit export, we screened 960 *ts*$^-$ mutants with the 5′ ITS1 localization assay. We identified 5 nucleoporin mutants and 2 Ran regulator mutants that are defective in small subunit export. Interestingly, no novel factors were identified. This could indicate that it may be difficult to generate conditional alleles of such factors. Alternatively, there may be inherent limitations to the assay presented here as screens of the same collection have yielded large numbers of mutants defective in assembly and export of the large ribosomal subunit (Bassler et al., 2001; Gadal et al., 2001; Milkereit et al., 2001). From our *ts*$^-$ screen, we identified three mutants in which the *ts*$^-$ mutation was not linked to the 5′ ITS1 mislocalization phenotype. After separation from the *ts*$^-$ mutation, the 5′ ITS1 mislocalizing mutants grew at normal rates at all temperatures (data not shown). Since the characteristics of these mutants do not match the phenotypes of known nuclear transport factors,

these mutants may correspond to novel factors involved in small ribosomal subunit export.

In total, we examined 17 nucleoporin mutants with the 5′ ITS1 localization assay. Twelve of these mutants are defective in the assembly of the small ribosomal subunit (Moy and Silver, 1999). These assembly defects could be caused by alterations in the nuclear export of mRNAs encoding ribosomal proteins or by defects in the nuclear import of ribosomal proteins, assembly factors, or ribosome biogenesis regulators. Seven of these nucleoporin mutants are also defective in the nuclear export of the small ribosomal subunit (Table 2). Two nucleoporin mutants are defective in small ribosomal subunit export, but do not have detectable defects in ribosome assembly. Overall, these results emphasize the multiple transport functions of nucleoporins and the importance of the NPC in ribosome biogenesis.

It is interesting to speculate on the role of certain nucleoporins in ribosome export. It could be that certain nucleoporins define docking sites on either side of the NPC that are critical for binding and/or release. They could also define contact sites within the NPC that the ribosome makes as it passes through the channel. The manner in which a large particle such as a ribosomal subunit passes through the NPC remains one of the outstanding questions in cell biology and further analysis of these and other transport mutants in combination with biochemical assays will help to further elucidate the process.

We thank all of the investigators who generously shared yeast strains and reagents. We thank Elissa Lei and Tetsuya Taura for critical reading of this manuscript and for their support. This work was funded by grants from the National Institutes of Health to P.A.S. and an National Cancer Institute training grant to the Dana-Farber Cancer Institute for T.I.M.

References

Aitchison, J. D., Blobel, G. and Rout, M. P. (1996). Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. *Science* **274**, 624-627.

Amberg, D. C., Fleischmann, M., Stagljar, I., Cole, C. N. and Aebi, M. (1993). Nuclear PRP20 protein is required for mRNA export. *EMBO J.* **12**, 233-241.

Amberg, D. C., Goldstein, A. L. and Cole, C. N. (1992). Isolation and characterization of RAT1: an essential gene of Saccharomyces cerevisiae required for the efficient nucleocytoplasmic trafficking of mRNA. *Genes Dev.* **6**, 1173-1189.

Askjaer, P., Jensen, T. H., Nilsson, J., Englmeier, L. and Kjems, J. (1998). The specificity of the CRM1-Rev nuclear export signal interaction is mediated by RanGTP. *J. Biol. Chem.* **273**, 33414-33422.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1997). Saccharomyces cerevisiae. In *Current Protocols in Molecular Biology*, pp. 13.0.1-13.13.7. New York: John Wiley & Sons.

Bassler, J., Grandi, P., Gadal, O., Lessmann, T., Petfalski, E., Tollervey, D., Lechner, J. and Hurt, E. (2001). Identification of a 60S Preribosomal Particle that is closely linked to nuclear export. *Mol. Cell* **8**, 517-529.

Ben-Efraim, I. and Gerace, L. (2001). Gradient of increasing affinity of importin beta for nucleoporins along the pathway of nuclear import. *J. Cell Biol*. **152**, 411-417.

Burke, D., Dawson, D. and Stearns, T. (2000). *Methods in Yeast Genetics*. Plainview, NY: Cold Spring Harbor Laboratory Press.

Corbett, A. H. and Silver, P. A. (1996). The NTF2 gene encodes an essential, highly conserved protein that functions in nuclear transport in vivo. *J. Biol. Chem.* **271**, 18477-18484.

Davis, L. I. (1995). The nuclear pore complex. *Annu. Rev. Biochem.* **64**, 865-896.

Doye, V., Wepf, R. and Hurt, E. C. (1994). A novel nuclear pore protein Nup133p with distinct roles in poly(A)+ RNA transport and nuclear pore distribution. *EMBO J.* **13**, 6062-6075.

Englmeier, L., Fornerod, M., Bischoff, F. R., Petosa, C., Mattaj, I. W. and Kutay, U. (2001). RanBP3 influences interactions between CRM1 and its nuclear protein export substrates. *EMBO Rep.* **2**, 926-932.

Fabre, E. and Hurt, E. (1997). Yeast genetics to dissect the nuclear pore complex and nucleocytoplasmic trafficking. *Annu. Rev. Genet.* **31**, 277-313.

Feng, W., Benko, A. L., Lee, J. H., Stanford, D. R. and Hopper, A. K. (1999). Antagonistic effects of NES and NLS motifs determine S. cerevisiae Rna1p subcellular distribution. *J. Cell Sci.* **112**, 339-347.

Ferrigno, P., Posas, F., Koepp, D., Saito, H. and Silver, P. A. (1998). Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. *EMBO J.* **17**, 5606-5614.

Finley, D., Bartel, B. and Varshavsky, A. (1989). The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* **338**, 394-401.

Fornerod, M., Ohno, M., Yoshida, M. and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-1060.

Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M. and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**, 308-311.

Gadal, O., Strauss, D., Kessl, J., Trumpower, B., Tollervey, D. and Hurt, E. (2001). Nuclear export of 60s ribosomal subunits depends on Xpo1p and requires a nuclear export sequence-containing factor, Nmd3p, that associates with the large subunit protein Rpl10p. *Mol. Cell. Biol.* **21**, 3405-3415.

Goldstein, A. L., Snay, C. A., Heath, C. V. and Cole, C. N. (1996). Pleiotropic nuclear defects associated with a conditional allele of the novel nucleoporin Rat9p/Nup85p. *Mol. Biol. Cell* **7**, 917-934.

Görlich, D. and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Dev. Biol.* **15**, 607-660.

Gorsch, L. C., Dockendorff, T. C. and Cole, C. N. (1995). A conditional allele of the novel repeat-containing yeast nucleoporin RAT7/NUP159 causes both rapid cessation of mRNA export and reversible clustering of nuclear pore complexes. *J. Cell Biol.* **129**, 939-955.

Heath, C. V., Copeland, C. S., Amberg, D. C., del Priore, V., Snyder, M. and Cole, C. N. (1995). Nuclear pore complex clustering and nuclear accumulation of poly(A)+ RNA associated with mutation of the Saccharomyces cerevisiae RAT2/NUP120 gene. *J. Cell Biol.* **131**, 1677-1697.

Ho, J. H. and Johnson, A. W. (1999). NMD3 encodes an essential cytoplasmic protein required for stable 60S ribosomal subunits in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **19**, 2389-2399.

Ho, J. H., Kallstrom, G. and Johnson, A. W. (2000a). Nascent 60S ribosomal subunits enter the free pool bound by Nmd3p. *RNA* **6**, 1625-1634.

Ho, J. H., Kallstrom, G. and Johnson, A. W. (2000b). Nmd3p is a Crm1p-dependent adapter protein for nuclear export of the large ribosomal subunit. *J. Cell Biol.* **151**, 1057-1066.

Hodge, C. A., Colot, H. V., Stafford, P. and Cole, C. N. (1999). Rat8p/Dbp5p is a shuttling transport factor that interacts with Rat7p/Nup159p and Gle1p and suppresses the mRNA export defect of xpo1-1 cells. *EMBO J.* **18**, 5778-5788.

Huber, J., Cronshagen, U., Kadokura, M., Marshallsay, C., Wada, T., Sekine, M. and Luhrmann, R. (1998). Snurportin1, an m3G-cap-specific nuclear import receptor with a novel domain structure. *EMBO J.* **17**, 4114-4126.

Hurt, E., Hannus, S., Schmelzl, B., Lau, D., Tollervey, D. and Simos, G. (1999). A novel in vivo assay reveals inhibition of ribosomal nuclear export in ran-cycle and nucleoporin mutants. *J. Cell Biol.* **144**, 389-401.

Hurwitz, M. E. and Blobel, G. (1995). NUP82 is an essential yeast nucleoporin required for poly(A)+ RNA export. *J. Cell Biol.* **130**, 1275-1281.

Jakel, S. and Görlich, D. (1998). Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. *EMBO J.* **17**, 4491-4502.

Koepp, D. M. and Silver, P. A. (1996). A GTPase controlling nuclear trafficking: running the right way or walking RANdomly? *Cell* **87**, 1-4.

Kressler, D., de la Cruz, J., Rojo, M. and Linder, P. (1997). Fal1p is an essential DEAD-box protein involved in 40S-ribosomal- subunit biogenesis in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **17**, 7283-7294.

Kressler, D., Linder, P. and de la Cruz, J. (1999). Protein trans-acting factors involved in ribosome biogenesis in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **19**, 7897-7912.

Larimer, F. W. and Stevens, A. (1990). Disruption of the gene XRN1, coding for a 5′----3′ exoribonuclease, restricts yeast cell growth. *Gene* **95**, 85-90.

Li, O., Heath, C. V., Amberg, D. C., Dockendorff, T. C., Copeland, C. S., Snyder, M. and Cole, C. N. (1995). Mutation or deletion of the Saccharomyces cerevisiae RAT3/NUP133 gene causes temperature-dependent nuclear accumulation of poly(A)+ RNA and constitutive clustering of nuclear pore complexes. *Mol. Biol. Cell* **6**, 401-417.

Lindsay, M. E., Holaska, J. M., Welch, K., Paschal, B. M. and Macara, I. G. (2001). Ran-binding protein 3 is a cofactor for Crm1-mediated nuclear protein export. *J. Cell Biol.* **153**, 1391-1402.

Maurer, P., Redd, M., Solsbacher, J., Bischoff, F. R., Greiner, M., Podtelejnikov, A. V., Mann, M., Stade, K., Weis, K. and Schlenstedt, G. (2001). The nuclear export receptor Xpo1p forms distinct complexes with NES transport substrates and the yeast Ran binding protein 1 (Yrb1p). *Mol. Biol. Cell* **12**, 539-549.

Milkereit, P., Gadal, O., Podtelejnikov, A., Trumtel, S., Gas, N., Petfalski, E., Tollervey, D., Mann, M., Hurt, E. and Tschochner, H. (2001). Maturation and intranuclear transport of pre-ribosomes requires Noc proteins. *Cell* **105**, 499-509.

Moy, T. I. and Silver, P. A. (1999). Nuclear export of the small ribosomal subunit requires the ran-GTPase cycle and certain nucleoporins. *Genes Dev.* **13**, 2118-2133.

Mueller, L., Cordes, V. C., Bischoff, F. R. and Ponstingl, H. (1998). Human RanBP3, a group of nuclear RanGTP binding proteins. *FEBS Lett.* **427**, 330-336.

Murphy, D. J., Hardy, S. and Engel, D. A. (1999). Human SWI-SNF component BRG1 represses transcription of the c-fos gene. *Mol. Cell. Biol.* **19**, 2724-2733.

Nehrbass, U., Kern, H., Mutvei, A., Horstmann, H., Marshallsay, B. and Hurt, E. C. (1990). NSP1: a yeast nuclear envelope protein localized at the nuclear pores exerts its essential function by its carboxy-terminal domain. *Cell* **61**, 979-989.

Neville, M. and Rosbash, M. (1999). The NES-Crm1p export pathway is not a major mRNA export route in Saccharomyces cerevisiae. *EMBO J.* **18**, 3746-3756.

Neville, M., Stutz, F., Lee, L., Davis, L. I. and Rosbash, M. (1997). The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. *Curr. Biol.* **7**, 767-775.

Noguchi, E., Hayashi, N., Nakashima, N. and Nishimoto, T. (1997). Yrb2p, a Nup2p-related yeast protein, has a functional overlap with Rna1p, a yeast Ran-GTPase-activating protein. *Mol. Cell. Biol.* **17**, 2235-2246.

Ohno, M., Segref, A., Bachi, A., Wilm, M. and Mattaj, I. W. (2000). PHAX, a mediator of U snRNA nuclear export whose activity is regulated by phosphorylation. *Cell* **101**, 187-198.

Paraskeva, E., Izaurralde, E., Bischoff, F. R., Huber, J., Kutay, U., Hartmann, E., Luhrmann, R. and Gorlich, D. (1999). CRM1-mediated recycling of snurportin 1 to the cytoplasm. *J. Cell Biol.* **145**, 255-264.

Ribbeck, K., Kutay, U., Paraskeva, E. and Gorlich, D. (1999). The translocation of transportin-cargo complexes through nuclear pores is independent of both Ran and energy. *Curr. Biol.* **9**, 47-50.

Rose, M. D., Novick, P., Thomas, J. H., Botstein, D. and Fink, G. R. (1987). A Saccharomyces cerevisiae genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* **60**, 237-243.

Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y. and Chait, B. T. (2000). The yeast nuclear pore complex: composition, architecture, and transport mechanism. *J. Cell Biol.* **148**, 635-651.

Schlenstedt, G., Saavedra, C., Loeb, J. D., Cole, C. N. and Silver, P. A. (1995). The GTP-bound form of the yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly(A)+ RNA in the cytoplasm. *Proc. Natl. Acad. Sci. USA* **92**, 225-229.

Stade, K., Ford, C. S., Guthrie, C. and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell* **90**, 1041-1050.

Stage-Zimmermann, T., Schmidt, U. and Silver, P. A. (2000). Factors affecting nuclear export of the 60S ribosomal subunit in vivo. *Mol. Biol. Cell* **11**, 3777-3789.

Stevens, A., Hsu, C. L., Isham, K. R. and Larimer, F. W. (1991). Fragments of the internal transcribed spacer 1 of pre-rRNA accumulate in Saccharomyces cerevisiae lacking 5′-3′ exoribonuclease 1. *J. Bacteriol.* **173**, 7024-7028.

Taura, T., Krebber, H. and Silver, P. A. (1998). A member of the Ran-binding protein family, Yrb2p, is involved in nuclear protein export. *Proc. Natl. Acad. Sci. USA* **95**, 7427-7432.

Taura, T., Schlenstedt, G. and Silver, P. A. (1997). Yrb2p is a nuclear protein that interacts with Prp20p, a yeast Rcc1 homologue. *J. Biol. Chem.* **272**, 31877-31884.

Trapman, J., Retel, J. and Planta, R. J. (1975). Ribosomal precursor particles from yeast. *Exp. Cell Res.* **90**, 95-104.

Udem, S. A. and Warner, J. R. (1973). The cytoplasmic maturation of a ribosomal precursor ribonucleic acid in yeast. *J. Biol. Chem.* **248**, 1412-1416.

Venema, J. and Tollervey, D. (1995). Processing of pre-ribosomal RNA in Saccharomyces cerevisiae. *Yeast* **11**, 1629-1650.

Verschoor, A., Warner, J. R., Srivastava, S., Grassucci, R. A. and Frank, J. (1998). Three-dimensional structure of the yeast ribosome. *Nucleic Acids Res.* **26**, 655-661.

Wente, S. R., Rout, M. P. and Blobel, G. (1992). A new family of yeast nuclear pore complex proteins. *J. Cell Biol.* **119**, 705-723.

Winston, F., Dollard, C. and Ricupero-Hovasse, S. L. (1995). Construction of a set of convenient Saccharomyces cerevisiae strains that are isogenic to S288C. *Yeast* **11**, 53-55.

Zabel, U., Doye, V., Tekotte, H., Wepf, R., Grandi, P. and Hurt, E. C. (1996). Nic96p is required for nuclear pore formation and functionally interacts with a novel nucleoporin, Nup188p. *J. Cell Biol.* **133**, 1141-1152.