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Yeast Shuttling SR Proteins Npl3p, Gbp2p, and Hrb1p Are Part of the Translating mRNPs, and Npl3p Can Function as a Translational Repressor

Merle Windgassen,[1]† Dorothée Sturm,[1]† Iván J. Cajigas,[2] Carlos I. González,[2] Matthias Seedorf,[3] Holger Bastians,[1] and Heike Krebber[1]*

Institut für Molekularbiologie und Tumorforschung der Philipps-Universität Marburg, Marburg,[1] and Zentrum für Molekulare Biologie der Universität in Heidelberg, Heidelberg,[3] Germany, and Department of Biology, University of Puerto Rico—Río Piedras, San Juan, Puerto Rico[2]

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A major challenge in current molecular biology is to understand how sequential steps in gene expression are coupled. Recently, much attention has been focused on the linkage of transcription, processing, and mRNA export. Here we describe the cytoplasmic rearrangement for shuttling mRNA binding proteins in *Saccharomyces cerevisiae* during translation. While the bulk of Hrp1p, Nab2p, or Mex67p is not associated with polysome containing mRNAs, significant amounts of the serine/arginine (SR)-type shuttling mRNA binding proteins Npl3p, Gbp2p, and Hrb1p remain associated with the mRNA-protein complex during translation. Interestingly, a prolonged association of Npl3p with polysome containing mRNAs results in translational defects, indicating that Npl3p can function as a negative translational regulator. Consistent with this idea, a mutation in *NPL3* that slows down translation suppresses growth defects caused by the presence of translation inhibitors or a mutation in eIF5A. Moreover, using sucrose density gradient analysis, we provide evidence that the import receptor Mtr10p, but not the SR protein kinase Sky1p, is involved in the timely regulated release of Npl3p from polysome-associated mRNAs. Together, these data shed light onto the transformation of an exporting to a translating mRNP.

In eukaryotic cells, mRNA biogenesis is a highly conserved process in which the messenger ribonucleoprotein (mRNP) complex composition changes constantly. The assembly starts cotranscriptionally and requires several processing steps until the export-competent mRNP is transported into the cytoplasm, where translation finally occurs (5, 9, 27, 41). During the whole process, mRNAs are covered by a multitude of proteins that associate and dissociate at given times during the course of the journey. Only some RNA binding proteins remain associated during export from the nucleus and are therefore termed shuttling mRNA binding proteins. At which step these proteins finally leave the mRNP is unclear.

In yeast, some of the shuttling RNA binding proteins are recruited early during transcription, whereas others join the mRNP at later steps of processing. In addition to Cbp80p and Cbp20p, which form the cap binding complex at the 5′ end of an mRNA (35, 46), and Pab1p (PABP in metazoans), which associates with the 3′ poly(A) tail (1, 54), several shuttling mRNA binding proteins are known to date: a cotranscriptional recruitment has been proposed for Dbp5p/Rat8p (DBP5 in metazoans), a DEAD-box helicase that has been suggested to unwind the mRNA during translocation through the nuclear pore complex (NPC) (7, 39, 44), and for Npl3p, an essential mRNA export factor that might have a function in proper packaging of the mRNP (23, 25). Npl3p has been suggested to be guided to the mRNA via RNA polymerase II (26). Gbp2p and Hrb1p were recently identified as novel shuttling RNA binding proteins with similarity to Npl3p (16, 48). Interestingly, in contrast to Npl3p, the recruitment of Gbp2p and Hrb1p to the mRNA in the nucleus is dependent on components of the THO complex involved in transcription elongation (16, 19). All three proteins contain RNA recognition motives and a serine/arginine (SR)-rich domain. The phosphorylation states of the SR domains of both Npl3p and Gbp2p are regulated by the cytoplasmic SR-specific protein kinase Sky1p (SRPK1 and SRPK2 in mammals), and phosphorylation of both SR proteins has been shown to influence their RNA binding activity and their cellular localization (11, 48, 52). In addition, the SR domains of both proteins are important for the nuclear reimport mediated by the import receptor Mtr10p (transportin SR [TRN-SR1 and TRN-SR2] in mammals) (34, 48).

mRNA biogenesis further requires the recruitment of the shuttling mRNA binding proteins Nab2p and Hrp1p. Nab2p is involved in 3′ processing events, and Hrp1p is the cleavage and polyadenylation factor cleavage factor IB (CF IB) that, upon arrival in the cytoplasm, is either replaced from the mRNA after an initial round of translation or, by detection of a premature termination codon, triggers the nonsense-mediated decay (13, 15, 17). Prior to export, mature mRNAs are subsequently recognized by Mex67p (NXF1/TAP in metazoans), which functions as an export receptor by mediating the interaction between the mRNP and the NPCs (31). Mex67p is recruited to the RNA via Npl3p and Yra1p (Aly in metazoans); however, it is presently unclear whether Yra1p accompanies the mRNA into the cytoplasm (10, 40).

Here, we present data describing the rearrangement of mRNP organization for shuttling mRNA binding proteins before and during translation. We show that significant amounts

* Corresponding author. Mailing address: Institut für Molekularbiologie und Tumorforschung (IMT) der Philipps-Universität Marburg, Emil-Mannkopff-Str. 2, 35037 Marburg, Germany. Phone: 49 6421 286 6773. Fax: 49 6421 286 5932. E-mail: krebber@imt.uni-marburg.de.
† M.W. and D.S. contributed equally to this work.

TABLE 1. Plasmids

Plasmid	Features	Source or reference
pHK13	P_{MTR10}:*MTR10* in Yep24 *URA3* 2μm	A. M. Tartakoff (Cleveland, Ohio)
pHK20	P_{MEX67}:*MEX67-GFP LEU2 CEN*	33
pHK33	P_{NOP1}:*GFP-MTR10 LEU2 CEN*	34
pHK45	P_{NPL3}:*NPL3 URA3 CEN*	26
pHK231	P_{GAL1}:*GFP URA3* 2μm	35
pHK258	P_{CBP80}:*CBP80-GFP URA3* 2μm	36
pHK356	P_{NAB2}:*NAB2-GFP URA3 CEN*	6
pHK358	P_{GAL1}:*NAB2-GFP LEU2 CEN*	6
pHK367	P_{GBP2}:*GBP2-GFP URA3 CEN*	48
pHK418	P_{NPL3}:*GFP-NPL3 LEU2 CEN*	11
pHK537	P_{HRB1}:*HRB1-GFP URA3 CEN*	16
pHK611	P_{ADH1}:*npl3-27 URA3 CEN*	This work
p4036	P_{PGK1}:*mini-PGK1 URA3* 2μm	13

of the SR proteins Npl3p, Gbp2p, and Hrb1p remain associated with the mRNP during early steps of translation elongation. Further, prolonged association of Npl3p with polysome containing mRNAs results in translational defects, suggesting that Npl3p can function as a negative translational regulator that needs to be unloaded in time for translation elongation. Finally, we present data indicating that Mtr10p, but not Sky1p, is involved in dissociating Npl3p from the mRNA in the cytoplasm, thus providing novel insights into the cytoplasmic mRNP rearrangements.

MATERIALS AND METHODS

Plasmids and yeast strains. All plasmids used in this study are listed in Table 1. The yeast strains are listed in Table 2. The *npl3-27* strain (HKY16) was backcrossed to the *sky1*::*TRP1* strain (HKY267) to create the *npl3-27 sky1*::*TRP1* strain (HKY379). The *npl3-27* strain (HKY16) was backcrossed three times to HKY36 to create the *npl3-27* strain (HKY351) and its isogenic wild type (HKY352).

Sucrose density gradient fractionation. Sucrose density gradient fractionation was done as described previously (8). Yeast cells were grown at 25°C in the appropriate medium to an optical density at 600 nm (OD_{600}) of 0.3 to 0.5. If indicated, a temperature shift to 37°C was done before cycloheximide was added to a final concentration of 100 μg/ml. Cultures were grown for another 15 min in the presence of cycloheximide prior to their harvest. Cell pellets were resuspended to a concentration corresponding to an OD_{600} of 7.5/100 μl in ice-cold low-salt buffer (20 mM HEPES-KOH, pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 1 mM ditiothreitol, 100 μg of cycloheximide/ml, 0.1 mM phenylmethylsulfonyl fluoride, complete protease inhibitor mix [prepared according to the manufacturer's instructions; Roche Molecular Biochemicals]). After addition of two cell volumes of glass beads, cells were lysed by vigorous shaking for 5 min at 4°C. Lysates were obtained by centrifugation for 20 min at 6,000 × *g*. One hundred thirty microliters of the supernatant (corresponding to an OD_{600} of 10) was loaded on a linear 12-ml 15 to 50% (wt/vol) sucrose gradient in low-salt buffer. Gradients were centrifuged at 40,000 rpm in a SW40 rotor (Beckman) at 4°C for 90 min and consequently fractionated into 600-μl fractions using an ISCO640 gradient fractionator, with continuous monitoring of absorbance at 254 nm. Each fraction was precipitated with 10% (wt/vol) trichloroacetic acid. The resulting pellet was washed with 80% (wt/vol) acetone, dried, and finally resuspended in 65 μl of sodium dodecyl sulfate (SDS) sample buffer. In the experiment where 1 mM puromycin–GTP was added, cycloheximide was omitted, and those samples as well as the control samples without puromycin were incubated for 15 min at 22°C prior to lysis. Samples were separated on SDS–9% polyacrylamide gels and analyzed by Western blotting using different antibodies. Quantification was done using image gauge version 3.1.

Immunofluorescence and GFP localization. Immunofluorescence and green fluorescent protein (GFP) localization were carried out as described previously (23, 48). For fluorescent microscopy, strains were grown to logarithmic phase (1×10^7 to 5×10^7 cells/ml) at 25°C and then shifted to 37°C for the indicated times. The GFP signal was analyzed directly by fluorescence microscopy, and pictures were taken from fixed cells. For this purpose, 5 ml of the cultures was mixed with 350 μl of 37% formaldehyde. Cells were collected immediately by centrifugation and washed once in 0.1 M K_2HPO_4–KH_2PO_4 at pH 6.5 and once in P solution (0.1 M K_2HPO_4–KH_2PO_4, pH 6.5, 1.2 M sorbitol).

In situ poly(A)$^+$ RNA hybridization. Localization of poly(A)$^+$ RNA by in situ hybridization was performed as described previously (23), with the following modifications. Instead of using a digoxigenin-labeled oligo$(dT)_{50}$ probe, a Cy3-end-labeled oligo$(dT)_{50}$ probe (0.2 pmol/μl) that made superfluous the use of the anti-digoxigenin antibody was used. Therefore, this step was skipped and the protocol was continued with DAPI (4′,6′-diamidino-2-phenylindole) staining.

Northern analysis and RT-PCR. Total RNA was extracted using peqGOLD RNAPure (Peqlab). The RNA was either used in reverse transcriptase PCR (RT-PCR) using the OneStep RT-PCR kit from QIAGEN or spotted onto a Hybond N$^+$ membrane (Amersham Pharmacia). The radiolabeled ^{32}P-poly(dT) probe was produced as described previously (26). Prehybridization and hybridization were performed in 0.5 M sodium phosphate (pH 7.5), 7% SDS, 1 mM EDTA, and 50 μg of salmon sperm DNA/ml for 4 h and 16 h at 55°C, respectively. The membrane was washed in 0.04 M sodium phosphate (pH 7.2)–0.1% SDS for 30 min at 55°C. The signal was quantified with a PhosphorImager. Full-length *GFP* and partial *NAB2* or *ACT1* were amplified via RT-PCR by adding the primers HK293 (5′ ATGGCTAGCAAAGGAGAAG 3′) and HK294 (5′ TTTGTATAGTTCATCCATG 3′) for *GFP*, HK267 (5′ GCGCCTGTCGA CAACA 3′) and HK268 (5′ CTCGAGTGCTTTTGCTAAC 3′) for *NAB2*, and HK300 (5′ CCCAAGATCGAAAATTTACTG 3′) and HK301 (5′ GGGTGTT CTTCTGGGGC 3′) for *ACT1*. The reverse transcriptase reaction was carried out with 100 ng of total RNA for 30 min at 50°C, followed by a 15-min denaturation at 95°C and standard PCR conditions, i.e., 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C for 40 cycles. For Fig. 6C, 1 μg of total RNA was used.

Nonsense-mediated decay. The experiments for the mRNA decay measurements were essentially done as described previously (13). DNA probes of a 1.3-kb BamHI/HindIII fragment from plasmid p4036 were used to monitor the decay of the *mini-PGK1*, and *PGK1* transcripts were labeled to high specific activity with [α-^{32}P]dCTP. The results of these experiments were quantitated by using a Bio-Rad model GS-800 Imaging Densitometer.

RESULTS

Significant amounts of all three yeast shuttling SR proteins are associated with mRNAs during translation. To get insight into the transformation of an exporting mRNP into a translating mRNP, we analyzed the ability of yeast shuttling mRNA binding proteins to cosediment with polysomes. To be able to compare all shuttling mRNA binding proteins in the same strain, we used a wild-type yeast strain carrying plasmids en-

TABLE 2. Yeast strains

Strain	Genotype	Source or reference
HKY16	*MAT***a** *ura3 leu2 his3 lys ade2 ade8 can1 npl3-27*	23
HKY35	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63*	49
HKY36	*MAT*α *ura3-52 leu2Δ1 his3Δ200*	49
HKY77	*MAT***a** *ura3 his3 trp1 rpb1-1*	26
HKY124	*MAT*α *ura3-52 leu2Δ1 his3Δ200 rat7-1*	14
HKY267	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 trp1-1 ade2-1 ade3 can1-100 sky1*::*TRP1*	38
HKY268	*MAT***a** *ura3-52 leu2-3,112 his3-11,15 trp1-1 ade2-1 ade3 can1-100*	38
HKY270	*MAT*α *ura3 leu2 his3 trp1 ade2 mtr10*::*HIS3* + (p*mtr10-7 TRP1 CEN*)	34
HKY271	*MAT*α *ura3 leu2 his3 trp1 ade2 mtr10*::*HIS3* + (p*MTR10 URA3 CEN*)	34
HKY280	*MAT***a** *ura3-52 leu2 trp1 pep4-3 pre1-407 prb1-1122 PAB1-GFP*::*KAN*	53
HKY351	*MAT*α *ura3 leu2 his3 trp1 ade npl3-27*	This study
HKY352	*MAT*α *ura3 leu2 his3 trp1*	This study
HKY405	*MAT***a** *ura3-52 tyr1 his3-200 ade2-101 tif51A* (*ts1159*)	55
KC2*upf*Δ	*MAT***a** *ura3-52 trp1 leu2-2 tyr7-1 his4-38 met14 upf1*Δ	47

coding functionally tagged versions of the analyzed proteins (Table 1 and 2). It should be noted that this slight overexpression might have an influence on the behavior of the proteins; however, comparison of endogenous Npl3p to GFP-Npl3p expressed from a *CEN* plasmid in addition to the wild-type copy resulted in a nearly identical protein distribution on the polysome gradients (data not shown). Therefore, we analyzed endogenous Npl3p as an additional internal control in all experiments. Log-phase cells were lysed, and extracts were fractionated by sucrose density centrifugation to separate ribosomes from ribosomal subunits and unbound proteins. The rRNA profiles, measured at OD_{254}, and the distribution of the small subunit protein Rps3p indicate the position of ribosomal subunits, monosomes, and polysomes (Fig. 1A). Western blot analysis of all fractions revealed that significant amounts of the SR proteins Npl3p, Gbp2p, and Hrb1p, as well as Pab1p and to a lesser extent Mex67p, were detectable in polysome-containing fractions. In contrast, almost all of Nab2p, Hrp1p, Mtr10p, and Cbp80p were detected in ribosome-free fractions. The amounts of the proteins in the ribosome-free fractions were compared to those determined in the ribosome-bound fractions. The ratios from typical experiments (Fig. 1A) and a statistical analysis of three independent experiments (Fig. 1B) are shown. The slight upshift of the proteins in the first fractions is a result of the high protein concentration in the samples, because the shift vanished when less was loaded onto the gel (data not shown). However, to be able to compare all lanes of the Western blot, equal amounts of all fractions were loaded.

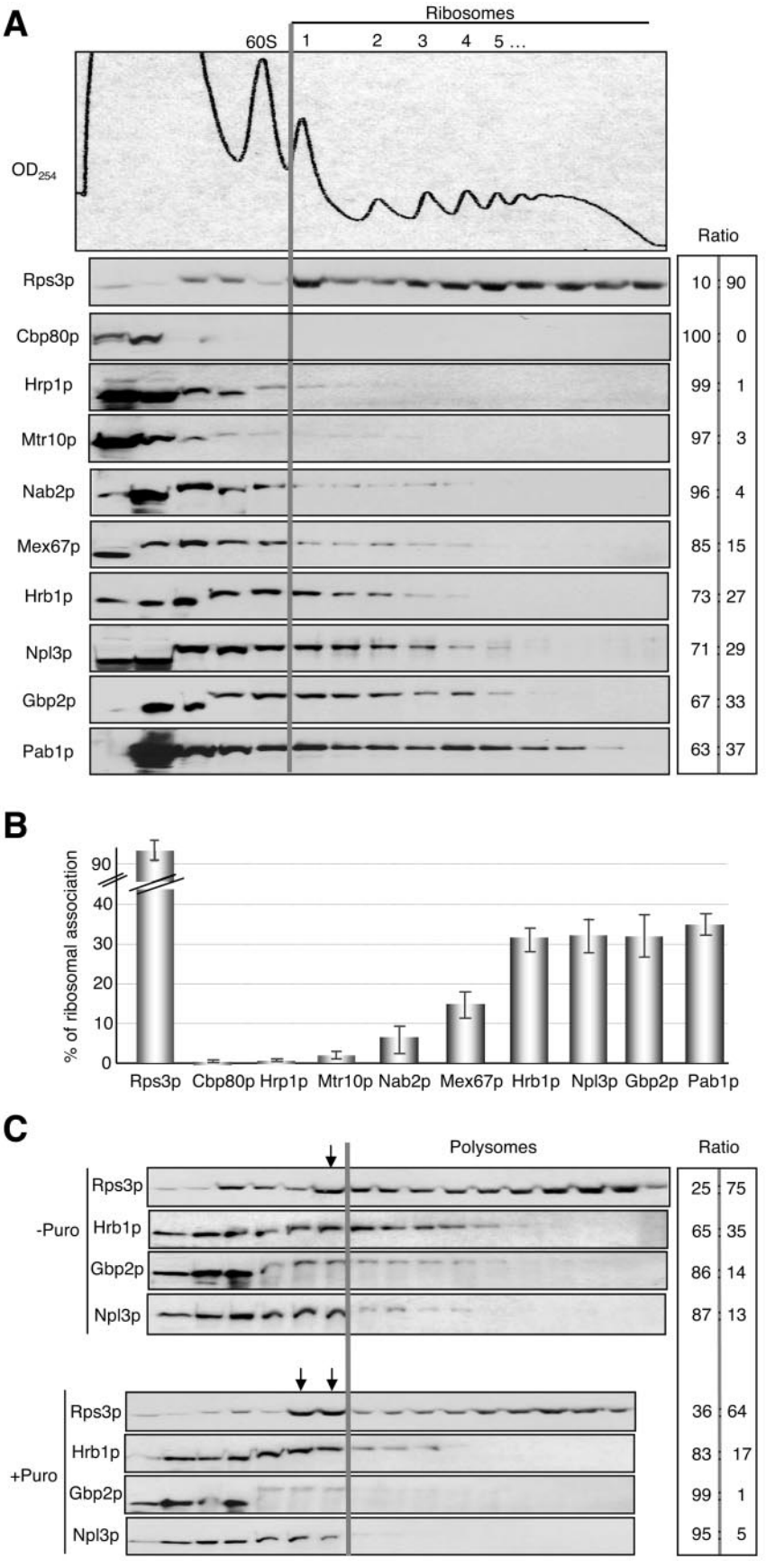
To confirm that Npl3p, Gbp2p, and Hrb1p cosediment with polysomes and do not represent other high-molecular-weight RNPs, we used the antibiotic puromycin to specifically disrupt polysomes (3). Lysates of wild-type cells expressing *GBP2-GFP* or *HRB1-GFP* were treated with puromycin-GTP for 15 min at 22°C or not treated, followed by separation in density gradient centrifugations and Western blotting (Fig. 1C). Due to the incubation time, the polysome association of the proteins was already slightly decreased in the no-puromycin control experiment compared to the results shown in Fig. 1A. However, upon addition of puromycin, significant amounts of Rps3p, Npl3p, Gbp2p, and Hrb1p were removed from the polysomal fractions, verifying their cosedimentation with polysomes. Extended incubations with puromycin resulted in further removal of Rps3p from the polysomes (data not shown); however, they also led to increasing protein degradation of the SR proteins. Since the monosomes remain intact after puromycin treatment, ratios were now calculated from the polysome fractions compared to the fractions containing the unbound proteins and the monosomes. Together, these experiments revealed that significant amounts of the three SR proteins in *Saccharomyces cerevisiae*, Npl3p, Gbp2p, and Hrb1p, are part of the translating mRNPs.

Mtr10p, but not Sky1p, is involved in dissociation of Npl3p from mRNAs during translation. Mutations in both Sky1p and Mtr10p have been shown to result in an increased association of Npl3p to poly(A)$^+$ RNA (11). Interestingly, although both mutant strains mislocalize Npl3p (11, 34), the extent of the cytoplasmic mislocalization is more pronounced in *mtr10* than in *sky1* mutant strains (Fig. 2A). To address whether in both cases the retention at polysomes causes this increased mRNA association of Npl3p, we repeated the polysome cosedimentation experiments with *sky1* and *mtr10* mutant strains. Strikingly, while deletion of *SKY1* did not influence the sedimentation behavior of Npl3p (Fig. 2B), the protein was enriched in the polysomal fractions of *mtr10-7* cells (Fig. 3A). This result indicates that Mtr10p but not Sky1p is required for efficient cytoplasmic release of Npl3p from polysomes. Longer temperature shifts of *mtr10-7* cells to the nonpermissive temperature result in mRNA export defects; however, after 30 min, no mRNA export defect is detectable (Fig. 3B). Therefore, the extended polysomal association of Npl3p in *mtr10-7* cells might not be a secondary effect of the mRNA export defect.

The shift of Npl3p to the high-molecular-weight fractions in the sucrose density gradients might be caused by an impaired dissociation of this protein from the mRNA. To test whether this in turn could impair the translation of mRNAs that are not released from Mtr10p import substrates, the expression of an inducible reporter gene (GFP) was investigated in *mtr10-7* cells. As shown in Fig. 3C, compared to wild type, only a very low level of GFP can be detected in the *mtr10* mutant, indicating a defect in gene expression, while no mRNA export defect is detectable. Comparison of the reporter mRNA level in *mtr10-7* cells with that in wild-type cells revealed that the GFP mRNA is slightly less well transcribed in the mutant, which could indicate that the reduction in protein expression might be generated in part by a transcriptional defect or vice versa (data not shown). Together, these data indicate that Mtr10p, besides being an import receptor for the SR proteins, might also regulate their dissociation from translating mRNAs. However, the increased association of Npl3p with polysomes might also be an artifact of its cytoplasmic mislocalization. To investigate whether the cytoplasmic relocalization of an SR protein per se results in an increased polysome association, we analyzed the comigration behavior of Gbp2p in the *sky1* deletion strain, because in contrast to Npl3p, this protein is cytoplasmic at steady state in the *sky1*Δ strain (Fig. 4A) (48). As shown in Fig. 4B, the absence of Sky1p does not influence the association of Gbp2p with polysomes, indicating that an elevation of the cytoplasmic protein level does not lead to an increased polysomal association of this SR protein.

Together, these data indicate the following things: (i) the dissociation of the SR proteins Npl3p and Gbp2p is independent of Sky1p, (ii) dissociation of Npl3p from polysomes is dependent on Mtr10p, and (iii) gene expression is defective in *mtr10-7* cells.

Release from translating polysomes is defective in the *npl3-27* strain. Since Mtr10p is the import receptor of several proteins, it is difficult to further dissect the defects of this mutant. Therefore, we took advantage of an *NPL3* strain mutant, the *npl3-27* mutant, that has a slowed nuclear import rate (23). The observed phenotype of this mutant is very similar to that of wild-type Npl3p in *mtr10* mutants (Fig. 2A). Further, the cytoplasmic accumulation of npl3-27p is suppressed by overexpression of *MTR10* (Fig. 2A), and although the import rate is slowed for npl3-27p, the protein is still capable of shuttling between the nucleus and the cytoplasm, as it accumulates within minutes after a temperature shift in the nucleus of temperature-sensitive strains defective in the export of mRNA (reference 23 and data not shown). Further, npl3-27p shows an increased binding to poly(A)$^+$ RNA (48). Most important,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99 : 1
95 : 5

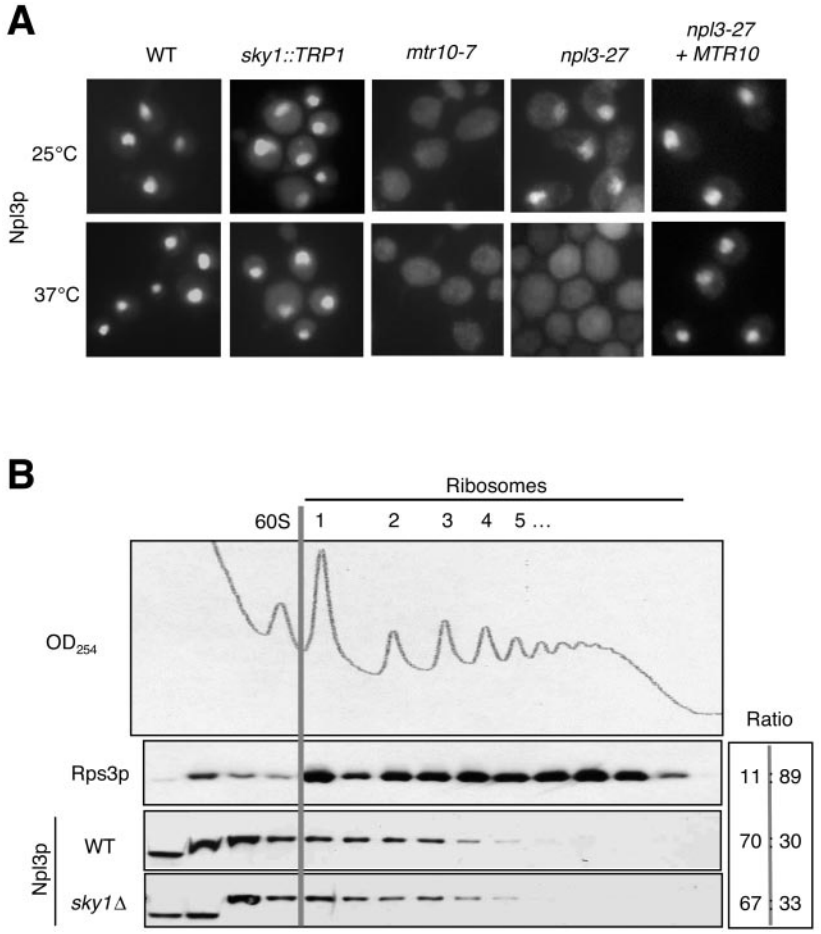


FIG. 2. Deletion of *SKY1* has no influence on the dissociation of Npl3p from polysome-associated mRNAs. (A) Mislocalization of Npl3p is similar in *mtr10-7* and *npl3-27* strains and less pronounced in *SKY1* deletion strains. Wild-type *sky1*::*TRP1* and *mtr10-7* cells expressing *NPL3-GFP* and *npl3-27* carrying either an empty vector or 2μm *MTR10* were grown in uracil-free medium to log phase. Half of the cultures were retained at 25°C, and the other half were shifted to 37°C for 30 min before they were fixed and analyzed either directly by fluorescence microscopy or indirectly by immunofluorescence using anti-Npl3p antibodies. (B) The absence of Sky1p has no influence on the amount of Npl3p associated with polysome containing mRNAs. The *sky1*::*TRP1* strain and its isogenic wild-type strain were grown in YPD medium to log phase before extracts were fractionated through 15 to 50% sucrose gradients and subjected to Western blot analysis with anti-Npl3p and anti-Rps3p antibodies. Quantification of the amount of both proteins in nonribosomal fractions (left of the gray line) and in the ribosome-associated fractions (right of the gray line) is shown on the right. The experiment shown represents a typical result from three independent experiments.

however, the *npl3-27* mutant shows no mRNA export defects (23) (Fig. 5A).

npl3-27p is defective in the Mtr10p-induced release from translating mRNAs. Sucrose density fractionation experiments comparing the ribosomal association of the protein in wild-type and *npl3-27* strains and *npl3-27* cells overexpressing *MTR10* were carried out. As shown in Fig. 5B, a significant shift of npl3-27p into the polysome fractions was detected (~65% of total Npl3p in *npl3-27* cells versus ~35% in the wild type). Interestingly, the increased association was reversed by simultaneous overexpression of *MTR10* (Fig. 5B), indicating that Mtr10p is responsible for the dissociation of Npl3p and

FIG. 1. Significant amounts of SR-type shuttling mRNA binding proteins associate with polysomes. (A) Extracts of wild-type strains expressing either *Cbp80-GFP*, *GFP-MTR10*, *NAB2-GFP*, *MEX67-GFP*, *GBP2-GFP*, *HRB1-GFP*, or *PAB1-GFP* were fractionated through 15 to 50% sucrose gradients and subjected to Western blot analysis. Absorbance at 254 nm shows the distribution of ribosomes (top). The corresponding fractions (bottom) were separated on SDS–9% polyacrylamide gels, and the proteins of interest were detected by Western blotting using either anti-GFP, anti-Hrp1p, anti-Npl3p, or anti-Rps3p antibodies. Quantification of the amount of all proteins tested in nonribosomal fractions (left of the gray line) and in the ribosomal fractions (right of the gray line) is shown on the right. (B) Quantification of three independent experiments shown in panel A. (C) Rapid sedimentation of Npl3p, Gbp2p, and Hrb1p requires polysome integrity. Extracts of wild-type cells expressing *GBP2-GFP* or *HRB1-GFP* were either mock treated (top) or treated with puromycin (bottom) for 15 min at 22°C to disrupt polysomes prior to sucrose gradient sedimentation. Because puromycin activity requires progression through an elongation cycle, the elongation inhibitor cycloheximide was omitted, and GTP was added. The arrows indicate the monosome fractions that are increased in puromycin-treated cells (two arrows) because of the disruption of the polysomes. Quantification of the protein amounts in the polysome fractions (right of the gray line) is indicated on the right.

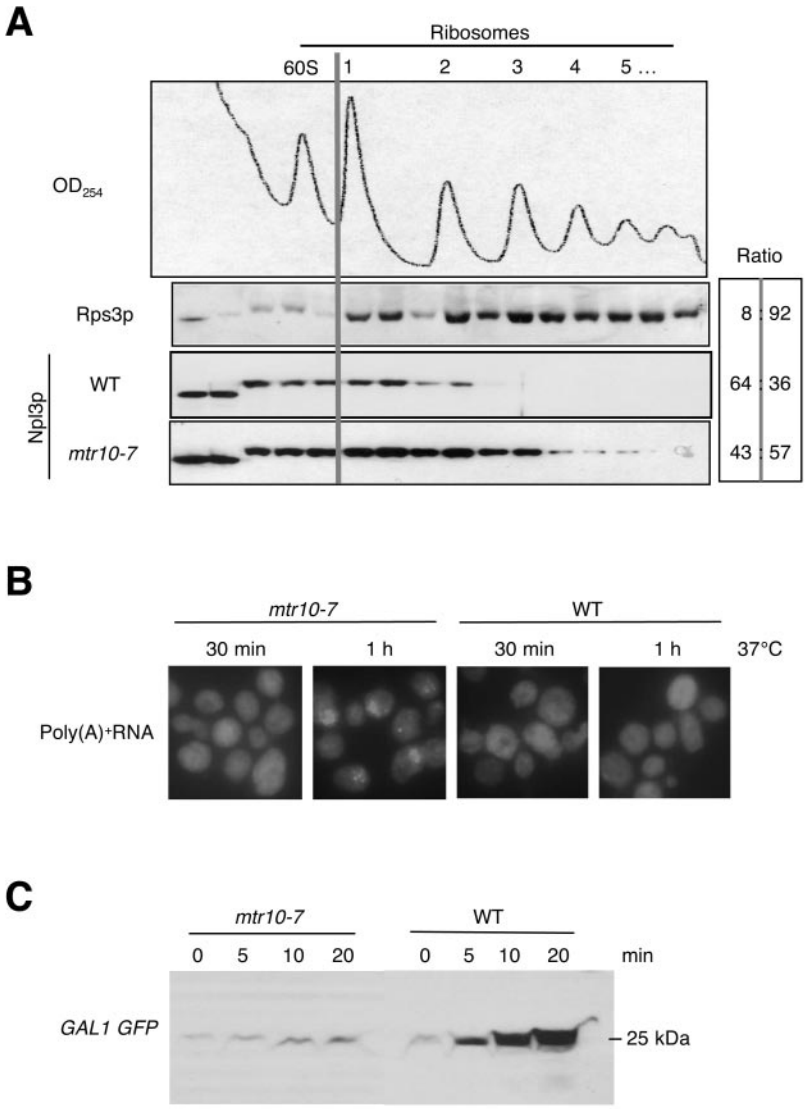


FIG. 3. Mtr10p is involved in the dissociation of Npl3p from polysome-associated mRNAs. (A) The *mtr10-7* strain is defective in the dissociation of Npl3p from the polysome containing mRNA. The *mtr10-7* strain and its isogenic wild-type strain were grown in YPD medium to log phase and shifted to 37°C for 30 min before extracts were fractionated through 15 to 50% sucrose gradients and subjected to Western blot analysis with anti-Npl3p and anti-Rps3p antibodies. Quantification of the amount of both proteins in nonribosomal fractions (left of the gray line) and in the ribosome-associated fractions (right of the gray line) is shown on the right. The experiment shown represents a typical result from three independent experiments. (B) The *mtr10-7* strain has no mRNA export defect after a 30-min temperature shift. Samples of log-phase cells were shifted to 37°C for 30 min or 1 h before samples were analyzed by in situ hybridization experiments with Cy3-labeled oligo$(dT)_{50}$ probes. (C) New synthesis of a reporter protein is significantly reduced in the *mtr10-7* strain. Wild-type and *mtr10-7* cells carrying a galactose inducible GFP reporter plasmid were grown in raffinose-containing medium to log phase at 25°C. Cells were then shifted to 37°C for 15 min before galactose was added to induce the production of the reporter gene. Equal samples with OD_{600}s of 1.0 were lysed after 0, 5, 10, and 20 min of induction and analyzed by Western blotting using anti-GFP antibodies.

possibly also the other shuttling SR-type proteins from the mRNPs, as it acts as the import receptor for this type of protein (16, 34, 48).

Translation is impaired in the *npl3-27* strain. The increased association of Npl3p with translating mRNAs in the *mtr10* or the *npl3* mutant strains suggests a possible linkage between this SR protein and translation. If so, one might expect that a slowed dissociation of Npl3p impairs translation because displacement could be a prerequisite for translation elongation. A first hint to this hypothesis was the observed gene expression defect of *mtr10-7* cells (Fig. 3C); however, since mutations in *MTR10* cause pleiotropic defects, we monitored the translation efficiency in *npl3-27* cells by expression of a galactose inducible reporter gene. As shown in Fig. 6A, wild-type cells accumulate the reporter protein significantly faster and reach higher expression levels than *npl3-27* cells at similar mRNA levels (Fig. 6B) and similar rates of reporter mRNA degradation after promoter shutdown by the addition of glucose (Fig. 6C). This result demonstrates an impaired reporter-RNA translation in *npl3-27* cells. Moreover, the overall protein content is significantly reduced in the *npl3-27* strain, as shown in Fig. 6D.

To exclude the possibility that the translational defect seen

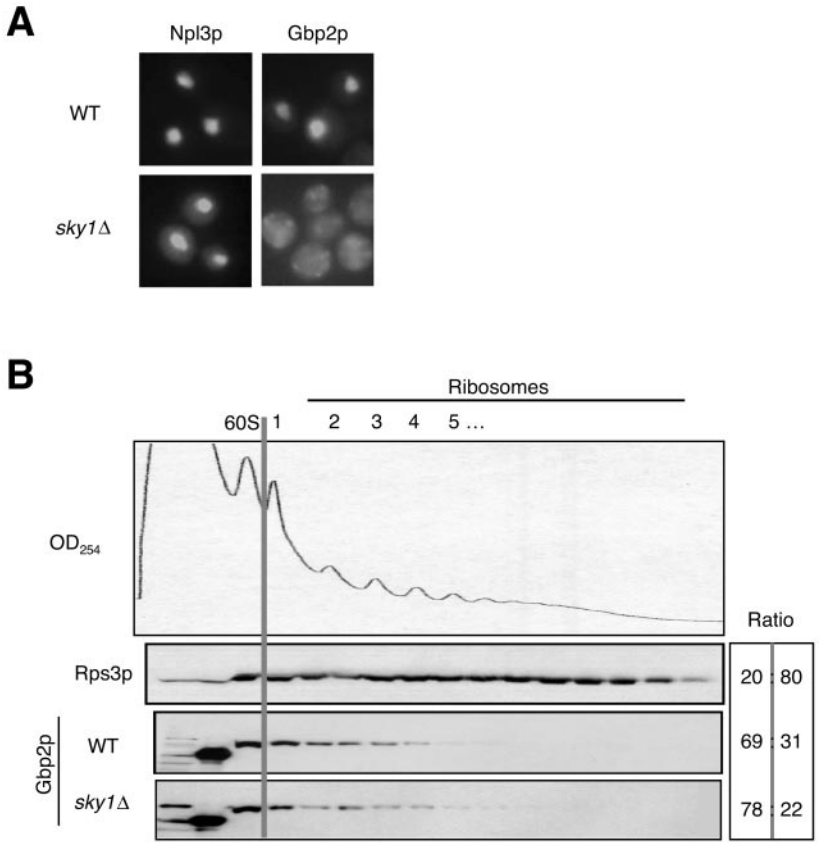


FIG. 4. The cytoplasmic mislocalization of Gbp2p in *sky1*Δ cells does not lead to an increased association of the protein with polysomes. (A) Wild-type and *sky1*::*TRP1* cells expressing *NPL3-GFP* and *GBP2-GFP* were grown in URA-free medium to log phase at 25°C before they were fixed and analyzed by fluorescence microscopy. (B) The absence of Sky1p has no influence on the amount of Gbp2p associated with polysome containing mRNAs. The *sky1*::*TRP1* strain and its isogenic wild-type strain carrying *GBP2-GFP* were grown in URA-free medium to log phase before extracts were fractionated through 15 to 50% sucrose gradients and subjected to Western blot analysis with anti-GFP and anti-Rps3p antibodies. Quantification of the amount of both proteins in nonribosomal fractions (left of the gray line) and in the ribosome-associated fractions (right of the gray line) is shown on the right. The experiment shown represents a typical result from three independent experiments.

in the *npl3-27* strain is a result of upstream events, we tested this strain for general defects in transcription, ribosome biogenesis, and nonsense-mediated mRNA decay. While the poly(A)$^+$ RNA level is reduced by ~80% in a mutant strain of the RNA polymerase II, the *rpb1-1* mutant, the mRNA level of the *npl3-27* strain matched that of the wild type (Fig. 7A), indicating that *npl3-27* has no effect on transcription. Further, the 18S and 25S rRNA levels in both strains were found to be very similar at both temperatures (Fig. 7B), and since no additional 27S rRNA band can be detected, which would be the case for defects in the processing of the 5.8S rRNA precursor (28), no obvious rRNA processing defects are detectable in the *npl3-27* strain. Further, the *npl3-27* strain does not accumulate nonsense-containing mRNAs (Fig. 7C), in contrast to *upf1* mutants defective in nonsense-mediated decay (13). Finally, mRNA export defects are not observed in the *npl3-27* strain (Fig. 5A), suggesting that translational defects are responsible for the impaired protein expression in the *npl3-27* strain.

Since high-copy-number Mtr10p rescues both the cytoplasmic localization of npl3-27p (Fig. 2A) and its increased association with polysomes (Fig. 5B), one would expect that overexpression of Mtr10p would also abolish the translational defects observed in the *npl3-27* strain. Therefore, *npl3-27* and wild-type cells were analyzed for the expression of a reporter gene (*Nab2-GFP*). As shown in Fig. 6E, we found the translational defect of the *npl3-27* strain to be substantially rescued by overexpression of *MTR10*.

Together, these data indicate that translation is slowed down in the *npl3-27* strain by inefficient dissociation of npl3-27p from translating mRNAs. A slowed translational activity should be advantageous for a cell when it has to cope with situations in which translation is inaccurate or defective. Therefore, one would expect that *npl3-27* strains could have growth advantages in situations where translation is impaired, for example in media containing translational inhibitors. Thus, growth of the *npl3-27* strain and its isogenic wild-type strain were tested on plates containing cycloheximide and paromomycin, two translation elongation inhibitors that inhibit translation by binding to the large and the small ribosomal subunit, respectively. While both strains show similar growth rates on yeast extract-peptone-dextrose (YPD) plates, the growth rate of the wild type is significantly reduced on plates containing the translation inhibitors, whose effects are suppressed by the presence of *npl3-27* (Fig. 8A). In expectance of the *npl3-27* strain to be a

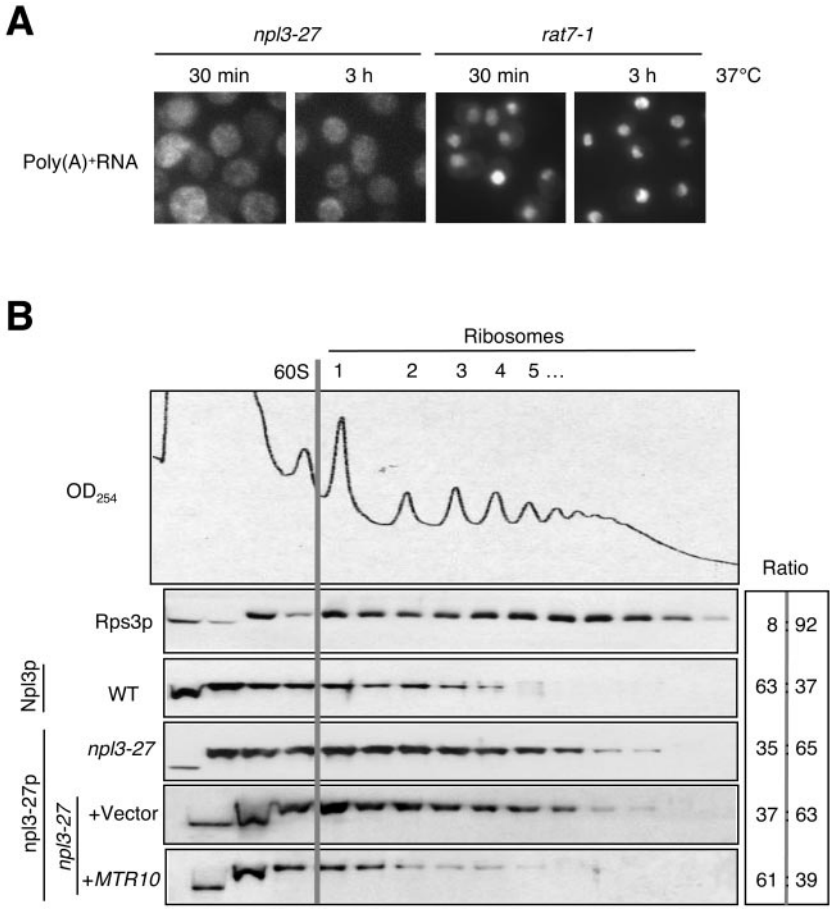


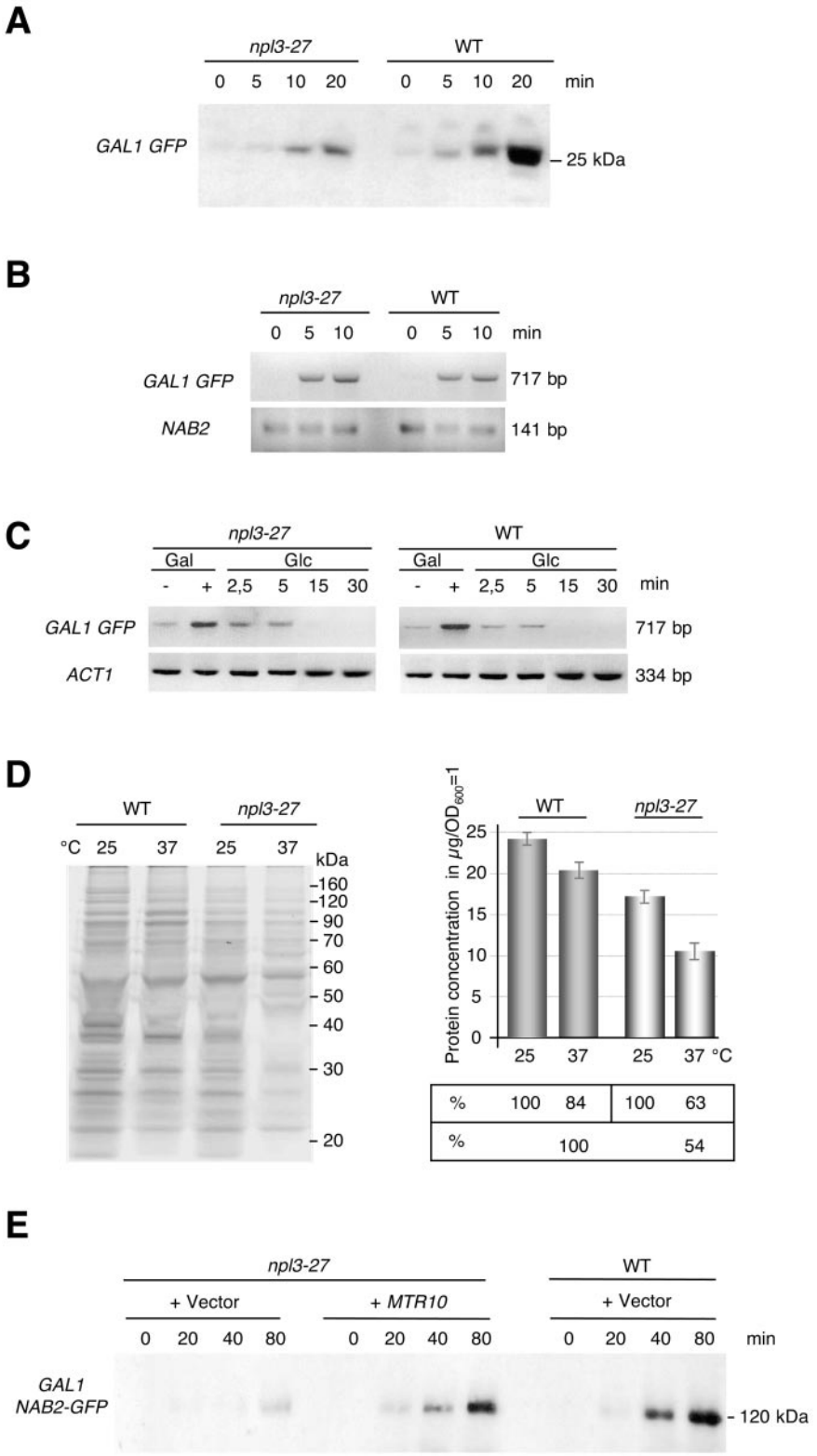
FIG. 5. The *npl3-27* mutant has no mRNA export defect, but npl3-27p is defective in dissociation from polysome-associated mRNAs. (A) The *npl3-27* mutant has no mRNA export defect. The *rat7-1* mutant, encoding a defective nucleoporin required for mRNA export, and the *npl3-27* mutant were grown to log phase in YPD medium and then shifted to 37°C for 30 min and 3 h. Cells were fixed and subjected to in situ hybridization experiments using a Cy3-labeled oligo$(dT)_{50}$ probe. (B) npl3-27p is enriched in the polysome fractions, and this effect is abolished by high-copy-number *MTR10*. The *npl3-27* strain and its isogenic wild-type strain were grown in YPD medium, and *npl3-27* cells carrying either an empty vector or *MTR10* on a 2μm *URA3* plasmid were grown in URA-free medium to log phase and shifted to 37°C for 1 h before extracts were fractionated through 15 to 50% sucrose gradients and subjected to Western blot analysis with anti-Npl3p and anti-Rps3p antibodies. Quantification of the amount of both proteins in nonribosomal fractions (left of the gray line) and in the ribosome-associated fractions (right of the gray line) is shown on the right.

dominant mutant, because its defect in being inefficiently released from mRNA should not be altered by the presence of the wild-type protein, we expressed large amounts of *npl3-27* from a plasmid driven by the strong *ADH1* promoter in a wild-type strain and spotted serial dilutions onto cycloheximide-containing YPD plates. As shown in Fig. 8B, high-copy-number *npl3-27* was indeed able to suppress the growth defects caused by the drug. Interestingly, it was also capable of suppressing the growth defects of a temperature-sensitive mutant of *tif51A* encoding the eukaryotic initiation factor 5A (eIF5A) (Fig. 8B). These findings indicate the capability of *npl3-27* to suppress the inhibition of translation caused by addition of translational inhibitors and a mutation in eIF5A, further supporting the idea that Npl3p could function as a negative translational regulator.

DISCUSSION

In this study, we present a comprehensive analysis of the cytoplasmic mRNP rearrangement for shuttling mRNA binding proteins in yeast and unravel striking differences in their behavior in leaving the mRNA after nuclear export. Consistent

FIG. 6. The *npl3-27* strain exhibits defects in translation. (A) New synthesis of a reporter protein is significantly reduced in the *npl3-27* strain. Wild-type and *npl3-27* cells containing a galactose-inducible GFP reporter plasmid were grown in raffinose-containing media to log phase at 25°C. Cells were then shifted to 37°C for 30 min before galactose was added to induce the production of the reporter that was analyzed by Western blotting (A) and RT-PCR (B) after the indicated times. (C) RNA degradation of the reporter is not inhibited in *npl3-27* cells. Wild-type and *npl3-27* cells containing a galactose (Gal) inducible GFP reporter plasmid were grown in raffinose-containing media to log phase at 25°C. Cells were then shifted to 37°C for 30 min (−Gal lane) before galactose was added for 5 min to induce the production of the reporter (+Gal lane). The synthesis was subsequently blocked by the addition of glucose (Glc), and further samples were collected and subjected to RT-PCR analysis after the indicated times. (D) *npl3-27* cells contain less total protein. Wild-type and *npl3-27* cells were grown to log phase, split into two equal portions, and



either retained at 25°C or shifted to 37°C for 3 h. Equal samples with OD_{600}s of 0.6 were lysed in SDS sample buffer, and proteins were separated on an SDS–12% polyacrylamide gel that was stained with Coomassie blue (left panel). Total protein amounts of both strains were calculated from three independent experiments and are shown in a graph (right panel). (E) Overexpression of *MTR10* suppresses the reporter gene expression defects in the *npl3-27* strain. Wild-type and *npl3-27* cells carrying a galactose inducible *NAB2-GFP* reporter plasmid and 2μm *MTR10* or an empty vector were grown in raffinose-containing medium to log phase at 25°C. Cells were then shifted to 37°C for 30 min before galactose was added to induce the production of the reporter gene. All samples (in panels A, B, and C) have an OD_{600} of 1.0 for Western blot analyses using anti-GFP antibodies. For the RT-PCR, primers that amplify GFP and endogenous *NAB2* or *ACT1* were used.

with earlier studies which have shown that cap binding complex is replaced from the mRNA by the eIF4F complex prior to cap-dependent initiation of translation (12), we do not find Cbp80p associated with the mRNA during translation. Further, it has been reported that the functional association of the 3′ end of an mRNA with its 5′ end, mediated through the interaction of the poly(A) tail-binding protein Pab1p with the eIF4F complex, promotes translation (42, 43). Thus, Pab1p is found in the polysome fractions of sucrose gradients (Fig. 1) (18). For the initial round of translation, Hrp1p has been suggested to remain on the RNA to detect defective mRNAs and trigger their degradation, referred to as nonsense-mediated decay (13). Consistently, we do not find Hrp1p to be part of the polysomes, whereas 35 to 40% of Pab1p is associated with ribosomes.

In contrast to cap binding complex, Hrp1p, and Pab1p, much less is known about the cytoplasmic proceedings of other yeast shuttling mRNA binding proteins. Interestingly, we find different dissociation behaviors for them. While the bulk of Nab2p dissociates from the mRNP prior to translation, small amounts of Mex67p remain associated with the mRNP during translation. Consistent with this finding, the mammalian homologue of Mex67p, TAP, has been reported to promote the translation of unspliced mRNAs (20). Most strikingly, however, significant amounts (~30%) of the SR-type proteins Npl3p, Gbp2p, and Hrb1p remain bound to the mRNA during translation elongation. This sets this group of proteins apart from the other shuttling mRNA binding proteins, of which only a small percentage, if any, comigrates with polysomes. Thus, in sucrose density gradient experiments the behavior of the three SR proteins is highly similar to that of Pab1p, which is known to play a role in translation. In fact, we demonstrate that translation is affected by Npl3p in situations where it persists on the RNA, as shown with a mutation of its import receptor *MTR10* or a mutation in *NPL3* itself (the *npl3-27* strain). It is tempting to speculate that a timely regulated dissociation of Npl3p could provide a mechanism by which the cell might tune its translation activity. We have obtained additional evidence for such a model by the fact that the *npl3-27* strain suppresses the growth defects caused by the presence of translational inhibitors. In these situations, the slowed translation might ease off the situation and lead to the generation of correct translation products. Further, we show that mutations in *TIF51A* encoding eIF5A are suppressed by the presence of *npl3-27*. eIF5A was initially identified as a candidate translation factor associated with polysomes (2, 22). However, subsequent studies revealed that the protein might rather play a role in mRNA turnover, as mutations in the gene lead to an accumulation of short-lived and uncapped mRNAs (45, 55). Nevertheless, its depletion in yeast results in an approximately 30% decrease in protein synthesis (21, 55) and an increase of G_1-arrested cells, which raised the hypothesis that eIF5A might have a role in the translation of a specific subset of mRNAs (21, 29, 51). eIF5A localization is largely cytoplasmic in puromycin-sensitive structures that might represent rough endoplasmic reticulum-bound polysomes (37), and eIF5A interacts with the ribosomal protein L5 (32), further supporting a possible function of eIF5A in translation. An influence of *npl3-27* on eIF5A is conceivable for translation and mRNA decay, although the presence of npl3-27p does not stabilize the GFP-reporter mRNA (Fig. 6C), which might rather point to a suppression of a translational defect in the *tif51A* mutant.

Both the *npl3-27* and *mtr10-7* mutants show similar phenotypes for Npl3p (cytoplasmic mislocalization and extended binding to polysome containing mRNAs). However, the *npl3-27* strain is able to survive these defects at higher temperatures, while the *mtr10-7* strain does not (Fig. 8) (34). This result is possibly due to the fact that cells can tolerate the dissociation defect of one Mtr10p substrate from mRNA and survive with less total protein, while impaired dissociation of several Mtr10p substrates might simply be too much to take.

In analogy to our findings with the yeast SR proteins, the mammalian shuttling SR protein SF2/ASF has recently been shown to associate with polysomes (30). In contrast, only small amounts of a second mammalian shuttling SR protein, SRp20, were detected in the ribosomal fractions but not in the polysomal fractions (30). Interestingly, our studies reveal a very similar association profile for all three shuttling SR proteins in yeast. Further, SF2/ASF has been shown to stimulate the translation of a reporter protein when tethered to the reporter or when overexpressed (30). It seems to be in contrast with our model that tethering of this protein to the reporter stimulates translation in higher eukaryotes, since, as we show here, a prolonged association of the yeast SR protein causes the opposite. One possible explanation for this apparent contradiction could be that the SR proteins not only tune translation by their timely regulated dissociation but also by recruiting certain translation initiation factors. In any case, this type of protein modulates translation, and it will be interesting to identify interacting partners that help to unravel this process further.

Previous studies of Npl3p revealed that during heat shock or other stress situations the protein dissociates from the mRNAs (23). A model was proposed in which Npl3p is involved in packaging the mRNAs into export-competent mRNPs. This dissociation was suggested to inhibit mRNP export and in this way participate in keeping the NPCs and—as a downstream effect—the ribosomes accessible for the transport and translation of heat shock RNAs necessary for cell survival. Our findings presented here suggest an involvement of Npl3p in passing the mRNAs on to the ribosomes. In stress situations it seems efficient for a cell to impact all steps in the life cycle of an mRNA efficiently, and Npl3p seems to be predestined to be such a trigger protein, since it persists on the RNA from transcription to translation. It will be interesting to further investigate this idea, as manipulation of the dissociation of Npl3p from mRNA might represent a general regulatory step in translation.

Earlier work has established that members of the importin transport receptor family bind their import cargoes only in the cytoplasm, dependent on the RanGDP/GTP cycle. The separation of the two pools of Ran (nuclear RanGTP and cytoplasmic RanGDP) is thought to act as a molecular switch in the binding and release steps of the importin transport receptor family (also termed transportins or karyopherins) and their cargoes upon translocation through the NPC (9). The nuclear dissociation of Npl3p from Mtr10p (an importin family member) in the presence of RanGTP has been shown to be significantly enhanced in the presence of RNA (34). Similarly, a combination of RanGTP and RNA acts cooperatively to dissociate both Nab2p and Hrp1p from their transport receptor,

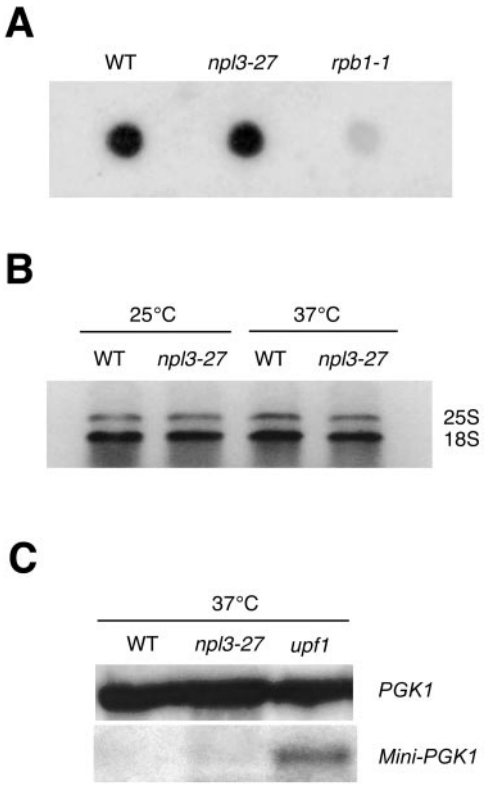


FIG. 7. The *npl3-27* strain has no obvious defects in transcription, rRNA synthesis, and nonsense-mediated decay. (A) The *npl3-27* strain has no transcriptional defect. Northern blot hybridization of total poly(A)$^+$ RNA of wild-type, *npl3-27*, and *rpb1-1* cells. Cells were grown to log phase at 25°C before they were shifted to 37°C for 30 min. Equal samples with OD_{600}s of 2.5 were lysed, and total RNA was isolated, spotted onto a nylon membrane, and hybridized with a ^{32}P-labeled poly(dT) probe. (B) The *npl3-27* strain has no obvious defects in rRNA synthesis. Cells were grown to log phase at 25°C before they were shifted to 37°C for 30 min. Equal samples with OD_{600}s of 8.0 were lysed, and total RNA was isolated and separated on a 1% agarose gel. (C) The *npl3-27* strain is not impaired in nonsense-mediated mRNA decay. Wild-type, *npl3-27*, and *upf1* strains expressing a nonsense-containing *mini-PGK1* gene were grown to log phase and shifted to 37°C for 30 min. Total cellular RNA was isolated, and the amounts of the endogenous *PGK1* mRNA (loading control) and the accumulation of the *mini-PGK1* mRNA were determined by Northern blot analysis.

Kap104p, in the nucleus (24), indicating that the shuttling mRNA binding proteins are directly passed on to the mRNA in the nucleus. Conversely, the cytoplasmic displacement of Nab2p and Hrp1p from the exported mRNA has been proposed to be promoted solely by Kap104p, as both proteins can be dissociated from single-stranded DNA-cellulose specifically by the addition of Kap104p in vitro (24). Here, we provide in vivo evidence for the Mtr10p-induced dissociation of Npl3p from mRNA in the cytoplasm. Consistently, we do not find Mtr10p to be present in the polysome containing mRNAs (Fig. 1A). It is interesting that the cytoplasmic release of the bulk of Hrp1p, Nab2p, and Npl3p from the mRNA is not accomplished at the same time. This result could simply reflect their accessibilities upon refolding of the mRNP for translation.

Despite their continuous shuttling, the SR proteins Npl3p, Gbp2p, and Hrb1p are nuclear at steady state. However, these proteins accumulate in the cytoplasm in import receptor Mtr10p mutants and in SR-specific protein kinase Sky1p mutants (11, 16, 48, 52). Further, in both mutants an increased association of Npl3p and Gbp2p to poly(A)$^+$ RNA has been demonstrated in UV-cross-linking experiments. From these results a model has been proposed in which Sky1p and Mtr10p both contribute to the release of the SR proteins from mRNAs (11, 48). Glc7p has been identified as the antagonistic nuclear phosphatase to Sky1p that dephosphorylates Npl3p. Dephosphorylated Npl3p associates with the export receptor Mex67p for mRNP export (10). However, this model still leaves some open questions. It seems puzzling why the deletion of *sky1* does not lead to mRNA export defects but a mutation in *glc7* results in nuclear mRNA accumulation. Further, the *glc7* mutation

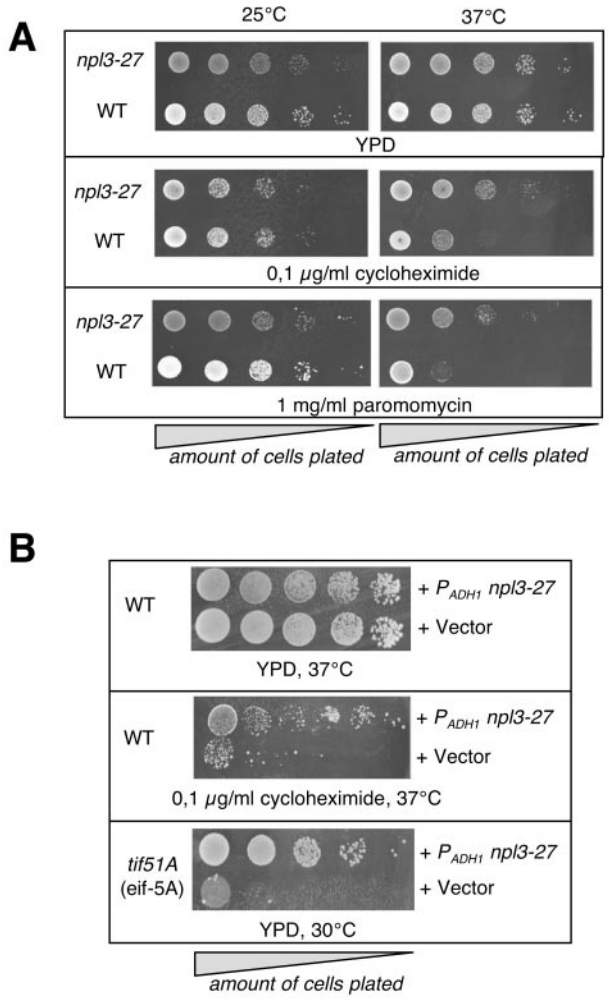


FIG. 8. npl3-27p suppresses growth defects caused by the presence of translation elongation inhibitors or by a mutation in eIF5A. (A) Ten-microliter yeast suspensions with similar cell numbers were spotted in 10-fold serial dilutions onto YPD medium and YPD medium containing 0.1 μg of cycloheximide/ml or 1 mg of paromomycin/ml. The *npl3-27* strain was spotted in the top lanes, and its isogenic wild type was spotted in the bottom lanes. (B) Growth of serial dilutions of wild-type and *tif51A* mutant cells is shown on YPD plates containing cycloheximide where indicated. The strains were carrying either an empty vector (bottom lanes) or a plasmid containing *npl3-27* under the strong *ADH1* promoter (top lanes). All plates were incubated for 3 to 5 days at the indicated temperatures.

cannot be suppressed by preventing phosphorylation of Npl3p (10). Also, mutations in both *sky1* and *mtr10* lead to similar increases in mRNA binding (11, 48) but different extents of cytoplasmic mislocalization for Npl3p. Finally, *npl3-27* has been reported to be synthetically lethal with *mtr10*Δ (23), whereas the combination of *npl3-27* with *sky1*Δ is viable (our unpublished observation). Here, we have presented data showing that mutations in *SKY1* do not have an influence on the polysome association of Npl3p, whereas mutations in *MTR10* do. These novel data suggest that Npl3p is dissociated from actively translated mRNAs by the action of Mtr10p and independent of Sky1p.

The role of Sky1p and the phenotypes observed for *sky1* mutant strains might be explained if one assumes that Npl3p and possibly also Gbp2p and Hrb1p are phosphorylated after their Mtr10p-induced dissociation from mRNA in the cytoplasm, for example to prevent an unspecific association of the shuttling SR proteins to any nuclear mRNA. It has been shown previously that phosphorylation of SR domains reduces the nonspecific binding of the otherwise highly positively charged proteins to RNA (50). Thus, the cytoplasmic accumulation of Npl3p in the *sky1*Δ strain could simply result from a faster return of the protein into the cytoplasm by nonspecific association with mRNAs already on their way out. Consequently, most of the increased mRNA binding of Npl3p in the *sky1*Δ strain, detectable in UV-cross-linking experiments, would be nuclear. This could further explain why less cross-linking product in the *sky1*Δ strain can be detected for Gbp2p than for Npl3p (48), since its cytoplasmic mislocalization is more pronounced. Consistent with this, synthetic lethal interactions for the *sky1*Δ strain have been described recently for *PRP8* and *PRP17*, both of which are involved in a rather nuclear event: splice site selection during pre-mRNA splicing (4). Further, it has been reported that binding of phosphorylated Npl3p to Mtr10p is increased (11), which could be beneficial for nuclear import. This increased binding might apply for the other shuttling SR proteins too, especially for Gbp2p, but it cannot be excluded that unphosphorylated Gbp2p might be exported more rapidly.

An alternative model for the role of the phosphorylation cycle of the shuttling SR proteins, which would not be mutually exclusive with the idea that this modification could alter the RNA binding specificity, could be envisioned for an interaction with Mex67p. It has been suggested that dephosphorylated Npl3p recruits Mex67p to the mRNA prior to export. Conversely, it seems possible that the cytoplasmic phosphorylation of the SR proteins could support the displacement of the export receptor Mex67p after the dissociation of the complex from the RNA. Alternatively, phosphorylation of Npl3p could simply prevent an early complex formation with the export receptor upon arrival in the nucleus prior to its recruitment to the RNA.

Together, our work presented here broadens the view of the cellular journey of an mRNP by doing the following: (i) presenting a comprehensive analysis of the cytoplasmic remodeling of yeast shuttling mRNA binding proteins prior to and during translation; (ii) identifying significant amounts of all three shuttling SR proteins, Npl3, Gbp2p, and Hrb1p, in association with polysomes; (iii) providing mechanistic insights into the dissociation of Npl3p from translating mRNAs; and (iv) linking mRNA synthesis, maturation, and export to translation, as the belated dissociation of Npl3p represses translational activity. It will be interesting to investigate further these final cytoplasmic steps of the shuttling mRNA binding proteins.

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