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Cosmin Saveanu[1], Jean-Claude Rousselle[2], Pascal Lenormand[2], Abdelkader Namane[2], Alain Jacquier[1], and Micheline Fromont-Racine[1]*

[1] Unité de Génétique des Interactions Macromoléculaires, CNRS-URA2171, and [2] Plate-Forme Protéomique, Institut Pasteur, 75724 Paris Cedex 15, France

* Corresponding author. Mailing address: Génétique des Interactions Macromoléculaires, Institut Pasteur, 25 rue du docteur Roux, 75724 Paris Cedex 15, France; Phone: +33 140613432; Fax: +33 14568790; E-mail: mfromont@pasteur.fr

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Abstract

Ribosome biogenesis is driven by a large number of pre-ribosomal factors that associate to and dissociate from the pre-ribosomal particles along the maturation pathway. We have previously shown that budding yeast Mak11, whose homologues in other eukaryotes were described as modulating a p21-activated protein kinase function, accumulates in Rlp24 associated pre-60S complexes when their maturation is impeded in *S. cerevisiae*. The functional inactivation of Mak11 interfered with the 60S ribosomal RNA maturation, led to a cell cycle delay in G1 and blocked Rpl25-GFP in the nucleoli of yeast cells, indicating an early role of Mak11 in ribosome assembly. Surprisingly, Mak11 inactivation also led to a dramatic destabilization of Rlp24. The suppression of the thermosensitive phenotype of a *mak11* mutant by *RLP24* overexpression and a direct *in vitro* interaction between Rlp24 and Mak11 suggest that Mak11 acts as an Rlp24 co-factor during early steps of 60S ribosomal subunit assembly. Moreover, we found that Skb15, the Mak11 homologue in *S. pombe*, also associated with pre-ribosomes and affected 60S biogenesis in fission yeast. It is thus likely that the previously observed phenotypes for *MAK11* homologues in other eukaryotes are secondary to the main function of these proteins in ribosome formation.

Introduction

Eukaryotic ribosome biogenesis begins in the nucleolus with the association of ribosomal and pre-ribosomal proteins to a nascent rRNA precursor transcribed by the RNA Polymerase I. This precursor consists, in yeast, of the sequences for mature 18S, 5.8S and 25S ribosomal RNAs, separated by two ITS (Internal Transcribed Spacers) and flanked by additional 5' end and 3' end sequences. The rRNA precursor, as a large ribonucleoprotein particle, is next matured in well-ordered processing steps. Many auxiliary factors are required all along the pathway but except for a few enzymatic functions, the molecular roles of these factors remain unclear. A cleavage in the region separating the precursor of the 18S rRNA and the 5.8S and 25S rRNA leads to the generation of pre-60S and pre-40S intermediate particles. The third RNA component of the large ribosomal subunit, the 5S rRNA, associates in a precursor form with the pre-60S particles and both pre-60S and pre-40S particles are further processed and exported to the cytoplasm, where final maturation events take place and generate the ribosomal subunits (56, 58).

Studies with budding yeast allowed the identification and functional characterization of a surprisingly large number of proteins and RNAs that participate in the maturation of eukaryotic ribosomes. Since 2001, generic purification methods such as TAP, in association with developments in mass spectrometry, allowed the identification of about 200 preribosomal factors (15, 16, 20, 29). In contrast to the ribosomal proteins, which associate to the pre-particles and remain associated to the mature ribosomes, the pre-ribosomal factors associate transiently to the precursors (for reviews see 12, 55). When ribosome biogenesis is blocked, the changes in purified pre-ribosomal complexes composition may indicate the order of protein association, dissociation or subcomplex formation during the pathway (see for example 19, 35, 47). Such experiments can

1 also establish the requirement of a given factor for subsequent association of other proteins to the
2 precursors.

3 We identified Mak11 in complexes purified in association with the pre-60S essential
4 protein Rlp24 and have shown that the amount of co-purified Mak11 starkly increased when
5 another essential pre-60S factor, the Nog1 GTPase, was depleted (47). Several known pre-60S
6 factors (Nop7, Tif6, Erb1, Nop2) were identified in association with Mak11 in a large-scale
7 experiment (20). Recent purification of a large number of macromolecular complexes in yeast
8 further confirmed the presence of Mak11 in predicted pre-60S complexes (15, 29). Moreover, the
9 Mak11 fusion with GFP was shown to localize to the nucleus and concentrated in the nucleolus
10 of yeast cells (22).

11 MAK11's name (MAintenance of Killer) comes from its identification in genetic screens
12 looking for mutations that would affect the maintenance of the M1 toxin-encoding dsRNA,
13 satellite of the L-A dsRNA yeast virus (54, 61, 62). The RNAs used for the synthesis of the viral
14 proteins have neither a typical 5' cap nor a poly(A)-tail (60). Many of the isolated *mak* mutants
15 showed polysome profiles typical for 60S ribosomal subunit biogenesis impairment (40). It is
16 likely that the identification of 60S ribosomal subunit biogenesis factors among the *mak* genes
17 was due to the fact that normal 60S ribosomal subunit levels, in contrast to 40S subunit levels, are
18 required for the translation of uncapped and non-polyadenylated RNAs like those encoding viral
19 proteins (45, 50). Several mutations discovered during the *mak* screens affected genes like *mak7*,
20 *mak8* or *mak18*, later identified to be coding for ribosomal proteins (Rpl8a, Rpl3 and Rpl42b
21 respectively) (40). Other mutations affected genes coding for proteins recently shown to be
22 physically associated with pre-60S particles by large-scale complex purification studies (15, 28).
23 Recently, the requirements of Mak21/Noc1(10, 36), Mak5 (64) and Mak16 (41) for 60S

ribosomal subunit formation were described. One of the mutations described in the genetic screen and having effects on 60S levels affected *MAK11*, a gene shown to be essential for viability and suspected to be required for 60S subunit biogenesis (40).

Based on the available data, we predicted Mak11 to be an essential factor involved in nuclear maturation of 60S ribosomal subunits. Puzzlingly, while Mak11 putative orthologues exist in many eukaryotes, both the *S. pombe* homologue Skb15 (26, 27) and the human homologue hPip1 (63) have been previously described as direct binders and inhibitors of p21 activated protein kinases (PAK), Shk1/Pak1 in fission yeast and Pak1 in *H. sapiens*. PAKs link different receptors with modification of protein substrates by phosphorylation in the mitogen activated protein-kinase (MAPK) pathway and are directly activated by small GTPases of the Rho or Rac families (for a review see 21). PAKs activation or inhibition affect major cellular pathways since PAK substrates play important roles in cell polarity and morphology, mitotic exit and cytokinesis and mediate cellular responses to external stimuli. These signaling pathways are highly conserved as demonstrated by the heterologous complementation of the absence of yeast Ste20 by its human homologue Pak1 (8).

It was surprising that Mak11 homologues were described as modulators of PAK activity affecting central signaling pathways in other eukaryotes, while physical association and functional data strongly suggested that Mak11 is a novel factor involved in 60S ribosomal subunit formation in *S. cerevisiae*. There were two explanations for this discrepancy: either Mak11 was not the homologue of Skb15 and hPip1 or the previously observed effects were secondary to the function of these factors in ribosome biogenesis. We thus tried to understand the role of Mak11 in ribosome biogenesis in *S. cerevisiae* and tested the putative ribosome biogenesis function for *S. pombe* Skb15.

We show here that the essential function of Mak11 is linked to an early, nucleolar step of 60S ribosomal subunit biogenesis, and we propose that it serves as an Rlp24 cofactor during the assembly of early pre-60S particles in *S. cerevisiae*. The ribosome biogenesis function of Mak11 was conserved during evolution since we could show that Skb15 is required for 60S formation in *S. pombe*. We conclude that previously observed effects of Mak11 homologues are likely to be secondary to the primary effects of these factors on ribosome biogenesis.

Materials and methods

Strains and plasmids.

The strains used in this study are listed in Table 1. Chromosomal insertions were obtained by homologous recombinaison using PCR fragments (1).

pCM190-RLP24 was obtained by subcloning the RLP24 sequence from the plasmid pGEX4-T-RLP24 (47) using *BamH*I and *Not*I into pCM190 (14). The *MAK11* open reading frame was cloned after PCR amplification in the pDONR 201 vector using the Gateway system (Invitrogen). The resulting entry clone was used to generate the pTG189-*MAK11* plasmid. pTG189, a Gateway compatible TAP vector was obtained from pCM189 (14) by cloning the N-terminal TAP cassette (43) downstream of the tetracyclin regulated promoter and upstream a RfA Gateway cassette. A Gateway compatible vector derived from pET32a (Novagen), a gift from E. Bertrand (IGM, Montpellier, France), was used to obtain a vector allowing $(His)_6$-Mak11 expression in bacteria.

Nmt1-Skb15 and SP870 *S. pombe* strains were obtained from S. Marcus (University of Alabama, USA). The Skb15-TAP strain was obtained by homologous recombination in the Pb185 strain (gift from B. Arcangioli, Institut Pasteur, Paris, France) using a method adapted from (57). Long recombination arms were generated by two successive PCR reactions from genomic DNA using oligonucleotides: A-BamHI ACG GGA TCC AGG CAA ATC TGT CTA CCC TGT TG; B-XmaI TGA AAA GGA CGA AGC ATG CCC CCGGG; C-SalI GGT CGA CCT TAA TAG GGA AAG GAC GGG and D-BamHI TGC TAG ATG AGC TAT TTG CCA CGG GAT CCA GG. The product of the second PCR reaction, was cloned in pCRBlunt (Invitrogen) and next subcloned in the pFA6a-CTAP-MX6 vector (52) using *Sal*I and *Xma*I restriction enzyme sites. The resulting plasmid was linearized by digestion with *BamH*I and used

to transform strain Pb185. Clones resistant to G418 were tested by immunoblotting for the presence of the fusion protein.

Tandem Affinity Purification and in vitro binding assay

Complex purifications were performed as described in (44) with a few modifications, starting with 4 liters of yeast culture. Buffers contained 0.1M NaCl. Eluted proteins were precipitated with methanol/chloroform, separated on a 5-20% polyacrylamide gradient-SDS gel and identified either by immunoblot or mass spectrometry. Mass spectrometry protein identification was done using MALDI-TOF as described (32). For RNA associated determination, only the first step of purification was performed, in presence of vanadyl ribonucleoside complexes as RNAse inhibitor. The associated RNAs were extracted twice with phenol/chloroform. To investigate the association of different proteins with purified complexes, TEV eluates were separated on denaturing polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad) and probed with rabbit polyclonal antibodies against Nog2 (46), Nog1 and Rlp24 (47), Nsa2 (30) Arx1 (31) and Mak11 (this work) used at a 1:5 000 dilution. The peroxidase activity of secondary antibodies was detected using either the ECL+ (GE Healthcare) or the Immobilon Western (Millipore) chemiluminescence kit.

In vitro binding was tested as previously described (47) using plasmids expressing GST-tagged Rlp24, Rpl3 and Rpl5 (controls) and $(His)_6$-Mak11. The presence of Mak11 in the eluate was estimated by immunoblot with anti-Mak11 antibodies.

Sucrose gradient sedimentation

Polysomal extracts were obtained using glass beads vortexing. Polysomes were separated on a 10-50% sucrose gradient and centrifuged at 39 000 rpm for 2h. 45 min. at 4°C in a SW41-Ti

rotor. Fractions were recovered with an ISCO fractionator and the 254 nm absorbance was measured. For protein identification by immunoblot, the proteins from each fractions were precipitated with 10% TCA and separated on polyacrylamide gels. TAP-tagged proteins were revealed with a 1:10 000 dilution of peroxidase-antiperoxidase complex (PAP, Sigma).

RNA extraction, Northern blot and primer extension

RNA extractions were performed using glass beads and phenol/chloroform. Large size rRNAs were denatured with glyoxal and separated on 1% agarose gels and small size rRNAs were separated on 5% acrylamide-urea denaturing gels. Northern blots and primer extensions were performed using ^{32}P-labelled oligonucleotides. The sequence of the oligonucleotides specific of *S.cerevisiae* were previously described (46). Those related to *S.pombe* were based on the detected pre-rRNA intermediates previously described (18) and were the following: CS151, TGT CGG AAA GCA TAG CAA GC, for U2 snRNA, used as a control; CS148, AAC AAA TTT TCG TTC AAC ACC TCA TC, used to detect 27S and 7S intermediates, CS153, CGT TAA GGT TCA AAT ATA AAA GAG specific for the 27S intermediates.

Screen for mak11 *ts alleles*

For the selection of thermosensitive *MAK11* alleles, we used mutagenic PCR combined with gap repair (37). A region of genomic *S. cerevisiae* DNA encompassing the *MAK11* ORF and 243 nucleotides upstream the ATG sequence as well as 95 nucleotides downstream the stop codon was amplified by PCR using oligonucleotides: GCT CTA GAA GAC ATT TTT CTA GCT ACA TAA; AGG CGC GCC ATC ATC TTT AAC GAT TAA GATA. The resulting PCR product was cloned in the centromeric vector pFL38 (URA3 marker) using *Xba*I and *Asc*I sites and verified by sequencing. The obtained pFL38-Mak11 plasmid was able to fully complement *MAK11* deletion and the resulting haploid strain was further used as LMA260. A PCR-based

strategy was used for random mutagenesis of the *MAK11* sequence. PCR reactions were performed with oligonucleotides AGA TGC GTA AGG AGA AAA TAC CGC ATC and CGA CTG GAA AGC GGG CAG TGA using pFL38-Mak11 as template. In one case the ratio of dATP to other nucleotides was 1:5 and we used 4 mM $MgCl_2$ combined with 0.5 mM $MnCl_2$ and in another case the ratio of dATP to other nucleotides was 1:10 and the reaction mixture contained 10% DMSO. The strain LMA260 was transformed with PCR products and pFL36CII linearized by digestion with *Xba*I and *Hind*III. A total of 16 000 clones were obtained with around 5 000 of these being able to grow at 25°C on a selective medium containing 5FOA. After replica plating on selective medium we obtained clones that were able to grow at 25°C but not at 35°C. Plasmids were extracted from nine candidates, and tested by retransformation. The strongest *ts* phenotype was observed for the candidate *mak11-2*, further used in this study. Sequencing of the *mak11-2* allele showed several predicted amino acid changes depicted in Figure 7.

High copy number suppressor genetic screen

The *mak11-2 ts* mutant strain (LMA263-2) was transformed with a yeast genomic high copy library (generated in the pFL44L 2µ URA3 vector) a gift from F. Lacroute, CGM, Gif-sur-Yvette, France. The transformants were selected on minimal medium without uracil and leucine. After 24 h at 25°C the plates containing the transformants were incubated at 37°C and the clones growing at this restrictive temperature were selected. Plasmids were recovered and used to transform the LMA263-2 strain. The growth phenotype was compared with the growth phenotype of LMA264 transformed with an empty vector. After verification of the suppressor phenotype, the DNA inserts were amplified and the ends were sequenced. The complementation of the *mak11-2* ts phenotype was verified using the pCM190-RLP24 vector.

Fluorescence microscopy

Cells transformed with a centromeric plasmid expressing RPL25-eGFP or cells expressing chromosomal TAP-tagged fusion proteins were cultured in minimal medium. The protein A part of the TAP tag was detected with anti-protein A antibodies and Cy3 secondary antibodies (42). Observation of the cells by epifluorescence was done as described (31).

FACS analysis

To analyze the cell cycle distribution of yeast cells population we used exponentially growing cells in rich medium at 27°C or shifted for up to 6 hours at 37°C. Aliquots of 2 ml were retrieved and fixed with 70% cold ethanol for 1 hour at room temperature. RNA was digested with RNAse A (1 mg/ml in Tris HCl 0.2 M pH 7.5, EDTA 20 mM for 1 h. at 37°C) and DNA was stained with 50 µg/ml propidium iodide in 0.1 ml PBS overnight at 4°C. The cell suspension was diluted to 1 ml with PBS before analysis. Flow cytometry analysis was done using a FACS Calibur (BD Biosciences). One hundred thousand events were used to estimate the ratio between 1n and 2n DNA containing cells.

Rlp24-TAP half-life estimation

For the analysis of Rlp24-TAP half-life we used a procedure derived from (2). Addition of cycloheximide was done at the same time with a shift of the cultures from 27°C to 37°C. Immunoblotting was performed using peroxidase-anti-peroxidase complexes (Sigma) and the Immobilon Western (Millipore) chemiluminescence kit. Images were obtained with a cooled digital camera (GeneGnome, Syngene) and quantitated using ImageJ (version 1.38a, Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, [http://rsb.info.nih.gov/ij/]).

Results

Mak11 is required for the maturation of 27SB pre-ribosomal RNA in the nucleolus

MAK11 is essential in yeast (24) and a mutant allele of *MAK11* leads to free 60S ribosomal subunits decrease and formation of halfmer polysomes (40). To investigate the involvement of Mak11 in ribosome biogenesis, and specifically in 60S ribosomal subunit formation, we tested the relative amounts of different mature and precursors rRNA in yeast cells depleted for Mak11. We used a strain deleted for the chromosomal copy of *MAK11* where the expression of an N-terminal TAP fusion of *MAK11* was placed under the control of a tetracycline repressible promoter on a centromeric plasmid. In the absence of the repressor, the growth of the cells was comparable to the growth of a wild-type strain. Cell aliquots were collected at different time points after doxycyclin addition and total RNA was extracted and tested by primer extension or Northern blot with specific oligonucleotides (Fig. 1). The 25S mature rRNA decreased whereas the 18S was only slightly affected (Fig. 1B), in agreement with the previously observed decrease of free 60S subunit levels. The only pre-ribosomal RNA species that showed a relative accumulation when Mak11 was depleted was the 27SB precursor and, as observed for other 60S mutants, the 35S precursor (Fig. 1B-D). The 27SB did not accumulate when compared with U2 or U5 snRNAs but were increased when compared with the 27SA2 precursor. The apparent increase in U2 and U5 snRNA levels was due to the relative decrease of ribosomal RNA in the total RNA samples that were analyzed.

A decrease in the amounts of 7S pre-rRNA as compared with earlier 27S intermediates could be detected after 6 hours of Mak11 depletion, in correlation with the rapid decrease in the amount of Mak11 as judged by immunoblotting with antibodies binding to the N-terminal protein

tag (not shown). The aberrant 23S rRNA processing intermediate, generated by cleavage at position A3 before A2, was detected at 10h of depletion and strongly accumulated at later time points (21 hours of shift to glucose) when 7S became undetectable. This aberrant cleavage was accompanied by a decrease of the 20S pre-rRNA levels (Fig. 1B).

To perform additional functional studies, we searched for variants of Mak11 that, while supporting growth in yeast cells, were responsible for a thermosensitive phenotype. By mutagenic PCR coupled with plasmid gap repair, we obtained several alleles of *mak11* that complement the deletion of the gene at 25°C but support only very slow growth at 37°C. The *mak11-2* strain began to grow noticeably slower than a wild type strain at 4 hours after a shift of temperature from 25 to 37°C (Fig. 2A). The observed growth defect was associated with an increase in the G1 cell cycle phase length as determined by FACS analysis (Fig. 2B). Such a G1 cell cycle delay when ribosome biogenesis was impaired could be seen when other pre-60S factors like Nog1 were depleted (not shown) and was previously reported for SSU processome factors depletion (3). Total RNA extracted from the wild-type and *mak11-2* strain after 4 and 12 hours of growth at 37°C showed a decreased amount of the 25S ribosomal RNA when compared with the 18S ribosomal RNA (Fig. 2C), in agreement with a role of *MAK11* in 60S ribosomal subunit formation.

Both protein depletion and *mak11* mutation led to 60S formation defects. To look for the cellular localization of these blocked pre-60S particles, we used the Rpl25-GFP fusion previously described as a suitable marker of 60S ribosomal particles precursors export defects (23). When expressed in a *mak11-2* strain at non-permissive temperature, Rpl25-GFP accumulated in the nuclei of yeast cells, in a region excluded from DNA staining (Fig. 2D). We concluded that

Mak11 is not only required for the progression of rRNA maturation at the 27SB step but also plays a role in the exit of the 60S precursors from the nucleolus.

As predicted from our previous work, Mak11 acts thus early after the formation of the 60S precursors in the nucleolus. For a mechanistic analysis of Mak11 action we first defined the composition of pre-ribosomal complexes that contained Mak11.

Complexes associated with Mak11 do not contain late pre-ribosomal factors

Mak11 was found associated with pre-60S complexes purified in association with Rlp24 and its levels increased in these particles when another pre-60S factor, Nog1, was depleted (47). To place Mak11 on the 60S assembly pathway, we purified the associated complexes and identified the proteins by mass spectrometry. Addition of the TAP tag (44) to Mak11 as an N-terminal (strain LMA326) or C-terminal (strain LMA375) fusion had no deleterious effects on Mak11 function since both versions supported growth to wild-type levels (not shown). Forty known and putative pre-60S factors, other than ribosomal proteins, were identified by mass spectrometry from TAP purifications using chromosomal C-terminal tagged Mak11 (Fig. 3A, left lane) as well as from TAP purifications using N-terminal tagged Mak11 expressed from a plasmid in a Δ*mak11* strain (Table 2). Notable absences in the list of the identified proteins are Arx1, Nog2, Nug1 and Nsa2 known to associate late in the nucleus to the pre-ribosomes (30, 38, 46). We verified by immunoblotting with specific antibodies that Arx1, Nog2 and Nsa2 were present in complexes purified using Rlp24-TAP but absent when the complexes were purified using Mak11-TAP (Fig. 3B). The reverse experiment confirmed these results; neither Nog2-TAP nor Arx1-TAP were able to co-purify Mak11 (Fig. 3C).

In view of the number of pre-ribosomal proteins found in association with Mak11, we wondered what species of pre-rRNA were present in these particles. The analysis of the different pre-rRNA species enriched in the TEV protease eluate when TAP-Mak11 was purified, revealed the presence of trace amounts of 27SA2, 7S and 35S with a specific enrichment of the 27SB precursor (Fig 3D, E). Altogether, these results revealed Mak11's association with 27SB pre-rRNA in nucleolar complexes and its requirement for an essential maturation step of 27SB containing pre-60S particles. Mak11 seems to bind only transiently to nuclear 60S precursors as it leaves these particles before the association of late pre-60S factors such as Nog2, Nsa2 or Arx1.

Mak11 is both associated with pre-60S particles and in a free form.

We observed that the amount of proteins purified in association with Mak11 was strongly decreased when the purification was performed in a strain depleted for Rlp24 and was increased when the purification was done after Nog1 depletion (Fig. 3A). To better understand what changed under these conditions, we separated by ultracentrifugation, total protein extracts from a wild type strain or strains depleted for Rlp24 or Nog1, on sucrose gradients. Mak11-TAP was detected in the different fractions by immunoblotting (Fig. 4). Surprisingly, even in a wild-type strain, the Mak11 sedimentation profile showed two peaks, a major one in fractions sedimenting in the upper part of the gradient and a minor one around the position of the 60S pre-ribosomal particles (Fig. 4A). The ratio between these two fractions changed in opposite directions when either Rlp24 or Nog1 were depleted. Most of Mak11 was found in the smaller complexes, under conditions of low Rlp24 levels (Fig. 4B), whereas Mak11 was abundant in the pre-60S fractions when Nog1 was limiting for ribosome biogenesis (Fig. 4C). These changes were correlated with the small amount of pre-ribosomal proteins purified with Mak11-TAP when Rlp24 was depleted while pre-ribosomal proteins accumulated in the complexes associated to Mak11 under Nog1

depletion. In addition, our previous observation of Mak11 accumulating in Rlp24-TAP complexes under Nog1 depletion indicated that pre-60S complexes containing Mak11 where accumulating (47).

We tried to better characterize the form of Mak11 that sedimented in the upper part of the gradient by combining TAP purifications and sucrose gradients and by testing the putative oligomeric state of Mak11. No other proteins were found to be associated with the lower sedimentation rate Mak11-TAP when purified complexes were separated on a sucrose gradient. Moreover, other tagged forms of Mak11 (HA and myc epitopes) have shown that the small weight fraction of the protein sedimented in the first fractions of the gradients (not shown). The sedimentation of purified recombinant Mak11 from *E. coli* matched the expected behavior for a monomeric protein (not shown). The relatively high sedimentation rate of the free TAP-tagged Mak11 form was thus probably due to interactions between Mak11 and the tag.

Mak11 seems to have a dynamic distribution between a free form and the pre-60S form, depending on the levels of different pre-60S intermediates. The free form might represent a "storage" of protein, readily available for variable 60S biogenesis demands.

Rlp24 cooperates with Mak11 for 60S ribosomal particles assembly

To obtain further hints about Mak11 function, we performed a high-copy suppressor genetic screen with the *mak11-2* thermosensitive strain. The large majority of the recovered plasmids (one hundred) contained the *MAK11* gene and were able to complement both the thermosensitivity of the test strain and the loss of a wild-type *MAK11* plasmid. Nine other isolated plasmids allowed only partial complementation of the thermosensitive phenotype of the *mak11-2* strain and contained sequences that had in common the *RLP24* genomic region, from -619 upstream the ATG to 1074 nucleotides downstream the stop codon. We further verified the

complementation by using a 2 micron plasmid derived from pCM190 (14) expressing *RLP24* under the control of a doxycyclin-repressible promoter. Only the pCM190-RLP24 plasmid could complement the *mak11-2* ts phenotype, while the complementation failed if the expression of *RLP24* was repressed (Fig. 5A).

We wondered if the complementation of the thermosensitive *mak11-2* phenotype by *RLP24* overexpression could be explained by a direct physical interaction between the two proteins. To this end, we tested *in vitro* the putative interaction between isolated Rlp24 and Mak11. After mixing total bacterial extracts from *E. coli* expressing GST-Rlp24 or two ribosomal proteins, used as negative controls, with an extract from bacteria expressing $(His)_6$-Mak11, GST tagged proteins were purified on glutathione-Sepharose. The amount of co-purified Mak11 was estimated by Western blotting. In contrast with the negative controls, GST-Rlp24 was able to co-purify a substantial amount of Mak11 (Fig. 5B). This result suggests that functional complementation of Mak11 inactivation by Rlp24 correlates with a direct physical interaction, presumably on pre-60S particles in the nucleus.

A direct interaction between Mak11 and Rlp24, and the decrease of the amount of Mak11 associated pre-ribosomal proteins when Rlp24 was depleted (Fig. 3A) suggested a possible role for Mak11 in Rlp24 association to pre-60S complexes. The amount of proteins purified in association with Rlp24 decreased dramatically during Mak11 depletion (not shown). The absence of a complex under these conditions was explained by a decrease in the total amount of the tagged Rlp24 (Fig. 6A). No change was observed when another tagged protein, Ssf1, was used in a similar experiment and only a slight decrease in total protein concentration was observed with Nog1-TAP (Fig. 6B, C). No change was observed in the levels of the mRNA for Rlp24 when tested by reverse transcription and quantitative real-time PCR (not shown). These results strongly

suggest that Mak11 is required at an early 60S assembly step either for Rlp24 association to precursor complexes or for the stability of these particles.

To further investigate the destabilization of Rlp24 that follows the functional inactivation of *MAK11*, we transformed a strain expressing TAP-tagged Rlp24 and glucose-repressible *MAK11* (LMA515) with a plasmid expressing *mak11-2* or a wild type allele. These yeast cells had a thermosensitive or a wild-type phenotype when grown on glucose-containing medium. The levels of Rlp24-TAP at 27°C in the *mak11-2* strain were estimated by immunoblotting to be at about 50% of the levels of Rlp24-TAP in the wild type strain (Fig. 6D, E). Strikingly, when we performed a shift of the *mak11-2* culture to the non-permissive temperature, Rlp24-TAP levels dropped to barely detectable levels after only one hour of shift to 37°C compared with the wild-type background (Fig. 6D). For a quantitative estimation of the effects of Mak11 inactivation on Rlp24 stability, we looked for the decrease in the amount of Rlp24-TAP after a shift to 37°C, simultaneously with the addition of the translation inhibitor cycloheximide (Fig. 6E). Rlp24-TAP levels decreased at a higher rate in the *mak11-2* background than in the wild-type strain with an estimated half-life of 38 minutes for the ts strain and 87 minutes for the wild type strain. Mak11 is thus specifically required to maintain Rlp24 levels in yeast.

RLP24 overexpression complemented the phenotype of the *mak11-2* strain, probably via a direct protein-protein interaction, and Rlp24 was destabilized in the absence of Mak11. These data strongly suggest that the essential function of *MAK11* in *S. cerevisiae* is directly linked to 60S ribosomal subunit formation. Since another function has been described for putative orthologues of *MAK11* in other organisms, we wondered whether these proteins were involved in the large subunit biogenesis in these organisms.

Skb15, the fission yeast Mak11 homologue is involved in 60S ribosomal subunit biogenesis

Mak11 fission yeast (Skb15) and human (hPip1) homologues were previously described as components of a MAPK signaling pathway, which delivers signals from the cell surface to different effectors (27, 63). Multiple alignments of the sequences for the human, fission yeast and budding yeast proteins indicated a moderate level of similarity, with about 17% identity between any two aligned sequences (Fig. 7). Reciprocal BLAST searches against entire proteome sequences identified putative orthologues of Mak11 in many eukaryotes.

We wondered whether the fission yeast or the human protein complemented the lethal phenotype of *S. cerevisiae* cells deleted for *MAK11*. To test this hypothesis, we cloned the sequences coding for Mak11, Skb15 and hPip1 in vectors that would allow the expression of N-terminal protein A fusions in budding yeast. After transformation of the diploid heterozygote strain containing the deletion of *MAK11*, sporulation of the diploids was induced and the tetrads were dissected. Only the vectors bearing the *MAK11* sequence were able to complement the absence of *MAK11* (not shown). Either the sequence divergence is too important to allow functional complementation of *MAK11* deletion by Skb15 or hPip1, or Mak11 function is different in budding yeast than Skb15 or hPip1 function in fission yeast or human cells.

Since we could not directly demonstrate the functional conservation of the Mak11 putative homologue Skb15 in budding yeast, we looked for Skb15 potential implication in ribosome biogenesis in *S. pombe.* We took advantage of a previously described fission yeast strain where the expression of *SKB15* is under the control of a repressible promoter (27) to assess 60S ribosomal subunit defects when Skb15 was depleted. We compared the polysome profile of extracts from the cells depleted for Skb15 with that of a corresponding wild type strain. As expected for a Mak11 orthologue, depletion of Skb15 led to a polysome profile typical for 60S

ribosomal subunit biogenesis defects with a large peak of free 40S, a decrease in the amount of free 60S and a drastic decrease in the polysomes levels (Fig. 8A, B). Skb15 is thus functionally linked to ribosome biogenesis in *S. pombe*.

To get more mechanistic insights into Skb15 function in fission yeast, we generated a *S. pombe* strain producing the Skb15 protein fused to the TAP tag. We performed the first step of a TAP purification (TEV eluate) using extracts from this strain and from a corresponding wild type strain as a control. The analysis of co-purified rRNA precursors showed an enrichment of both 7S and 27S fission yeast equivalents of budding yeast pre-rRNA intermediates in the Skb15 associated complex (TEV eluate) in comparison with the wild type control (Fig. 8C). These results indicate that Skb15 is a Mak11 orthologue in *S. pombe* that associates to pre-60S particles and is required for 60S ribosomal subunit biogenesis in fission yeast.

Discussion

Building pre-ribosomal complexes with WD40 repeats

Based on genetic, co-purification and direct physical links, we show here that Mak11 is an essential, conserved pre-ribosomal protein directly interacting with Rlp24 and affecting Rlp24 stability. Mak11 sequence contains several repeated motifs, with conserved tryptophan and aspartic acid residues and an average length of 40 amino acids (WD40). For the proteins with known structure that contain such motifs it has been shown that the repeated sequences form a circularized beta-propeller structure mediating protein-protein interactions (for review see 33, 51).

There are more than one hundred WD40-repeat proteins in yeast as listed by the Superfam database (34). Almost one fifth of these proteins (22 out of 111) are factors annotated as involved in ribosome biogenesis and assembly – a significant enrichment of this pathway ($p < 4*10^{-11}$) over a random selection (GO Termfinder, 4). Many of the annotated ribosome biogenesis WD40-repeat proteins were shown to directly interact with, or regulate the levels of, other proteins (25) or to be components of multiprotein subcomplexes. A profusion of WD40-repeat proteins is found in discrete 80S processome subcomplexes. Four out of seven components of the tUTP complex (13) contain WD40-repeat proteins and the UTP "B" complex (9, 29) is composed almost exclusively of WD40-repeat proteins (5/6). One of the first pre-60S subcomplexes to be isolated contains two WD40-repeat proteins, Ytm1 and Erb1, in association with Nop7 (19, 29, 35). Another subcomplex, transiently associated with late, nuclear pre-60S precursors is composed of four proteins and contains Ipi3 as a WD40-repeat member (29, 39).

Two examples of WD40-repeat ribosome biogenesis factors interacting directly with ribosomal proteins for 60S ribosomal subunit assembly are known. Rrb1 interacts with the

ribosomal protein Rpl3 in the nucleus and regulates its levels (25, 48) and Sqt1 interacts with Rpl10 in the cytoplasm (11, 59). Both these proteins have a role in the association of the corresponding ribosomal protein with the nascent 60S ribosomal subunits and might regulate the levels of the corresponding ribosomal protein. Only one ribosomal protein of the Rpl24e family exist in Archaea and its sequence is closer to the sequence of the pre-ribosomal factor Rlp24 than to the sequence of the yeast ribosomal protein Rpl24 (discussed in 47). Yeast Rlp24 is essential to ribosome assembly, does not participate in translation, and thus may be considered an 'assembly only' version of a ribosomal protein. While it is assumed that most of the ribosomal proteins associate early to the precursors of the ribosomal RNA during ribosome formation, little is known about the timing and potential roles of pre-ribosomal factors in coordinating this assembly process.

Our description of Mak11 as directly interacting with Rlp24 thus fits a pattern established by Sqt1 and Rrb1, factors that contain WD40-repeats and function as partners of ribosomal proteins. Both Sqt1 and Rrb1 were shown to exist mainly as free proteins or small complexes as demonstrated by their sedimentation on sucrose gradients (11, 48). However the dynamics of the sedimentation pattern for these proteins under mutant conditions has not been evaluated in these previous studies. We show here that Mak11 is present in the cell both free and in association with pre-60S particles. This association is dynamic, and the relative ratios of the two forms vary under mutant conditions. An increase in the amount of the pre-60S associated Mak11 was observed when Nog1 was depleted, in correlation with the change in Mak11 sedimentation pattern and the increase of the 60S associated fraction. A similar effect was observed for the amount of Sqt1 co-purified with pre-ribosomal particles isolated in association with the cytoplasmic GTPase Lsg1.

Blocked particles, containing a dominant negative form of Lsg1 contained higher amounts of Sqt1 and Rpl10 than particles derived from wild-type cells (59).

In conclusion, the WD-40 repeat structure is used by several proteins for binding specifically to ribosomal proteins during ribosome assembly. It would be interesting to test other pre-ribosomal factors having WD-40 repeats for direct physical and functional interactions with ribosomal proteins. It would be important to see whether transient, and possibly regulated interactions between specific pre-ribosomal factors and ribosomal proteins might play a role in the timing of ribosomal protein association with nascent ribosomes.

Functional conservation of Mak11 in eukaryotic ribosome biogenesis

The sequence of Mak11 shows low levels of similarity with sequences of putative orthologues from other eukaryotes. Experiments with Skb15, the Mak11 homologue in *S. pombe*, allowed us to show that Skb15 associates with precursors of the 60S ribosomal subunits in fission yeast and that depletion of the protein leads to a decrease in free 60S levels. It is thus likely that the putative orthologues of Mak11 are involved in ribosome biogenesis in other eukaryotes as well. Our additional unpublished results suggest that even if *SKB15* cannot complement *MAK11* deletion, the protein and its mammalian homologues have features similar with Mak11: i) Skb15, when expressed in *S. cerevisiae*, localized to the nucleus of budding yeast cells with a stronger signal in the nucleolus. With the use of a high-copy plasmid and a strong promoter, the signal for the human Mak11 homologue – hPip1 was also detectable in the nucleus of yeast cells, including the nucleolus. The localization of Skb15 in fission yeast was previously described and the published images of the GFP fusion protein localization (26) suggest the presence of the protein into the nucleolus of *S. pombe* cells. Moreover, hPip1 has been identified in highly purified nucleolar fractions of human cells (49). ii) Total extracts of *S. cerevisiae* cells expressing tagged

Skb15 were fractionated by sucrose gradient ultracentrifugation. While the tag alone was found exclusively in the upper part of the gradient, tagged Skb15 could also be detected in fractions around 60S – consistent with a weak but significant association of the protein with pre-ribosomal particles (not shown). Skb15 thus contains a nuclear localization signal functional in *S. cerevisiae* and associate weakly with 60S size particles in budding yeast. iii) In response to various environmental stresses, the changes in *SKB15* mRNA levels are similar to the changes in the levels of mRNAs coding for factors predicted to be involved in ribosome biogenesis and assembly in *S. pombe* (7).

Both Skb15 and hPip1 have been previously described as p21 activated kinase (PAK) inhibitors (27, 63). Skb15 was identified by a two-hybrid screen performed using a truncated form of a p21 activated protein kinase, Shk1. *SKB15* deletion in *S. pombe* is lethal. Depletion of Skb15 led to cells with altered morphology and to an increase in the activity of protein kinase Shk1, estimated by the level of Shk1 autophosphorylation. Interestingly, expression of a mouse Skb15 homologue was able to complement the deletion of *SKB15* indicating functional conservation between fission yeast and mammals (27).

While *in vitro* experiments were performed with the human Skb15 homologue hPip1 showing that hPip1 could inhibit the activity of human PAK1, no such experiments were reported for Skb15. We tried to reproduce these results with the *S. cerevisiae* pair of proteins. It has been previously shown that *STE20* is functionally equivalent with human PAK1 since its deletion in yeast is complemented by the expression of human PAK1 (6). We tested *in vitro* the effect of adding purified Mak11 on the protein kinase activity of isolated Ste20 using MBP (myelin basic protein) as a substrate. Ste20 activity was not affected by added purified Mak11 but we could

observe that Mak11 itself was efficiently phosphorylated by Ste20 during these assays (not shown).

A simple hypothesis for the differences between our results and the published studies on Mak11 homologues is that these homologues acquired secondary functions in other eukaryotes, such as protein kinase inhibition. An alternative explanation is that Mak11 homologues function in 60S ribosome formation, as demonstrated here. The stimulation of Shk1 protein kinase activity by Skb15 depletion might thus be a secondary effect of decreasing 60S ribosomal subunits levels. This is an appealing hypothesis since it would suggest that the activation or inhibition of protein kinases might be important in the adaptation of cells to reduced ribosome biogenesis levels.

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Figure legends

Figure 1. Mak11 depletion in yeast cells leads to a block in 27SB to 7S conversion. **A.** Simplified drawing of different steps in rRNA maturation with the position of the oligonucleotides used for Northern blotting or primer extensions. The amounts of large pre-ribosomal and ribosomal RNA were estimated by Northern blotting (1% agarose gel with glyoxal denaturation) (**B.**), by primer extension (**C.**) or by Northern blotting after separation on denaturant urea-polyacrylamide (5%) (**D.**) Equal amounts of total RNA extracted at the indicated time points after addition of doxycyclin from cells where endogenous *MAK11* was deleted and where plasmidic *TAP-MAK11* was under the control of a tetracycline-repressible promoter (strain LMA326). Oligonucleotides used to reveal the different RNAs are indicated in brackets.

Figure 2. The *mak11-2* ts mutant shows alterations in 25S synthesis, cell cycle and pre-ribosomes export. **A.** Growth curves at 37°C for the *mak11-2* mutant (open circles) as compared with a wild-type strain (crosses). The number of cells was estimated using the absorbance of the culture at 600 nm. **B.** The cell cycle distribution of yeast cells was estimated by FACS analysis. Wild type and *mak11-2* haploid cells in rich medium were shifted from 27°C to 37°C for 0, 1, 3 and 6 hours, aliquots were fixed with 70% ethanol and stained with propidium iodide. The ratios between cells having 1n DNA content and 2n DNA content was estimated in triplicate experiments (wild-type strain, white bars and *mak11-2*, grey bars), error bars are standard deviation of the measured ratios. **C.** Total RNA was extracted from a wild type strain or the *mak11-2* strain at time 0, 4 and 12 hours after shift to the nonpermissive temperature and separated on a 1% agarose gel (glyoxal denaturation) and stained with ethidium bromide. **D.** GFP fluorescence of the *mak11-2* ts or isogenic wild type cells, both expressing Rpl25-eGFP, was

detected after a shift to 37°C for 8 hours. DNA was stained with Hoechst 33342. Arrow heads indicate the relative position of the DNA stained region of the nuclei.

Figure 3. Mak11 associated complexes do not contain late pre-60S factors. **A.** Proteins purified in association with Mak11-TAP from wild-type cells or cells depleted for Rlp24 or Nog1 (14 hours in glucose medium) were separated on a 5-20% polyacrylamide gradient gel and stained with colloidal Coomassie Blue. Proteins identified by MALDI-TOF mass-spectrometry, listed in Table 2, are indicated. **B.** The presence or absence of proteins in TEV eluates from purifications using Mak11-TAP and Rlp24-TAP were tested by immunoblotting with specific antibodies. **C.** The presence of Mak11 in complexes purified using Rlp24-TAP, Nog2-TAP and Arx1-TAP was tested by immunoblotting, with Nog1 as a positive control. **D.** RNAs associated with Mak11 complexes were enriched in the TEV protease eluate from a TAP-Mak11 (LMA326) purification. The recovered RNAs were extracted with phenol-chloroform and tested by primer extension or Northern blotting (**E.**) with the same oligonucleotides as those used for Fig. 1.

Figure 4. Dynamic association of Mak11 to pre-60S particles. Sucrose gradient ultracentrifugation analysis of Mak11-TAP in wild type cells (**A**) and cells depleted for Rlp24 (**B**) or Nog1 (**C**) was followed by protein precipitation from the recovered fractions, and immunoblotting to detect the TAP tag.

Figure 5. Mak11 and Rlp24 are functionally and physically linked. **A.** *RLP24* is a high-copy suppressor of the *mak11-2* ts phenotype. Rlp24 was expressed from a high copy vector (pCM190-RLP24) under the control of a tetracycline repressible-promoter in wild type or *mak11-2* cells at permissive and non-permissive temperatures. Growth was estimated by 10 times serial dilutions on solid YPD medium with or without doxycyclin. **B.** Protein-protein interaction between $(His)_6$-Mak11 and GST-Rlp24 were detected by mixing total extracts of *E. coli*

overexpressing the proteins and pulling down the GST fusion proteins with Glutathione-Sepharose. Eluates were separated on 4-12% Novex polyacrylamide gels and Coomassie stained. Immunoblots were performed in parallel with rabbit polyclonal antibodies raised against Mak11.

Figure 6. Mak11 stabilizes Rlp24. **A.** Strains expressing Rlp24-TAP, Ssf1-TAP (**B**) and Nog1-TAP (**C**) with *MAK11* under the control of a *GAL1* promoter were grown on rich galactose medium and shifted to glucose for 8 and 18 hours. The amounts of the tagged proteins were estimated by immunoblot for the TAP tag; the total amounts of loaded proteins on each lane were estimated by Ponceau S staining of the nitrocellulose membranes. **D.** Strains expressing Rlp24-TAP and either the *mak11-2* allele or wild-type *MAK11*, were grown on YPD at 27°C and shifted to 37°C for up to 6 hours. The amount of Rlp24-TAP was estimated by immunoblot (upper panel) and the amount of loaded total protein was visualized by Ponceau Red staining (lower panel). **E.** The stability of Rlp24-TAP in the *mak11-2* (open circles) and wild type (crosses) strains was measured by simultaneously shifting the culture from 27°C to 37°C and adding 35 µg/ml cycloheximide. The immunoblot signal was plotted as a ratio to the initial Rlp24-TAP amount. The inset shows a typical image used for quantification. The standard deviation of the results for three independent cultures is indicated as error bars.

Figure 7. Multiple sequence alignment between Mak11 and putative human (hPip1) and *S. pombe* (Skb15) homologues. Sequences of yeast Mak11, *S. pombe* Skb15 and human hPip1 were aligned using ClustalW (53) and similar or identical residues were shaded using BOXSHADE so that residues that have similar properties and are found in the three sequences have a black background while those with similar properties in two out of three sequences have a grey background. The residues changes indicated in the alignment correspond to the observed mutations in the *mak11-2* strain. Boxed regions correspond to predicted WD40 motifs. The

residues changes indicated in the alignment correspond to the observed mutations in the *mak11-2* strain.

Figure 8. The Mak11 homologue in *S. pombe*, Skb15, is involved in 60S formation in fission yeast. Cells expressing *SKB15* under the control of the *nmt1* thiamin repressible promoter were grown in synthetic medium without thiamin (**A**) or with 5 µg/ml thiamine (**B**). Extracts were separated by sucrose gradient ultracentrifugation and the amounts of free 40S, free 60S and polysomes estimated by the 254 nm absorbance profile. **C.** A wild-type *S. pombe* strain and a strain expressing Skb15-TAP were used for the first step of TAP purification; RNAs were extracted by phenol-chloroform and tested by Northern blot or primer extension with oligonucleotides specific for pre-rRNAs or U2 as control.

Tables

Table 1. *S. cerevisiae* and *S. pombe* strains used in this study.

Strain	Genotype	Reference
MGD353–13D	*MATa trp1–289 ura3–52 ade2 leu2–3,–112 arg4*	(44)
BY4741	*MATa ura3Δ0 his3Δ1 leu2Δ0 met15Δ0*	(5)
BY4742	*MATα ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0*	(5)
Y24870	*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 Mak11::kanMX4/MAK11*	(17)
LMA160	*MATa trp1–289 ura3–52 ade2 leu2–3,–112 arg4 Rlp24–TAP-TRP1*	(47)
LMA260	*MATa ura3Δ0 his3Δ leu2Δ0 lys2? met15? Mak11::kanMX4* [pFL38-*MAK11*]	This study
LMA263-2	*MATa ura3Δ0 his3Δ leu2Δ0 lys2? met15? Mak11::kanMX4* [pFL36CII-*mak11-2*]	This study
LMA264	*MATa ura3Δ0 his3Δ leu2Δ0 lys2? met15? Mak11::kanMX4* [pFL36CII-*MAK11*]	This study
LMA326	*MATa ura3Δ0 his3Δ leu2Δ0 lys2? met15? Mak11::kanMX4* [pTG189-*MAK11*]	This study
LMA375	*MATa ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 Mak11-TAP-HIS3*	This study
LMA364	*MATa ura3Δ0 his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 P_{GAL1}::Nog1/KanMX6 Mak11-TAP-HIS3*	This study
LMA371	*MATa ura3Δ0 his3Δ1 leu2Δ0 met15Δ0 P_{GAL1}::Rlp24/KanMX6 Mak11-TAP-HIS3*	This study
LMA431	*MATa trp1-289 ura3-52 ade2 leu2-3,-112 arg4 Ssf1-TAP-URA3 P_{GAL1}::Mak11/KanMX6*	This study
LMA437	*MATa trp1-289 ura3-52 ade2 leu2-3,-112 arg4 Nog1-TAP-TRP1 P_{GAL1}::Mak11/KanMX6*	This study
LMA515	*MATa trp1-289 ura3-52 ade2 leu2-3,-112 arg4 Rlp24-TAP-TRP1 P_{GAL1}::Mak11/KanMX6*	This study
LMA600	*h^- (Msmt0) leu1-32 ura4-D18 adeM6-210 Skb15-TAP/KanMX6 (S. pombe)*	This study
Pb185	*h^- (Msmt0) leu1-32 ura4-D18 adeM6-210 (S.pombe)*	(B. Arcangioli)
nmt-Skb15	*h^{90} ade6-M210 leu1-32 ura4-D18 skb15::ura4::nmt1-skb15-ADE2 (S. pombe)*	(27)
SP870	*h^{90} ade6-M210 leu1-32 ura4-D18 (S. pombe)*	(27)

"?" – genotype not tested

Table 2. Non-ribosomal proteins identified in complexes purified in association with TAP-Mak11 (N) and Mak11-TAP (C).

Name	ORF	pI	kDa	Band*	Tag**
RRP5	YMR229C	6.1	193		N
UTP22	YGR090W	8.8	140	1	N+C
ERB1	YMR049C	4.9	92	2, 3	N+C
MAK5	YBR142W	8.7	87	5	C
DRS1	YLL008W	5.5	85	4	N+C
DBP7	YKR024C	10.0	83	8	C
NOC2	YOR206W	8.9	82	6	C
NOC3	YLR002C	5.8	76	8	C
PUF6	YDR496C	6.9	75	8	N+C
NOG1	YPL093W	9.4	74	10	N
NOP7	YGR103W	5.4	70	9	N+C
NOP2	YNL061W	4.8	70	7	N+C
DBP9	YLR276C	10.0	68	11	C
ROK1	YGL171W	9.9	64	14	C
SIK1/NOP56	YLR197W	9.6	57		N
HAS1	YMR290C	10.1	57		N
CBF5	YLR175W	9.5	55		N
MAK11	YKL021C	8.6	54	16	N+C
NOP12	YOL041C	10.2	52		N
NSA1	YGL111W	8.0	52	17	N+C
SSF1	YHR066W	10.1	52	15	N+C
YTM1	YOR272W	7.0	51	18	C
RRP14	YKL082C	10.4	50	13	C
EBP2	YKL172W	6.4	50	12	C
FPR3/NPI46	YML074C	4.2	47	15	N+C
FPR4	YLR449W	4.4	44		N
CIC1/NSA3	YHR052W	9.5	43	19	C
RLP7	YNL002C	10.2	37	20	N+C
MAK16	YAL025C	5.1	36	21	C
RPF1	YHR088W	10.3	35	24	N+C
NOP1	YDL014W	11.0	34	23	N+C
BRX1	YOL077C	10.0	34	25	N+C
RRP1	YDR087C	5.0	33	22	N+C
RRP15	YPR143W	5.5	28	22	C
MRT4	YKL009W	9.1	27	27	C
NOP16	YER002W	10.1	27	26	N+C
TIF6/CDC95	YPR016C	4.4	26	30	C
NOP15	YNL110C	10.1	25	27	C
RLP24	YLR009W	10.4	24	28	C
LOC1	YFR001W	11.1	24	29	C
NIP7	YPL211W	10.1	20	31	C

* - Band position is indicated for the proteins identified from the gel shown in Figure 3.

** - Protein identified in purifications performed using N-terminal (N), C-terminal (C) tagged Mak11 or in both (N+C)

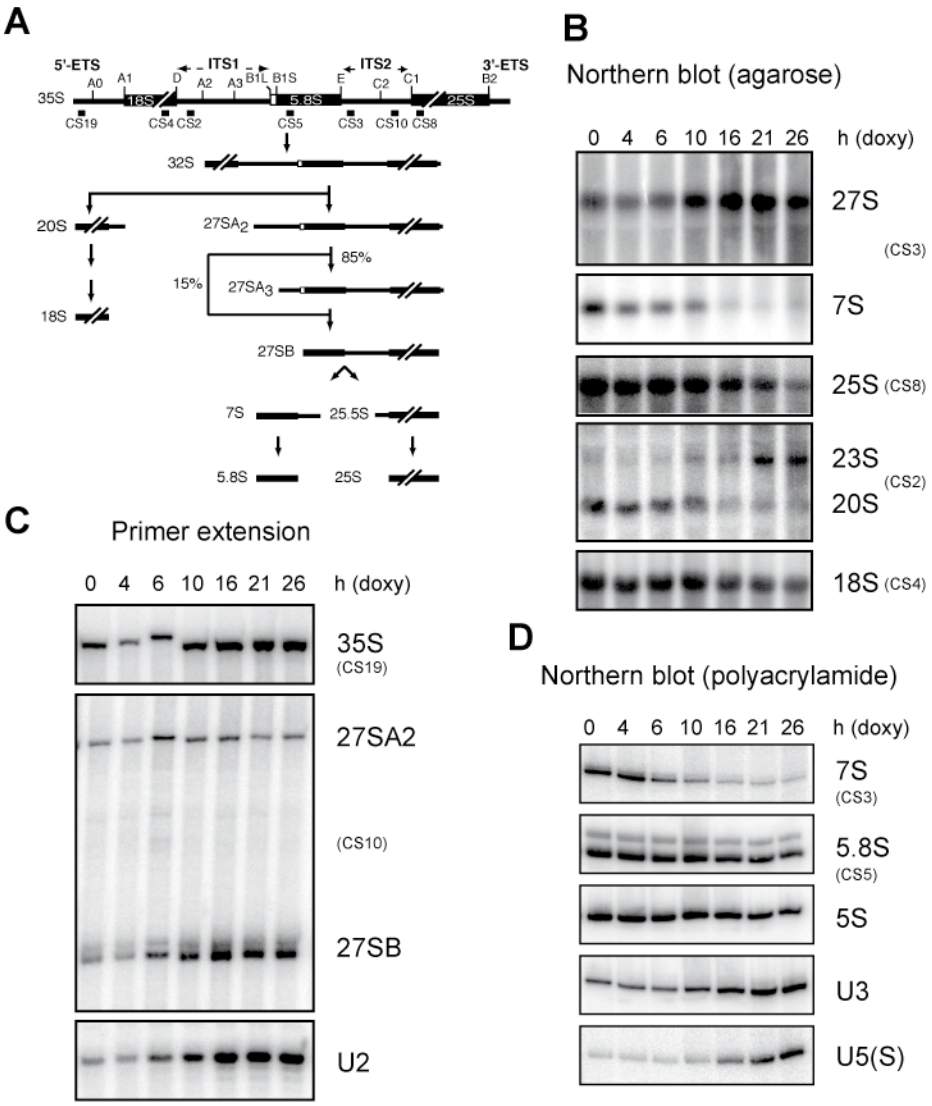

Figure 1, Saveanu et al.

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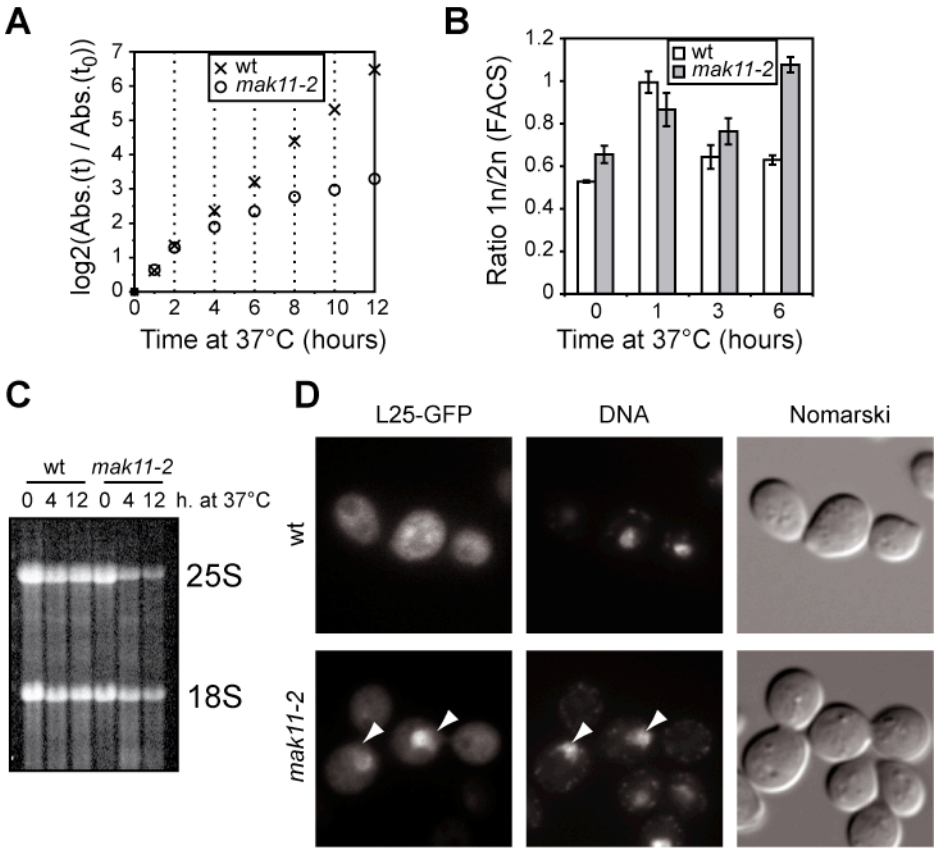

Figure 2, Saveanu et al.

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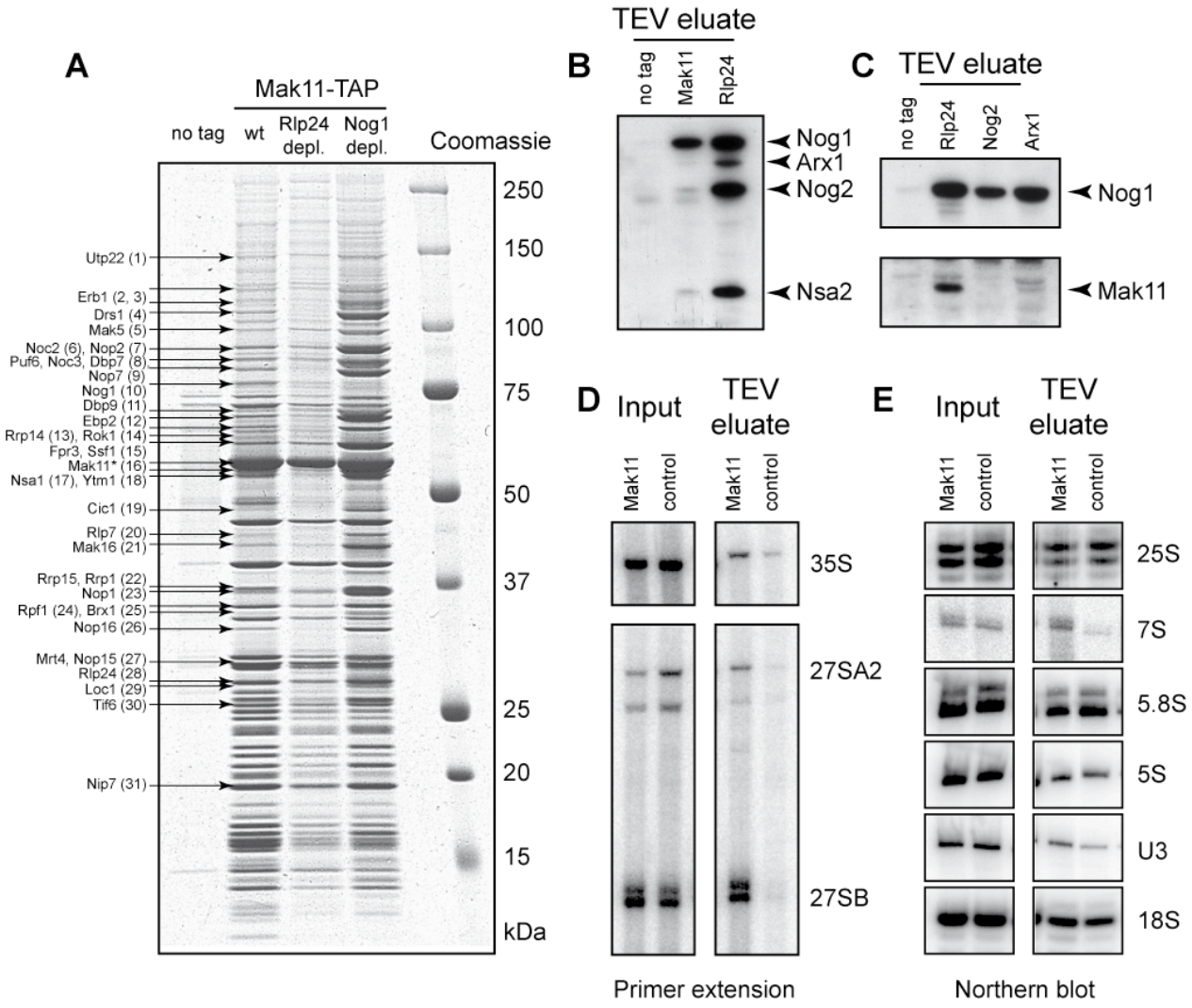

Figure 3, Saveanu et al.

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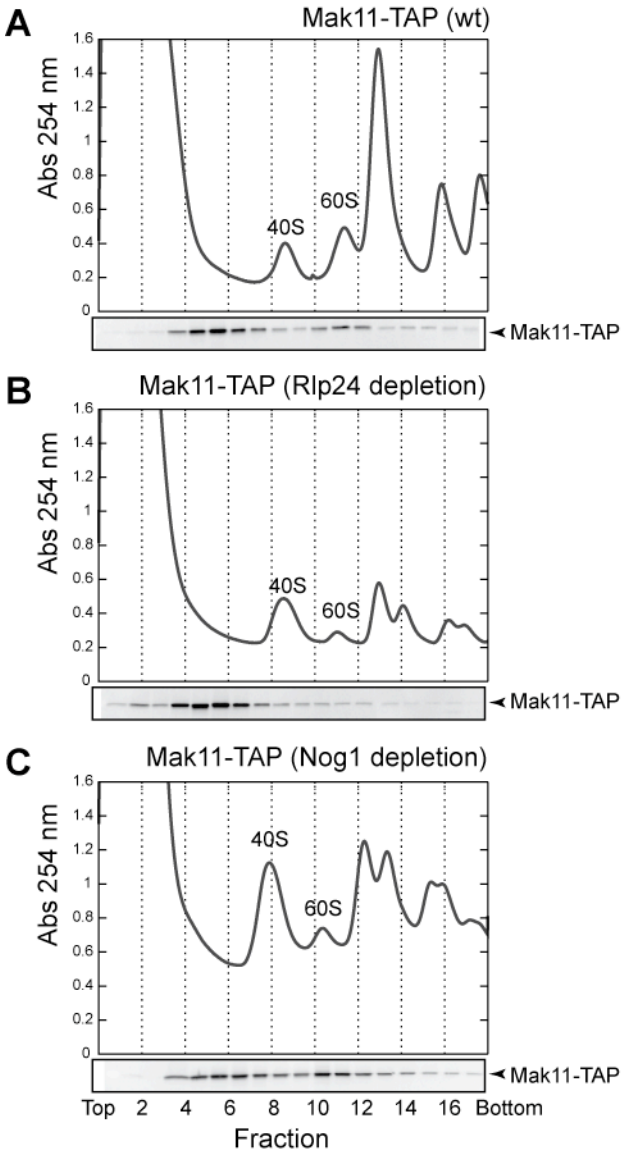

Figure 4, Saveanu et al.

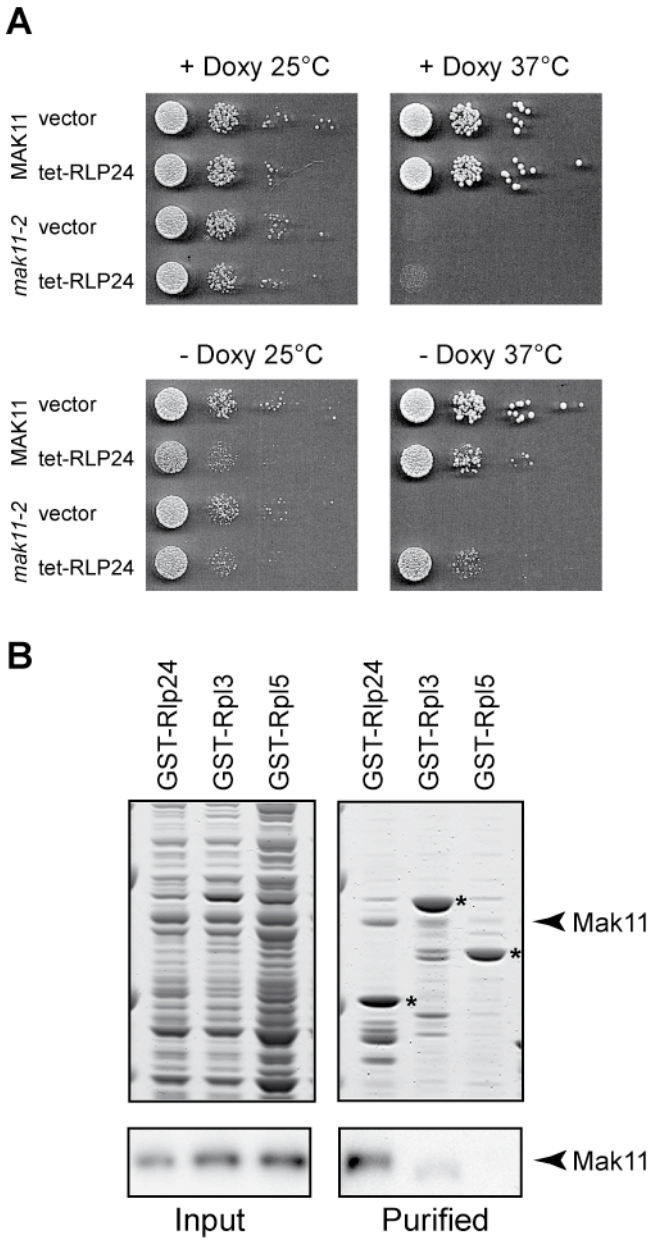

Figure 5, Saveanu et al.

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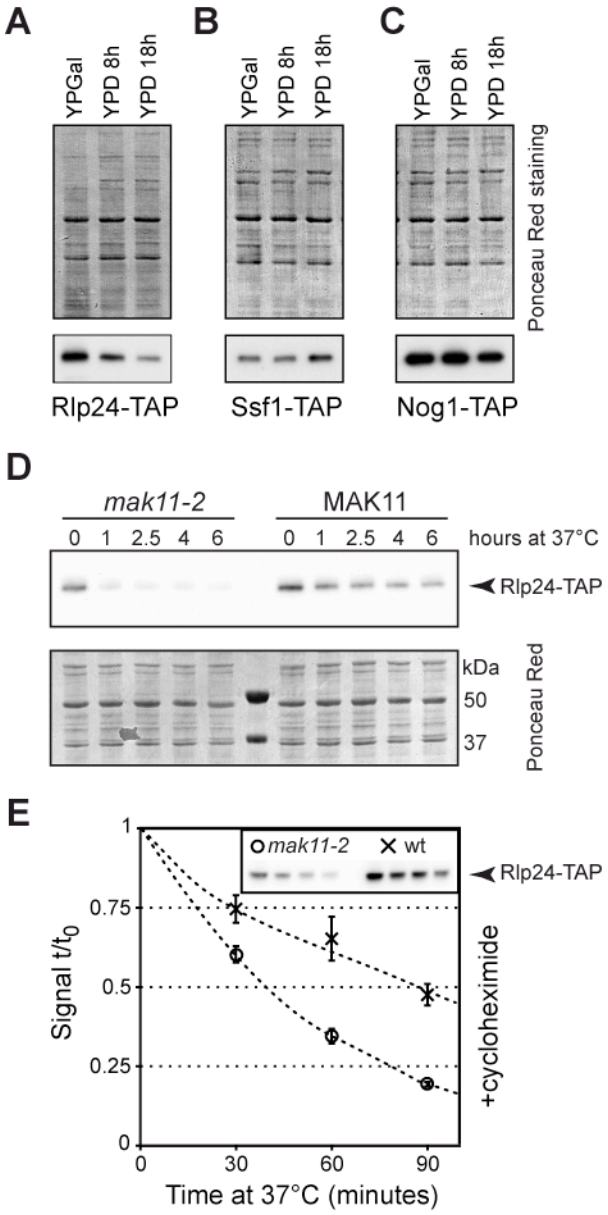

Figure 6, Saveanu et al.

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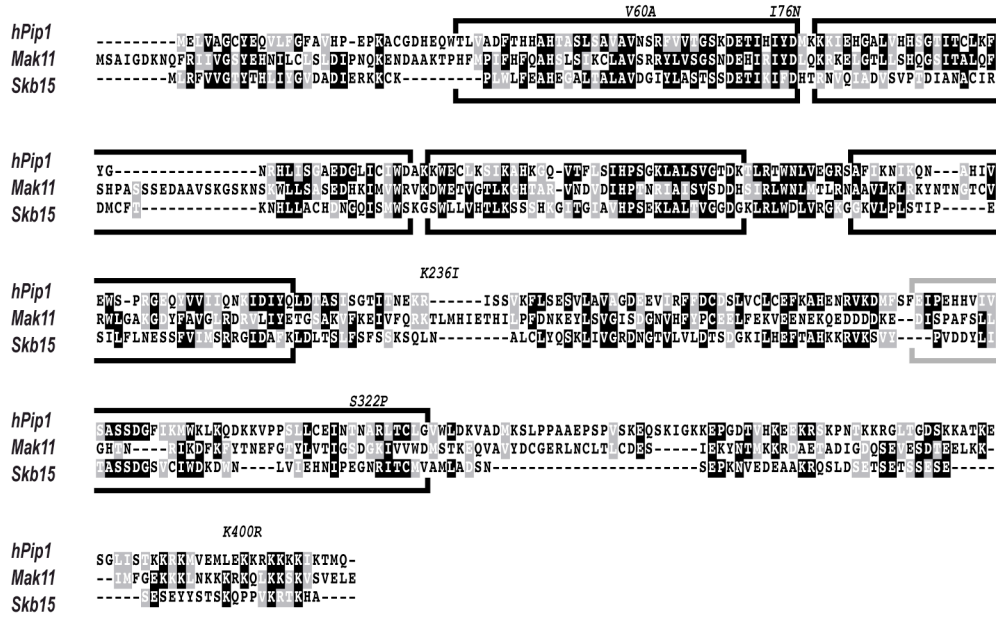

Figure 7, Saveanu et al.

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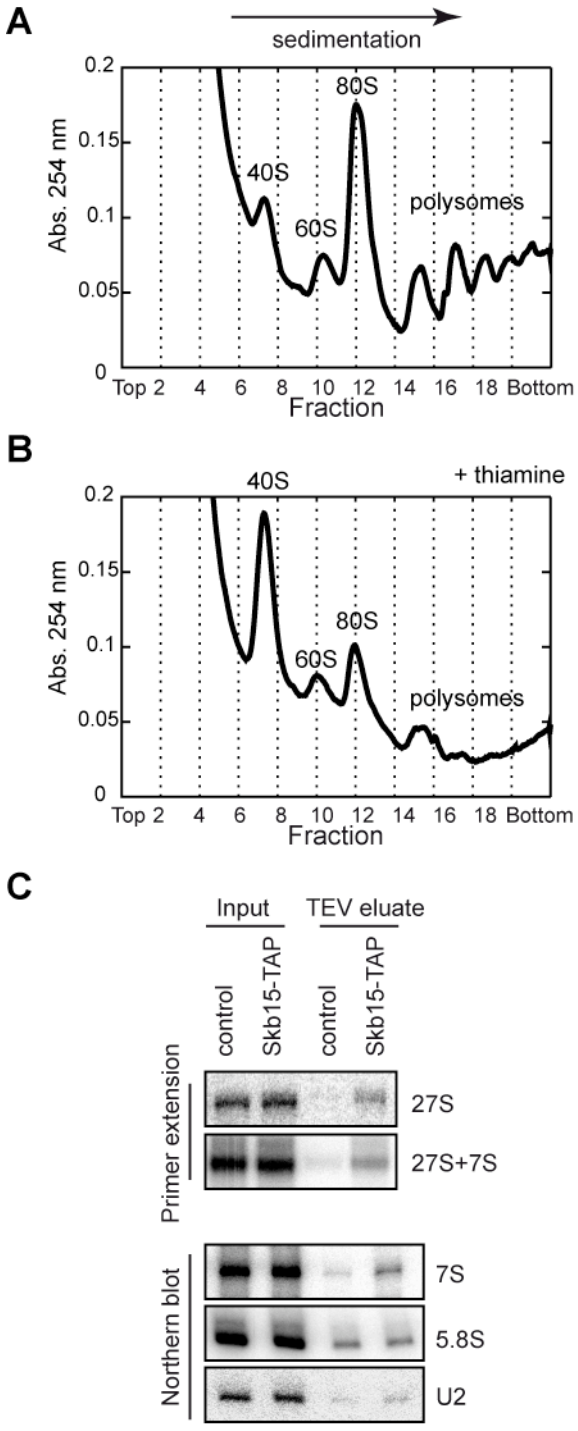

Figure 8, Saveanu et al.