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Formation and nuclear export of tRNA, rRNA and mRNA is regulated by the ubiquitin ligase Rsp5p

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The yeast ubiquitin-protein ligase Rsp5p regulates processes as diverse as polII transcription and endocytosis. Here, we identify Rsp5p in a screen for tRNA export (*tex*) mutants. The *tex23-1/rsp5-3* mutant, which is complemented by *RSP5*, not only shows a strong nuclear accumulation of tRNAs at the restrictive temperature, but also is severely impaired in the nuclear export of mRNAs and 60S pre-ribosomal subunits. In contrast, nuclear localization sequence (NLS)-mediated nuclear protein import is unaffected in this mutant. Strikingly, the nuclear RNA export defects seen in the *rsp5-3* strain are accompanied by a dramatic inhibition of both rRNA and tRNA processing, a combination of phenotypes that has not been reported for any previously characterized mutation in yeast. These data implicate ubiquitination as a mechanism coordinating the major nuclear RNA biogenesis pathways.

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INTRODUCTION

Eukaryotic gene expression requires the nuclear export of several classes of RNAs, including mRNA, tRNA and rRNA, the latter in the form of ribosomal subunits. While much has been learnt about the distinct export pathways for these individual types of RNAs, little is known how these pathways are coordinated (Lei & Silver, 2002). A highly conserved export receptor, Mex67-Mtr2 in yeast and TAP/NXF-p15/NXT in metazoans, is involved in the nuclear export of mRNAs, which is connected to intranuclear export factors that couple mRNA biogenesis with nuclear export (Reed & Hurt, 2002; Stutz & Izaurralde, 2003). The export of ribosomal RNA is strictly coupled to intranuclear pre-ribosome biogenesis, which involves a complex series of steps beginning with the nucleolar transcription of rDNA, followed by pre-rRNA processing and modification, assembly of pre-ribosomal particles and final export to the cytoplasm (Johnson *et al.*, 2002; Tschochner & Hurt, 2003). Nuclear export of the large 60S subunit requires the NES-containing adaptor Nmd3 and the Xpo1/Crm1/exportin-1 export receptor.

Although less complex than ribosome biogenesis, tRNA export also requires multiple maturation steps prior to export of the mature tRNA to the cytoplasm (Hopper, 1999; Wolin & Matera, 1999). Nuclear export of the mature tRNA is mediated by the karyopherin Xpo-t in vertebrates (Arts *et al.*, 1998; Kutay *et al.*, 1998) and Los1p in yeast (Hellmuth *et al.*, 1998; Sarkar & Hopper, 1998). However, the fact that yeast Los1p is not essential and that only a subset of tRNAs exhibit nuclear accumulation in *los1*$^{-}$ mutants suggests that at least one alternative nuclear tRNA export pathway exists (Grosshans *et al.*, 2000b).

Therefore, we set up a screen based on fluorescence *in situ* hybridization (FISH) to find novel tRNA export factors. In this screen for tRNA export (*tex*) mutants, we have identified Rsp5p, an essential ubiquitin ligase (Huibregtse *et al.*, 1995). Previously, Rsp5p has been implicated in several processes, for example, targeting to the vacuole (Helliwell *et al.*, 2001), remodelling of the actin cytoskeleton (Kaminska *et al.*, 2002), polII transcription (Huibregtse *et al.*, 1997) and activation of transcription factors (Hoppe *et al.*, 2000). Further characterization of the *rsp5-3* mutant revealed that it is strongly impaired in the nuclear export of mRNA and ribosomal 60S subunits. In a parallel work, it was recently reported that another *rsp5* mutant is impaired in mRNA export (Rodriguez *et al.*, 2003). In addition, our work showed that tRNA and rRNA export defects in the *rsp5-3* mutant are preceded by a severe inhibition of pre-tRNA and pre-rRNA processing. Thus, our data show a role of the ubiquitin ligase Rsp5p in controlling the major nuclear RNA biogenesis/export pathways in yeast.

RESULTS

To identify factors required for tRNA export, we performed a screen for temperature-sensitive (*ts*) mutants that accumulate tRNA inside the nucleus at the restrictive temperature. We screened a bank of randomly generated *ts* mutants for a *tex* phenotype. One mutant termed *tex23-1* showed a nuclear accumulation of intron-containing and intronless tRNA after a shift to the restrictive temperature (Fig. 1A). To characterize further

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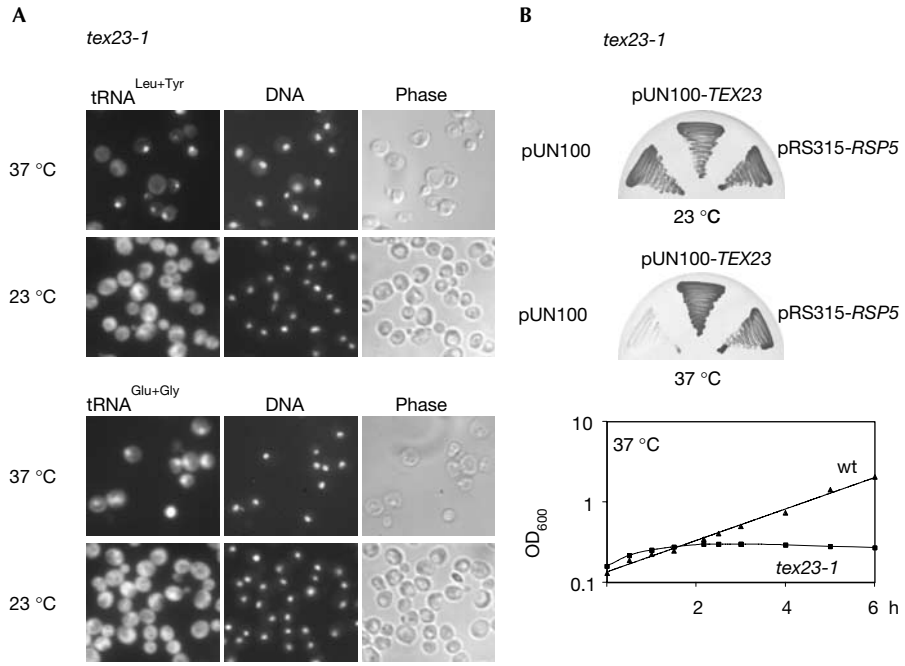


Fig. 1 | Genetic screen for tRNA export (*tex*) mutants identifies Rsp5p. (**A**) *tex23-1* cells grown at 23 °C were shifted to 37 °C for 3.5 h, and localization of $tRNA^{Leu+Tyr}$ and $tRNA^{Glu+Gly}$ was assessed by *in situ* hybridization. The DNA was stained with DAPI. (**B**) *tex23-1* cells were transformed with pUN100, with complementing pUN100-*TEX23* and with the *RSP5* gene (pRS315-*RSP5*). Transformants were grown for 3 days at 23 or 37 °C (upper panel). Growth curve of *rsp5-3* and wild-type cells in YPD medium after shift to 37 °C (lower panel).

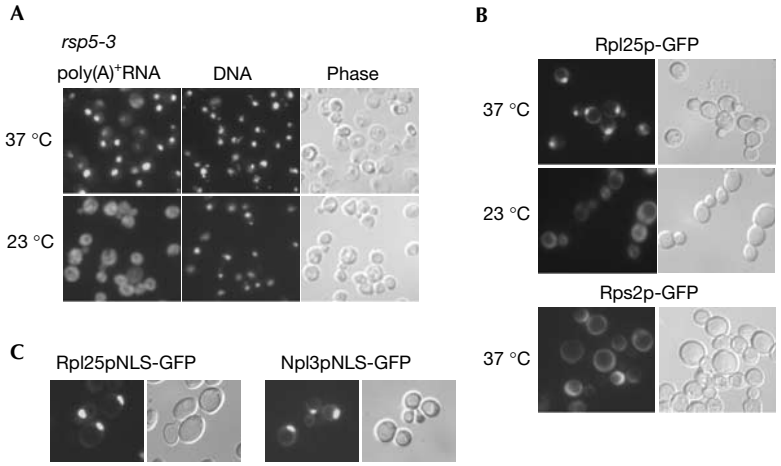
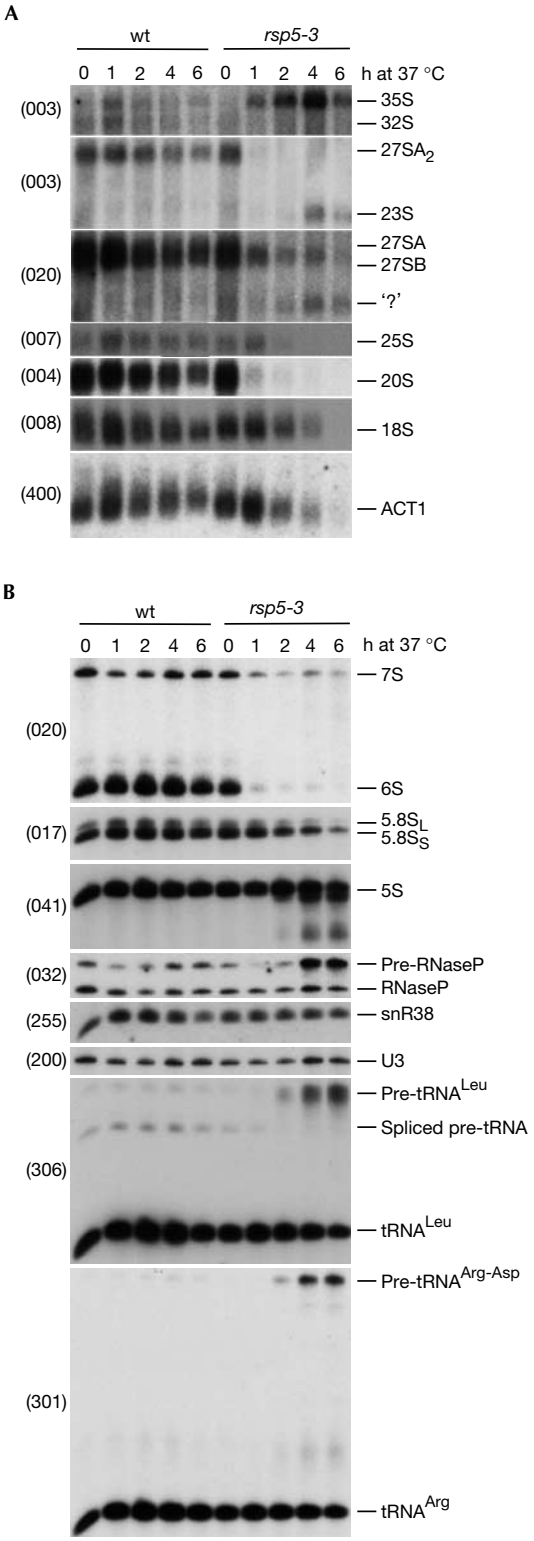


Fig. 2 | Poly(A)$^+$ RNA and pre-ribosomal subunit export is inhibited in *rsp5-3*. (**A**) *rsp5-3* cells grown at 23 °C were shifted to 37 °C for 3.5 h, and localization of poly(A)$^+$ RNA was assessed by *in situ* hybridization. (**B**) *rsp5-3* cells transformed with the respective reporter plasmids were observed in the fluorescence microscope for the localization of Rpl25p-GFP (upper panel), Rps2p-GFP (lower panel), and Rpl25pNLS-GFP and Npl3pNLS-GFP (**C**) after shifting for 3.5 h to 37 °C.

this putative tRNA export factor, the wild-type *TEX23* gene was cloned by complementation of the *tex23-1* phenotype (Fig. 1B). Unexpectedly, *TEX23* is identical to *RSP5*, a gene encoding the essential ubiquitin ligase Rsp5p. Accordingly, the *tex23-1* mutant was called *rsp5-3*. The *rsp5-3* allele was isolated and shown to contain the mutations T104A, E673G and Q716P. Notably, the Q716P mutation lies in helix 12 of the catalytic hect domain of Rsp5p (Huang *et al.*, 1999). To determine whether *rsp5-3* cells exhibit other defects in nucleocytoplasmic transport, both nuclear export of other classes of RNA and nuclear protein import were analysed. Interestingly, *rsp5-3* cells show a strong nuclear accumulation of poly(A)$^+$ RNA (Fig. 2A). To test for nuclear export of ribosomal subunits, functional green fluorescent protein (GFP)-tagged ribosomal protein reporters Rpl25p-GFP (large

subunit) and Rps2p-GFP (small subunit) were analysed by fluorescence microscopy (Fig. 2B). Nuclear export of the 60S subunit, but not of the 40S subunit, was inhibited significantly in the *rsp5-3* mutant at 37 °C. In contrast, NLS-reporter constructs continued to accumulate in the nucleus of the *rsp5-3* mutant (Fig. 2C). Altogether, the data suggest that Rsp5p is involved in the nuclear export of several classes of RNA.



To determine whether nuclear accumulation of rRNA, tRNA and mRNA in *rsp5-3* cells is a consequence of defects in RNA maturation, the processing of representatives of each of these classes of RNA was analysed by northern hybridization (Fig. 3) and primer extension (data not shown). These analyses revealed dramatic and unexpected defects in the maturation of both rRNAs and tRNAs. Northern analysis showed that the 35S pre-rRNA primary transcript accumulated strongly, accompanied by the appearance of the aberrant 23S RNA (Fig. 3A). In contrast, the normal products of 35S processing, the $27SA_2$ and 20S pre-rRNAs, were almost entirely lost within 1 h of transfer to 37 °C. These results show that the early pre-rRNA cleavages at sites A_0, A_1 and A_2 are strongly inhibited in the *rsp5-3* strain. Processing on the pathway of 5.8S and 25S synthesis is less completely inhibited, but levels of the 27SB, 7S and 6S pre-rRNAs were greatly reduced. Consistent with the pre-rRNA processing defects, the mature 18S, 25S and 5.8S rRNAs were depleted in the *rsp5-3* strain progressively. However, no alteration was seen in the relative levels of the $5.8S_L$ and $5.8S_S$ rRNAs (Fig. 3B), and primer extension analysis (data not shown) showed no alteration in the corresponding levels of the long and short form of the 27SB pre-rRNA. The generation of the short $27SB_S$ pre-rRNA and $5.8S_S$ rRNA requires the cleavage of processing site A_3 by the endonuclease RNase mitochondrial RNA processing (MRP), as does synthesis of the 23S RNA. We conclude that pre-rRNA cleavage by RNase MRP is not inhibited in the *rsp5-3* strain. In addition, truncated fragments of the 5S rRNA appeared, presumably representing degradation intermediates. The appearance of these truncated species is not a normal consequence of the inhibition of ribosome synthesis, and may represent a defect in the RNA degradation machinery. Polysome analyses were performed on lysates from the *rsp5-3* strain 5 h after transfer to 37 °C (data not shown). These showed little alteration in the ratios of free 40S and 60S to polysomes, and only a mild decrease in 80S monosomes to polysomes in the *rsp5-3* strain following transfer to 37 °C. This indicates that the residual subunits that are synthesized in the mutant are fully functional.

Pre-tRNA processing was also inhibited drastically in the *rsp5-3* strain. The dimeric pre-$tRNA^{Arg}$–pre-$tRNA^{Asp}$ (Fig. 3B) strongly accumulated with depletion of the mature $tRNA^{Arg}$. This pre-tRNA is normally cleaved by the endonuclease RNase P. Accumulation was also seen for the full-length pre-$tRNA_3^{Leu}$ (Fig. 3B), confirming the inhibition of processing by RNase P, and revealing an additional defect in pre-tRNA splicing. This was unexpected, since previous analyses have shown that pre-tRNA end maturation and splicing are independent events (O'Connor & Peebles, 1991). The RNA component of RNase P is processed from a 3′ extended

◀ **Fig. 3** | tRNA and rRNA processing is defective in the *rsp5-3* strain. Northern analysis was performed on RNA extracts from the *rsp5-3* and FY23 (wt) cells after shifting for 0–6 h from 23 to 37 °C. (**A**) High-molecular-weight RNAs separated on an agarose/formaldehyde gel. (**B**) Low-molecular-weight RNAs separated on a polyacrylamide/urea gel. RNA species detected are indicated on the right of the figure and oligonucleotide probes used are shown in parentheses. The identity of the aberrant processing intermediate indicated with '?' has not yet been established.

precursor, which was also accumulated in the *rsp5-3* strain at the non-permissive temperature.

In contrast to tRNA and rRNA synthesis, no clear defect was seen in the synthesis of the small nucleolar RNAs U3 or snR38 (Fig. 3B). Moreover, no defect was apparent in the splicing of the *ACT1* mRNA (Fig. 3A) or of pre-U3, which contains a pre-mRNA-like intron in yeast. Thus, pre-mRNA splicing seems not to be affected by the *rsp5-3 ts* mutant. The level of the *ACT1* mRNA was reduced in the *rsp5-3* strain during incubation at 37 °C, but this decline was much slower than that seen for the pre-rRNAs, suggesting that it may be an indirect effect of the growth arrest.

The defective nuclear RNA export in *rsp5-3* may be due to an inhibition of components of the respective RNA biogenesis/export machineries; however, it could also be indirect. Notably, Rsp5p is known to activate the transcription factor Spt23p via the ubiquitin/proteasome system (Hoppe *et al.*, 2000). Spt23p controls the fatty acid desaturase Ole1p, which is thought to regulate unsaturated fatty acid levels and accordingly could affect nuclear membrane/pore biogenesis. However, nuclear membrane abnormalities were not seen in the *rsp5-3* strain, and the nuclear pores appeared to be of normal size and structure in the electron microscope (Fig. 4A). Moreover, expression of a GFP-tagged nucleoporin in *rsp5-3* cells did not reveal alterations in the punctate nuclear envelope staining (data not shown). However, numerous electron-dense aggregates were observed in the nucleoplasm of *rsp5-3* cells (Fig. 4A). Similar electron-dense aggregates were seen in the nucleus of the *mex67-5* mutant, which is impaired in nuclear mRNA export (Segref *et al.*, 1997). We conclude that nuclear membrane/nuclear pore organization is not grossly altered in *rsp5-3* cells. Intranuclear aggregates may arise from the accumulation of aberrant RNA/RNPs resulting from defective processing, assembly and/or nuclear export.

We next sought to test whether *rsp5-3* is defective in lipid synthesis as a consequence of decreased Ole1p expression. Total lipids were extracted from the *rsp5-3* mutant. To analyse the unsaturated fatty acids in lipids, mass spectrometric (MS) analysis was performed and phosphatidyl ethanolamine (PE) profiles were obtained. As shown in Fig. 4B, *rsp5-3* cells grown at the restrictive temperature exhibit significantly reduced levels of the di-unsaturated PE species, PE 32:2 (*m*/*z* 688) and PE 34:2 (*m*/*z* 716). The decrease of di-unsaturated PE species can be suppressed by expressing the *OLE1* gene from a high-copy plasmid. Taken together, the data show that *rsp5-3* is also defective in the desaturation of fatty acids, most likely due to decreased Ole1p expression.

It is possible that an altered lipid composition with decreased levels of unsaturated fatty acids is a cause for the nuclear RNA export defects in the *rsp5-3* mutant. If this were true, nuclear RNA export should be defective in an *ole1* mutant. No inhibition of nuclear RNA export was seen in *ole1*Δ cells (Fig. 4C). Moreover, overexpression of the *OLE1* under a constitutive promoter (to circumvent Rsp5p/Spt23p-dependent regulation) did not abolish the mRNA (Fig. 4D) or 60S pre-ribosomal export defects in *rsp5-3* (data not shown). Thus, neither the lack of unsaturated fatty acids in the nuclear membrane nor the reduced expression of Ole1p in *rsp5-3* mutant cells is the cause of the observed RNA export defects.

If Rsp5p were directly involved in nuclear RNA formation, it might be expected to enter the nucleus. A functional GFP-Rsp5p was detected in both the nucleus and cytoplasm (Fig. 5A). In contrast, GFP-tagged aminoacyl-tRNA synthase is exclusively cytoplasmic and excluded from the nucleus (Fig. 5A; Galani *et al.*, 2001). In addition, GFP-Rsp5p was concentrated at the cell periphery of bud tips and growing daughter cells (Fig. 5A).

A functional Rsp5p-TAP fusion protein (TAP, tandem-affinity purification tag) was affinity-purified from yeast lysates. In agreement with its nuclear location and function, Rsp5p-TAP was associated with several subunits of RNA polII, the transcription factor Spt5p (Hartzog *et al.*, 1998) and the nuclear Sen1p, which is involved in tRNA splicing and snRNA/snoRNA maturation (Ursic *et al.*, 1997) (Fig. 5B). Rsp5p-TAP also co-precipitated Bul1p, a protein implicated in ubiquitin metabolism and associated with Rsp5p (Yashiroda *et al.*, 1996).

DISCUSSION

Here, we have shown that the ubiquitin ligase Rsp5p plays a role in regulating the major nuclear RNA biogenesis/export pathways in yeast. Remarkably, the *rsp5-3* mutant affects concomitantly the processing and/or nuclear export of tRNA, rRNA and mRNA without clearly affecting protein import. This combination of phenotypes has not been reported previously for nucleocytoplasmic transport mutants. It is conceivable that ubiquitination by Rsp5p of a single component of the nuclear pore complex that is required by all three nuclear export systems could affect global nuclear RNA export. This would not, however, readily explain the dramatic defects seen in RNA processing. Alternatively and more likely, Rsp5p may have multiple targets, which are part of the individual RNA biogenesis/export machineries. Since specific steps in rRNA and tRNA processing are severely affected in the *rsp5-3* mutant, it is likely that several substrates for Rsp5p participate in pre-rRNA and pre-tRNA processing. A high-throughput proteomic analysis recently identified many potentially ubiquitinated proteins in yeast (Peng *et al.*, 2003), including several ribosome synthesis factors and tRNA processing enzymes. Which of these ubiquitin residues are added directly by Rsp5p remains to be determined.

Pre-tRNA splicing is inhibited by mutation of the putative RNA helicase Sen1p, which was co-precipitated with Rsp5p-TAP, making it a potential target for Rsp5p activity. The abundances of numerous other RNAs are altered in *sen1-1* mutants, including rRNAs, small nuclear and small nucleolar RNAs, although the reported pre-rRNA processing defect does not resemble that of *rsp5-3* strains (Ursic *et al.*, 1997). Ubiquitinated pre-tRNA processing enzymes include the splicing endonuclease component Sen15p and the tRNA export factor Los1p/Exportin-t (Peng *et al.*, 2003). The observed pre-tRNA end processing defect must have a different basis, since this is independent of splicing (O'Connor & Peebles, 1991). An additional potential substrate for Rsp5p is a protein component of RNase P, since both the activity and maturation of this complex were inhibited. The depletion of the mature RNase P RNA did not, however, appear to be sufficient to account for the dramatic pre-tRNA accumulation observed, indicating that maturation and function are both affected.

Several early cleavages in the pre-rRNA were almost entirely blocked in the *rsp5-3* strain. This inhibition was seen within 1 h of transfer to the non-permissive temperature, and it seems probable that another direct substrate for Rsp5p can be found among the

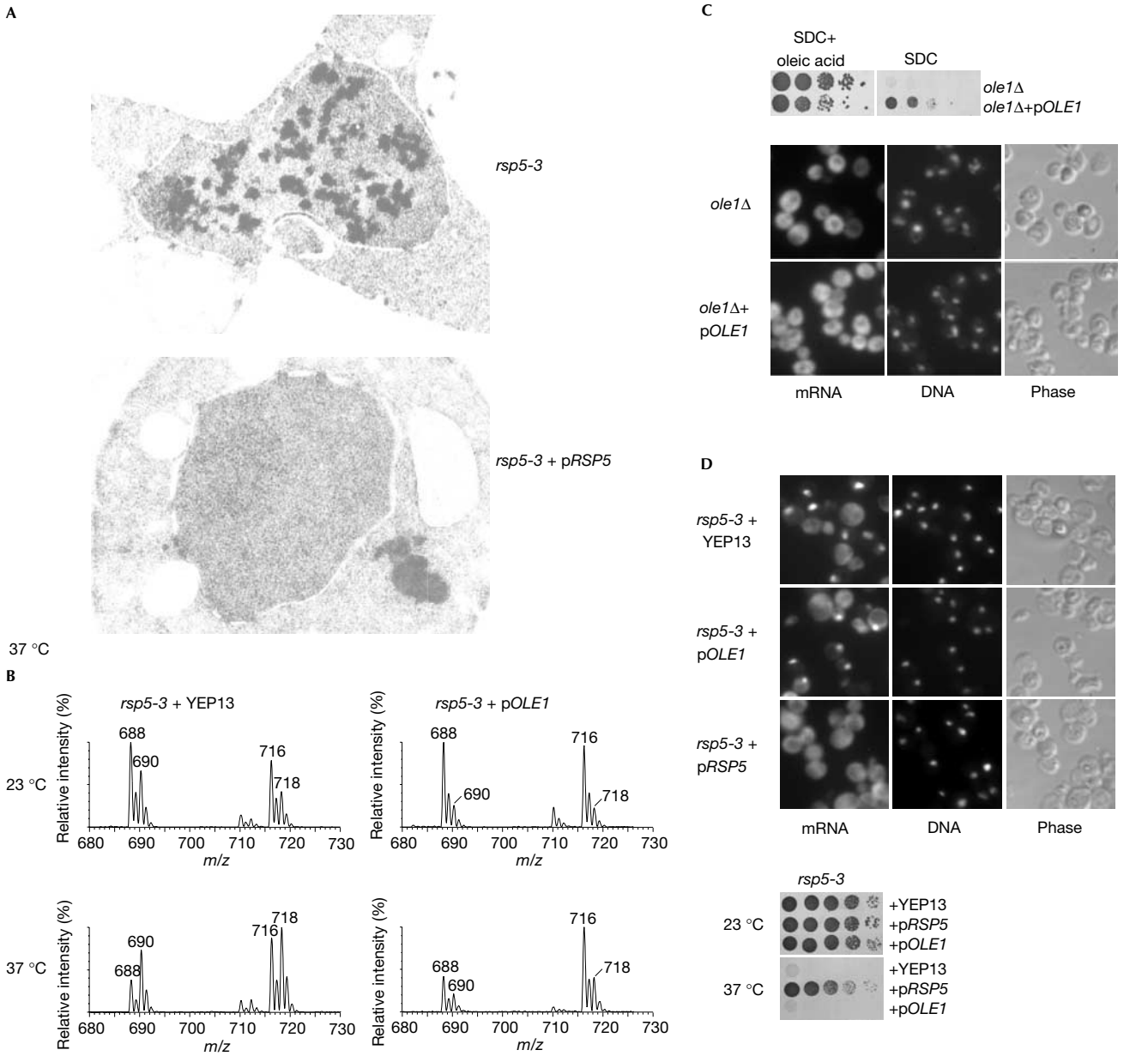


Fig. 4|The *rsp5-3* mutant is defective in nuclear structure and lipid synthesis. (**A**) *rsp5-3* cells with and without p*RSP5* grown at 23 °C and shifted to 37 °C were analysed by electron microscopy. (**B**) Phosphatidyl ethanolamine (PE) profiles of *rsp5-3* mutant cells and *rsp5-3* cells overexpressing Ole1p. (**C**) Growth of strains *ole1*Δ and *ole1*Δ complemented by p*OLE1* in SDC medium and SDC medium containing 0.5 mM oleic acid/1% tergitol (upper panel). *ole1*Δ strain shifted for 18 h to SDC medium without oleic acid was analysed for nuclear export of poly(A)$^+$ RNA (lower panel). (**D**) Analysis of mRNA export in the *ole1*Δ strain transformed with the indicated plasmids after shift for 3.5 h to 37 °C (upper panel). Cell growth was analysed after 3 days at 23 and 37 °C (lower panel).

many ribosome synthesis factors that are known to be required for these cleavages (Tschochner & Hurt, 2003), several of which are reported to be ubiquitinated (Peng *et al.*, 2003). However, pre-rRNA cleavage at site A_3, which is performed by RNase MRP, was not inhibited. RNase MRP is closely related to RNase P, and the defect in RNase P activity is therefore quite specific. Notably, two proteins required for 60S subunit export, Noc2p and Noc3p, were reported to be ubiquitinated, making them potential targets for Rsp5p (Milkereit *et al.*, 2001; Peng *et al.*, 2003).

In analogy, we expect that specific factors involved in pre-mRNA biogenesis, which link intranuclear mRNP formation with nuclear export, could be regulated by Rsp5p. Identification of these factors should further unravel the mechanism of how the ubiquitin ligase Rsp5p can control the three major RNA export pathways.

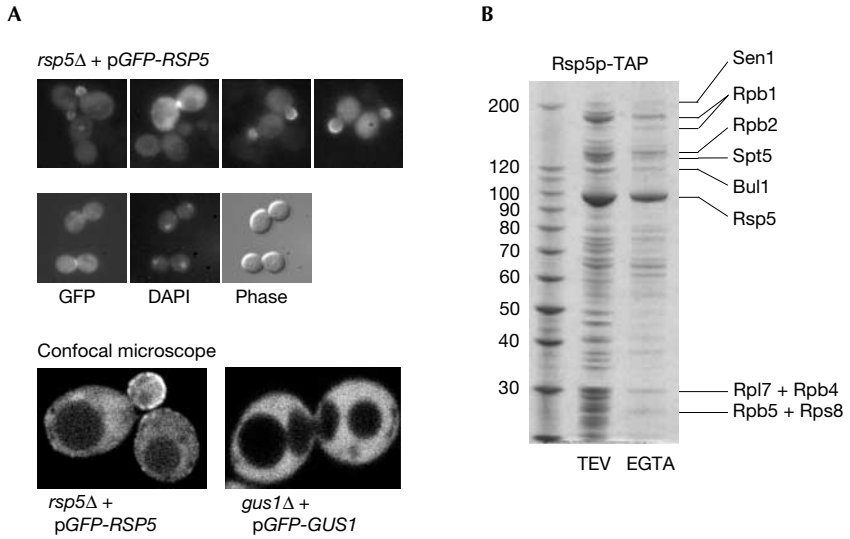


Fig. 5|Intracellular location and purification of Rsp5p. (**A**) *rsp5*Δ cells expressing GFP-Rsp5p were analysed in the fluorescence microscope (upper panel) and also stained with DAPI (middle panel). *rsp5Δ* cells expressing GFP-Rsp5p were analysed in the confocal microscope (lower panel). *gus1*Δ cells expressing GFP-Gus1p served as control. (**B**) TAP-tagged Rsp5p was purified by the tandem-affinity purification method. Co-purifying proteins were separated on an SDS–4–12% polyacrylamide gradient gel and stained with Coomassie. The bands were identified by mass spectrometry and are indicated. Both the TEV protease and EGTA eluate are shown.

Table 1|Yeast strains used

Name	Genotype	Origin
FY23	*MAT***a**, *ura3*, *trp1*, *leu2*	Derived from S288C
FY86	*MAT*α, *ura3*, *his3*, *leu2*	Derived from S288C
rsp5-3	*MAT***a**, *ura3*, *trp1*, *leu2*	Isolated from ts collection (Amberg *et al.*, 1992)
RSP5 shuffle	BY4743; *MAT***a**, *his3*, *leu2*, *ura3*, *rsp5::kanMX4*+p*URA3-RSP5*	Derived from Euroscarf strain Y26124
ole1Δ	BY4743; *MAT***a**, *his3*, *leu2*, *ura3*, *lys2*, *ole1::kanMX4*	Derived from Euroscarf strain Y24422
RSP5-TAP	*MAT*α, *ura3*, *leu2*, *trp1*, *his3*, *RSP5-CBP-TEV-protA::TRP1*	This work

METHODS

Yeast strains and plasmids. Yeast strains are shown in Table 1. Genomic integration of the TAP tag at the 3′-end of *RSP5* was performed as described previously (Grandi *et al.*, 2002). Used plasmids were pRS316-RPL25-GFP, pRPL25-NLS-GFP (Gadal *et al.*, 2001), pRS315-RPS2-GFP (Milkereit *et al.*, 2002) NPL3-NLS-GFP (Senger *et al.*, 1998) and pGFP-GUS1 (Galani *et al.*, 2001).

Lipid analysis. Lipids were extracted from yeast cells and analysed by MS (Brügger *et al.*, 1997). ESI-MS/MS analysis was performed on a Micromass QII triple-stage quadrupol tandem mass spectrometer equipped with a nano-ESI source from Micromass.

Screen for tRNA export (*tex*) mutants. A *ts* mutant collection (Amberg *et al.*, 1992) was screened for intranuclear accumulation of tRNA by FISH (intron-containing $tRNA^{Leu+Tyr}$ and intronless $tRNA^{Glu+Gly}$) at 37 °C (Grosshans *et al.*, 2000a).

Cloning of *TEX23*/*RSP5*. The complementing gene for *tex23-1* mutant cells was cloned by complementation of the *ts* phenotype through transformation with a yeast genomic library (Baßler *et al.*, 2001). The *rsp5-3 ts* allele, which was isolated by polymerase chain reaction and sequenced, was shown to restore the viability of *rsp5Δ* cells at 23 °C, but caused a *ts* phenotype at 37 °C.

Miscellaneous. Microbiological techniques, DNA manipulation, plasmid transformation and recovery, sporulation of diploids, tetrad analysis and fluorescence microscopy were performed according to standard protocols (Baßler *et al.*, 2001). Thin-section electron microscopy was carried out as described in Siniossoglou *et al.* (1998). Northern hybridization was performed as described previously (Tollervey *et al.*, 1993).

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