Maf1, REPRESSOR OF RNA POLYMERASE III, INDIRECTLY AFFECTS tRNA PROCESSING

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Running title: Indirect effect of Maf1 on tRNA processing

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Maf1 is negative regulator of RNA polymerase III in yeast. We observed high levels of both primary transcript and end-matured, intron-containing pre-tRNAs in the *maf1*Δ strain. This pre-tRNA accumulation could be overcome by transcription inhibition, arguing against a direct role of Maf1 in tRNA maturation and suggesting saturation of processing machinery by the increased amounts of primary transcripts. Saturation of the tRNA exportin, Los1, is one reason why end-matured intron-containing pre-tRNAs accumulate in *maf1*Δ cells. However, it is likely possible that other components of the processing pathway are also limiting when tRNA transcription is increased. According to our model, Maf1-mediated transcription control and nuclear export by Los1 are two major stages of tRNA biosynthesis that are regulated by environmental conditions in a coordinated manner.

INTRODUCTION

The existence of three RNA polymerases (Pol) is documented for all investigated eukaryotes. Pol I synthesizes ribosomal RNA (rRNA), Pol II produces mainly mRNAs, and Pol III generates tRNAs, 5S rRNA and other small noncoding RNAs. Each major category of nuclear-encoded RNA has unique processing, nucleus/cytoplasmic dynamics, and decay pathways. Generally, the regulation of individual categories of RNA occurs by mechanisms that are not shared with the other categories, probably providing a selective advantage by separately controlling mRNA, rRNA and tRNA synthesis in response to environmental changes. Recent studies have uncovered relationships between RNA transcription and RNA maturation. Co-transcriptional processing occurs during rRNA synthesis and most mRNAs (1-5), but it remains unknown whether there is co-regulation of Pol III transcription with any of the tRNA processing steps.

tRNA transcription is controlled by a general negative regulator, Maf1, conserved from yeast to man (6). Maf1 mediates several signaling pathways by repressing Pol III transcription in response to diverse stresses such as nutrient deprivation, rapamycin treatment, secretory defects, or DNA damage (7,8). Maf1 is the only negative Pol III regulator known in yeast; it is regulated by both PKA and TOR signaling pathways [for the review see (9,10)]. Under favorable growth conditions Maf1 is phosphorylated by PKA kinase, CK2 kinase, TORC1-dependent Sch9 kinase and TORC1 kinase itself (11-13). In conditions that slow growth Maf1 is dephospohorylated by PP2A phosphatase. The dephosphorylated form of Maf1 interacts with the Pol III complex and is thereby active as a repressor (8). Both, phosphorylation and dephosphorylation of Maf1 may occur inside the nucleus. Phosphorylated Maf1 is exported to cytoplasm by the Msn5 carrier, but nuclear export is not fundamental for appropriate regulation of Pol III (14).

Since Maf1 is a negative regulator of Pol III, its absence causes an increase in tRNA transcripts in yeast and mammals (6, 15). Elevated activity of mammalian Pol III is a recurring feature of mouse and human tumours (15). It has been shown that overexpression of Maf1 suppresses anchorage-independent growth of U87 glioblastoma cells (16). This suggests that

Maf1 is a potential tumour suppressor. As overexpression of the Pol III product, $tRNA_i^{Met}$, is sufficient to drive tumourigenesis in mice (17), it is therefore of great interest to better understand the involvement of Maf1 in global regulation of tRNA biosynthesis. We employed yeast as a powerful tool to study this regulation.

Inactivation of the *MAF1* gene in yeast leads also to increased translation fidelity (18). An explanation for how an excess of tRNA in *maf1*Δ affects translation remains unclear. The simplest hypothesis is that tRNAs transcribed in *maf1*Δ cells are incorrectly, or incompletely processed or they fail to be appropriately delivered to ribosomes.

tRNA processing involves multiple steps that occur in yeast at different subcellular locations [for review see (19)]. Initial transcripts are extended both at 5' and 3' termini and may contain introns. The 5' leader is removed from initial transcripts by RNase P endonuclease located in the nucleolus. Processing of 5' leader generally precedes trimming of 3' trailer which is followed by CCA addition at 3' termini. End-processed tRNAs are transported to the cytoplasm by the exportins Los1 and/or Msn5 (19-23). In yeast, 10 tRNA families are encoded by intron-containing genes. Introns are removed from end-processed transcripts by the splicing machinery consisting of the heterotetrameric tRNA splicing endonuclease located at the outer surface of mitochondria and the cytoplasmic pools of tRNA ligase and 2' phosphotransferase (24-26). tRNAs are modified and modifications are added throughout tRNA processing, both in the nucleus and in the cytoplasm. Thus, unlike pre-mRNA processing which is largely, if not completely co-transcriptional, pre-tRNA processing occurs in multiple subcellular locations.

To assess if tRNA transcription is coordinated with any part of the posttranscriptional tRNA biosynthesis process, we explored the possibility that perturbation of Maf1-mediated control of transcription could affect tRNA processing. Here we show accumulation of both the initial transcripts and end-processed, intron-containing tRNA precursors in the absence of Maf1, reflecting the imbalance between the rate of tRNA synthesis and efficiency of its maturation. These observations indicate that Maf1-dependent Pol III regulation plays an important role in synchronization of transcription with posttranscriptional events. Moreover, we have shown that saturation of Los1-dependent nuclear export of intron-containing tRNA precursors is responsible for the accumulation of pre-tRNA in *maf1Δ* nuclei.

EXPERIMENTAL PROCEDURES

Strains, Media, and Plasmids-Yeast strains BY4742 (*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0*) and isogenic BY4742 *maf1*Δ (BY4742 *maf1*::KanMX4) were purchased from Euroscarf. The *MSN5* locus in BY4742 was replaced by the natMX4 deletion cassette amplified from pAG25 plasmid (Euroscarf) creating BY4742 *msn5*Δ*::NAT*^{R}. *maf1*Δ *msn5*Δ was obtained by genetic cross. W303 *maf1*Δ (W303 *maf1*::KanMX4) was created in W303a (*MATα ade2-1 leu2-3 ura3-1 his3-11 trp1-1 can1-100*; Euroscarf). *sen2-42* mutant (*MAT a sen2*Δ*::NAT*^{R} *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) [*CEN6-URA3 sen-2-42*] was kindly provided by Dr. Yoshihisa. To express Los1-myc fusion protein, cassette encoding a 13 myc tag, an *ADH1* terminator, and a *kanMX6* selectable marker was amplified by PCR with target-gene-specific primers from pF6a-13myc-kanMx6 plasmid (27) and integrated immediately upstream of the stop codon of *LOS1* genomic locus in BY4742 strain. The *MAF1* gene was subsequently deleted in *LOS1*-myc strain by a *URA3* cassette (amplified from MB159-4D *maf1*Δ::URA3; 18). The sequences of primers used are available upon request.

Yeast media contained 1% yeast extract, 2% peptone and 2% glucose (YPD) or 2% glycerol (YPGly). The minimal medium (SC-ura) containing 0.67% yeast nitrogen base and 2% glucose or glycerol was supplemented with all necessary requirements without uracil (28). Solid media contained 2% agar. All reagents were from Difco. Temperature sensitive cultures of *sen2-42* were transferred to non-permissive temperatures at 37°C for 2h before RNA extraction. Induction of yeast cultures was done by galactose addition to 2% concentration and further incubation for 2h.

Plasmids used in this study were pFL44L (*URA3*, 2μ), YEp24 (*URA3*, 2μ), pLR233 (pFL44L-*RPR1*) (29) and YEpLOS1 (YEp24-*LOS1*) (30). The plasmids encoding inducible tRNA splicing endonuclease subunits and tRNA ligase were created in *Escherichia coli* strain XL1 blue using pRS412, pRS413, pRS414,

pRS415, and pRS416 (American Type Culture Collection) as backbones. Plasmids contained a *GAL1* promoter to allow inducible gene expression and GFP, both derived from pGP54a plasmid by restriction digest with *XhoI* and *EagI*, with the insertion of a stop codon after the GFP gene. Each digestion product was ligated into the pRS backbones. Sequences encoding splicing endonuclease subunit proteins and tRNA ligase were PCR amplified using HighFidelity TAQ polymerase (Invitrogen). Each PCR product was ligated into the pGEM-T (Promega) to obtain high quantities of the product, digested with *XmaI* and *EcoRI* and ligated into appropriate vectors. Vectors encoding only the galactose-inducible GFP were used as controls. All plasmid sequences were confirmed by DNA sequencing. Strains were transformed with all five inducible plasmids expressing tRNA splicing endonuclease subunits and tRNA ligase or with five control plasmids. Selection of yeast carrying the plasmids was accomplished by auxotrophic markers on the plasmids.

Northern analysis-Cells (30 ml of liquid culture; grown to 0.6 OD_{600nm}) were harvested by centrifugation and resuspended in 50 mM Na acetate, pH 5.3, 10 mM EDTA. Total RNA was isolated by heating and freezing the cells in the presence of SDS and phenol as described previously (31). For experiment presented in Fig. 5, small RNAs were extracted as described previously (32). RNA (10 µg per sample) was resolved by electrophoresis and transferred from gel to Hybond-N+ membrane (Amersham) with 0.5 x TBE by electroblotting and crosslinked by UV radiation (1200 mJ/cm^2) (33). The membrane was prehybridized in phosphate buffer (7% SDS, 0.5 M sodium phosphate pH 7.2, 1 mM EDTA pH 7.0, 1% BSA) and hybridized at 37°C in the same solution with oligonucleotide probes labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase (New England Biolabs). Following probes were employed: $tRNA^{Leu}$(CAA) intron: 5'-TATTCCCACAGTTAACTGCGGTCA-3; mature $tRNA^{Leu}$(CAA): 5'-CGATACCTGAGCTTGAATCAGG-3'; $tRNA^{Leu}$(CAA) 5'-GACCGCTCGGCCAAACAAC-3 (recognizing precursors as well as the mature form); $tRNA^{Phe}$(GAA): 5'-GCGCTCTCCCAACTGAG CT-3' (recognizing precursors as well as the mature form); $tRNA^{Ile}$(UAU): 5'-GTGGGGATTGAACCCACGACGGTCGCGT TATAAGCACGAAGCTCTAACCACTGGC TACA-3'; $tRNA_i^{Met}$(CAU): 5'-GTTTCGATCCGAGGACATCAG -3'; $tRNA^{His}$(GUG): 5'-GCCATCTCCTAGAATCGAAC-3'; $tRNA^{Gly}$(GCC): 5'-AAGCCCGGAATCGAACCGG-3'; 5.8S rRNA: 5'-GCGTTGTTCATCGATGC-3'; U3 snoRNA: 5'-GGATTGCGGACCAAGCTAA-3'; *RPR1*: 5'-GGCTCCACTGTGTTCCACCGAATTTCCCA C-3'.

After hybridization blots were washed 2 x 10 min with 1×SSC, 1% SDS and 3 x 10 min with 0.5× SSC, 0.1% SDS at 37°C, exposed to film or to a phosphoimager screen (Fujifilm). RNA was quantified using FLA-7000 PhosphoImager (Fujifilm). Band intensities were quantified using Multi Gauge V3.0 Software.

Fluorescence in situ hybridization (FISH)-Cells were grown at 30°C to early log phase in YPD, shifted to glycerol medium (YPGly) and cultivated at 37°C for the indicated period of time. Cells were prefixed, washed and converted to spheroplasts as previously described (34). Spheroplasts were attached to the poly-L-lysine coated slides. Prehybridization was conducted at 39°C for 3 h in a buffer containing 10% dextran sulfate, 2× SSC, 1x Denhardt's solution, 0.1% BSA, 0.1 mg/ml *E. coli* tRNA, 1mg/ml salmon sperm DNA and 0.8U/µl RNasin. Hybridization was conducted at 43°C overnight in the same buffer with addition of Cy3-labeled probes in concentration of 0.1 pmol/ul. The sequence of probes specific for pre-$tRNA^{Leu}$(CAA) was 5'-TATTCCCACAGTTAACTGCGGT CA-3. Cells were washed three times with 2× SSC at 50°C, once 1x SSC at 50°C and two times 1x SSC at room temperature. Cell nuclei were stained with 0.1 µg/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Samples were viewed using a Zeiss microscope and images were captured through a 100x objective.

Immunoluorescence-Cells were cultivated to early-log phase in YPD at 30°C. Immunofluorescence microscopy was performed according to a standard protocol (35). The primary anti-myc antibody (Millipore) at a 1:500 dilution and the

secondary anti-mouse Cy3 conjugated antibody (Millipore) at a 1:250 dilution were used for detection of Los1-myc fusion protein. Cell nuclei were stained with 0.1 μg/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Samples were viewed using a Zeiss microscope and images were captured through a 100x objective.

Quantitative β-galactosidase activity assay-Cells transformed with p180 reporter plasmid (p*GCN4-LacZ URA3 CEN*) (36) and grown overnight in SC-ura were inoculated to YPD, incubated to early log phase and split for two parts. One part of cells was harvested and the second part was shifted to YPGly and cultivated for 2h at 37°C. Protein extracts were prepared from cells and β-galactosidase activity was measured as described by previously (37).

Western analysis-Cells were broken and proteins isolated as described previously (14). Protein extracts were separated on 10% or 4-15% gradient SDS-PAGE and transferred to PVDF membrane by electroblotting. The membrane was blocked for 30 min in 10 mM Tris, 150 mM NaCl, and 0.05% Tween 20 containing 5% fat-free dry milk and then incubated with the appropriate antibodies. The following antibodies were used: rabbit Gcn4-specific antibody (Santa Cruz Biotechnology) at a 1:1000 dilution, mouse anti-Act1 antibody (Millipore) at a 1:2000, mouse anti-myc tag antibody (clone 9E10, Millipore) at a 1:5000 dilution; rabbit antibodies specific for phosphorylated Ser51 in eIF2α (Invitrogen) at 1:2000 and mouse anti-Vma1 antibody (Molecular Probes) at a 1:5000. To detect Act1, Los1-myc and Vma1 the membranes were incubated with the secondary HRP-conjugated anti-mouse antibody (DAKO). Gcn4 and eIF2α –P were detected by the use of secondary HRP-conjugated anti-rabbit antibody (DAKO). The proteins were visualized by chemiluminescence using the ECL detection kit (Millipore).

RESULTS

Inactivation of Maf1 results in accumulation of tRNA precursors-Despite its role as the sole negative regulator of Pol III transcription, Maf1 is not essential in yeast. The growth defect caused by *maf1*Δ is observed when cells are propagated on non-fermentable carbon sources at elevated temperatures (38). Initially, tRNA levels in *maf1*Δ cells incubated at non-permissive conditions were monitored by EtBr staining of RNA extracted from wild type and mutant cells (6). The increased levels were verified by analyses employing microarrays that contained sequences of all of the different yeast tRNA families and other genes transcribed by the Pol III (33). Comparison of Pol III transcribed RNAs from *maf1*Δ and wild type cells grown in medium with a non-fermentable carbon source shifted to elevated temperature by microarray analysis showed that the levels of RNAs were, on average, about 4-fold higher in the mutant cells. Importantly, not all tRNAs were elevated in *maf1*Δ to the same extent as they varied from over 10-fold to less than 2-fold different in abundance. Surprisingly, most of the tRNAs encoded by intron-containing tRNA genes were more elevated than were the tRNAs encoded by intron-lacking genes. For example, intron-containing $tRNA^{Phe}$(GAA) was increased over 11-fold, $tRNA^{Trp}$(CCA) – over 10-fold, $tRNA^{Leu}$(CAA) – nearly 9-fold but $tRNA^{Ile}$(UAU) only 2.5-fold (33).

Since both pre-tRNAs and mature tRNAs hybridize to the microarray and the absence of Maf1 seemed to preferentially affect tRNA products encoded by intron-containing genes, we wondered whether there might be a defect in splicing of these transcripts. To evaluate tRNA processing we employed Northern hybridization using tRNA-specific oligonucleotide probes. The levels of U3 snoRNA or 5.8S rRNA were comparable in *maf1*Δ and wild type cells and thus serve as internal controls. First, we monitored the processing of two representative intron-containing tRNAs, $tRNA^{Phe}$(GAA) and $tRNA^{Leu}$(CAA) (Fig. 1A, B). Both unprocessed initial transcripts (designated –▭–▭–) and end-matured intron-containing pre-tRNAs (designated ▭–▭) were increased in *maf1*Δ mutant relative to wild type cells. Noteworthy the relative amount of tRNA precursors varied depending on the carbon source utilized. Quantification of the Northern blots revealed that *maf1*Δ cells shifted to glycerol medium have, over 10-fold elevated levels of $tRNA^{Phe}$ initial transcript over its level in wild type cells cultivated under standard conditions in glucose medium. End-processed intron-containing pre-$tRNA^{Phe}$ was also elevated, about 2.5-fold, over

wild type. Lower fold of increase was detected in *maf1*Δ for both $tRNA^{Leu}$ precursors; however, the numbers varied depending on the probe specificity (compare Fig. 1A, and Fig. 1B). In contrast, the levels of spliced mature forms were only slightly affected by Maf1. It appears that, in the absence of Maf1, tRNA processing lags behind the elevated rate of Pol III transcription. Noteworthy, under restrictive conditions in glycerol medium, removal of pre-tRNA termini in *maf1*Δ lags behind more than in favorable conditions in glucose.

tRNAs encoded by genes lacking introns are also processed at 5' and 3' termini in the nucleus. Thus, we monitored by Northern hybridization the effect of Maf1 on end-processing for representative intron-free tRNAs: $tRNA_i^{Met}$(CAU), $tRNA^{His}$(GUG), $tRNA^{Gly}$(GCC) and $tRNA^{Val}$(AAC) Initial transcripts of $tRNA^{His}$ (Fig. 1C), $tRNA^{Gly}$ and $tRNA^{Val}$ (not shown) were hardly detectable by Northern blotting (even upon prolonged exposure) indicating efficient processing of 5' and 3' extensions. However, pre- $tRNA_i^{Met}$ precursors can be detected and they are significantly increased in *maf1*Δ cells (Fig. 1C). The results show that maturation of both categories of tRNAs encoded by intron-containing genes and intron-less tRNAs are defective in the absence of Maf1. Noteworthy, *maf1*Δ cells shifted to glycerol medium have about 3-fold elevated levels of mature $tRNA_i^{Met}$ and $tRNA^{His}$ (Fig. 1C).

Accumulation of pre-tRNA is correlated with loss of nuclear Maf1 function-Maf1 shuttles between nucleus and cytoplasm. The function of Maf1 in cytoplasm is unknown, but one possibility is that cytoplasmic Maf1 could be directly involved in tRNA splicing. To investigate this possibility we sought to examine pre-tRNA accumulation in *msn5*Δ cells in which localization of Maf1 is limited to the nucleus due to lacking of Maf1-specific exportin (14).

As assessed by Northern analyses, retention of Maf1 in the nucleus in *msn5*Δ cells resulted in a low level of pre-$tRNA^{Leu}$, especially when cells were cultivated under restrictive conditions (Fig. 2A, B). Since Msn5 is responsible for nuclear export of other proteins beside Maf1, theoretically their nuclear localization could indirectly cause increased tRNA processing in *msn5*Δ cells. We excluded this option by analyzing *maf1*Δ *msn5*Δ double mutant. The results showed that the double mutant accumulated elevated levels of both initial transcript and intron-containing end matured precursor (Fig. 2A, B). These results indicate that accumulation of pre-tRNA is correlated with loss of nuclear Maf1 function.

pre-tRNA accumulation in maf1Δ cells is due to elevated Pol III transcription-The increased pre-tRNA levels in *maf1*Δ cells raised the question as to how a lack of nuclear Maf1 affected tRNA maturation. In principle, Maf1 could function dually in the nucleus, by regulating tRNA transcription by Pol III and by regulating pre-tRNA processing. Alternatively, the observed pre-tRNA accumulation could be a secondary effect due to high levels of primary tRNA transcripts saturating the processing and/or the nuclear export machinery.

If the pre-tRNA processing defects in *maf1*Δ cells were caused by saturation of the processing or nuclear export machinery, they should be suppressed by a reduction of Pol III transcripts. To reduce *de novo* synthesis of tRNA, cells were treated with thiolutin, which inhibits RNA polymerases, including Pol III (39). As expected, the thiolutin treatment resulted in a gradual disappearance of pre-$tRNA^{Leu}$ in wild type cells (Fig. 3A) with no detectable changes of mature $tRNA^{Leu}$ levels (Fig. S1). Both primary transcripts and intron-containing end-matured $tRNA^{Leu}$ precursors, initially elevated in *maf1*Δ cells were reduced upon thiolutin treatment. Finally, after two hours of incubation in the presence of thiolutin, pre-$tRNA^{Leu}$ transcripts disappeared in *maf1*Δ cells similarly as in the control wild type (Fig. 3B). We thus concluded that tRNA precursors accumulate in *maf1*Δ cells because their transcription is elevated and maturation simply lags behind. Consequently, we focused our further study on the identification of the rate limiting steps in tRNA maturation.

Saturation of RNase P RNA is not a reason of pre-tRNA accumulation in the absence of Maf1-First, we explored the possibility that accumulation of untrimmed initial transcripts in *maf1*Δ cells is due to saturation of RNase P, which removes the 5' leader preceding removal of the 3' trailer (40). *RPR1*, the gene encoding the RNA subunit of RNase P, is transcribed by Pol III (41). It is known that Maf1 does not have the same effects on all Pol III genes. In fact, *RPR1* RNA levels are only slightly enhanced in *maf1*Δ cells compared to the substantially increased tRNA pool (33). Therefore, it is possible that the amount of *RPR1* required for RNase P activity could be disproportionately low in *maf1*Δ cells, causing inefficient processing of

5' termini. To increase levels of *RPR1 RNA*, we transformed wild type and *maf1*Δ cells with a multicopy plasmid carrying *RPR1*. The resulting transformants showed increased levels of *RPR1* precursor and mature forms as observed previously by others (42) (Fig. 4A). *RPR1* is a limiting factor for 5' end removal in wild type cells since *RPR1* overexpression resulted in a decrease of the initial 5'-extended $tRNA^{Leu}$ transcript (Fig. 4B). However, *RPR1* overexpression did not reduce the level of 5'-extended pre-tRNA transcripts in *maf1*Δ cells (Fig. 4B). Thus, the elevated level of 5'-extended pre-tRNA transcripts in *maf1*Δ cells is unlikely to be due to limiting quantities of *RPR1*. In contrast, a shortage of *RPR1* RNA was previously reported as the reason for initial transcript accumulation in a mutant defective in Pol III initiation (43). Although the elevated level of 5'-extended pre-tRNA transcripts in *maf1*Δ cells is unlikely to be due to limiting quantities of *RPR1*, it still could result from limiting one or more of the nine RNase P protein subunits.

*Cytoplasmic Sen-complex is not the limiting factor for processing of intron-containing pre-tRNA accumulating in maf1*Δ *cells*-In *Saccharomyces cerevisiae*, unlike in vertebrates, the intron-containing tRNA precursors are exported from the nucleus and pre-tRNA splicing occurs in the cytoplasm at mitochondrial membrane (25). Significantly, strains lacking Maf1 have growth defects in medium with a non-fermentable carbon source indicating a defect in mitochondrial function. This raised the possibility that accumulation of intron-containing tRNA in *maf1*Δ may be due to deficiency of mitochondrially-localized tRNA splicing endonuclease.

To address whether end-mature intron-containing pre-tRNAs might accumulate in *maf1*Δ cells due to the saturation of cytoplasmic splicing endonuclease, we expressed galactose inducible splicing endonuclease in wild type and *maf1*Δ cells. We generated strains bearing each of the four genes specifying GFP-tagged splicing endonuclease subunits, *SEN2*, *SEN15*, *SEN34*, *SEN54*, as well as the tRNA ligase *TRL1* on plasmids under galactose regulation. As anticipated, the tRNA splicing components are appropriately located in wild type and *maf1*Δ cells, Sen subunits to mitochondria and ligase to the cytoplasm (not shown). To ensure that splicing endonuclease proteins being expressed from the plasmids were functional, we transformed plasmids expressing inducible splicing endonuclease subunits into a temperature sensitive splicing mutant, *sen2-42*, that is known to accumulate intron-containing tRNAs at the non-permissive temperature (44). Under galacatose-induction, the *sen2-42* mutant expressing inducible Sen subunits and tRNA ligase no longer displayed an accumulation of end-matured, intron-containing pre-tRNAs at non-permissive conditions although this accumulation was observed when expression of splicing factors was not induced (Fig. 5, lane 10 compared to 12). Therefore the overexpressed splicing endonuclease proteins are functional. We then assessed whether overexpression of these genes alleviates the accumulation of intron-containing pre-tRNAs in *maf1*Δ cells. When expression of splicing endonuclease proteins was induced by the addition of galactose to the medium, *maf1*Δ cells continued to accumulate intron-containing pre-tRNAs at comparable levels to cells that were transformed with empty vectors or with not induced splicing machinery (Fig. 5, compare lanes 1, 2, 3 and 4). Wild type cells transformed with the same vectors did not show any difference in pre-tRNA processing when compared to these transformed with empty vectors. The data indicate that limitation of the cytoplasmic splicing machinery does not account for accumulation of intron-containing pre-tRNAs in *maf1*Δ cells.

*Saturation of nuclear export machinery in maf1*Δ-Since the yeast tRNA splicing factors are located in the cytoplasm, accumulation of intron-containing end matured precursors in *maf1*Δ cells may be caused by saturation of nuclear export machinery. Inefficient export of pre-tRNAs to the cytoplasm would cause intron-containing tRNAs to be physically separated from splicing machinery (45). Employing FISH analyses and probe specific to the intron of pre-$tRNA^{Leu}$ we showed elevated levels of pre-tRNA in nuclei of *maf1*Δ cells as compared to wild type cells (Fig. 6). We assumed that initial transcript together with the end-matured intron-containing tRNA transcript account for this effect since these pre-tRNA forms are indistinguishable by FISH. We thus explored whether elevated level of Los1 exportin in *maf1*Δ saturate the tRNA nuclear export machinery.

We overproduced Los1 by transformation of yeast with a *LOS1*-containing multicopy plasmid and examined the level of pre-tRNA accumulation by Northern blotting (Fig. 7). As anticipated (46), control *los1*Δ cells accumulated

end-matured intron-containing, pre-tRNA (Fig. 7A). Quantification of the Northern blot revealed 3-fold elevated levels of intron-containing end-matured pre-tRNA^{Leu} in *los1*Δ cells over that of the wild type when corrected for the U3 snoRNA levels (Fig. 7B). Plasmid-encoded Los1 fully compensated the elevated levels of pre-tRNA in *los1*Δ and did not affect cell growth. Importantly, *LOS1* overexpression partially suppressed pre-tRNA accumulation in *maf1*Δ cells grown in minimal glucose medium (Fig. 7A). Both, initial transcript and end-matured intron-containing pre-tRNA^{Leu}, were reduced in *maf1*Δ glucose cells by a *LOS1*-containing multicopy plasmid, respectively about 2- and 3-fold (Fig. 7B). We thus conclude that Los1 level is limiting for processing of intron-containing pre-tRNAs in *maf1*Δ cells under standard growth conditions. Noteworthy, increased amount of Los1 affects also levels of initial transcript by an unknown mechanism.

Under restrictive conditions in glycerol medium *LOS1*-containing multicopy plasmid had, however, no detectable effect on pre-tRNA^{Leu} accumulation in *maf1*Δ cells (Fig. 7A, B). We explored a possibility that growth in glycerol medium may influence expression or localization of Los1. It has been previously reported that Los1 is mislocalized to cytoplasm upon DNA damage causing increased nuclear accumulation of intron-containing tRNA (47). We therefore examined localization of C-terminally myc-tagged Los1 in control and *maf1*Δ cells grown under standard (YPD) or restrictive (YPGly, 37°C) conditions (Fig. 8). Los1-myc was detected primarily in the nucleus of cells cultivated on glucose and no obvious difference in its location was observed between wild type and *maf1*Δ cells (Fig. 8A). By contrast a large part of Los1-myc became redistributed to the cytoplasm in wild type and *maf1*Δ cell populations grown in glycerol medium (Fig. 8B). Noteworthy, neither growth conditions nor the absence of Maf1 effect Los1 expression (Fig. S2).

Thus, it is most likely that under normal conditions insufficient amount rather than inappropriate location of Los1 accounts for pre-tRNA accumulation in *maf1*Δ. Growth in medium with a nonfermentable carbon source additionally limits tRNA export by mislocalization of Los1 to cytoplasm and therefore its overproduction does not prevent pre-tRNA accumulation in *maf1*Δ.

*Induction of Gcn4 expression in maf1*Δ *cells*- Both, aberrant tRNA processing and limited tRNA export have marked effects upon the translation of the transcription factor, Gcn4. (47,48). Conditions that activate Gcn4 reduce the rate of general protein synthesis while simultaneously activating expression of proteins required under conditions of starvation and stress [reviewed in (49)]. Hence, we investigated whether accumulation of pre-tRNA in *maf1*Δ similarly results in elevated Gcn4 translation. We utilized a widely employed CEN-based reporter plasmid carrying a *GCN4* promoter and *GCN4* UTR translation regulatory sequences driving the expression of a *lacZ* gene that provided a quantitative measure for Gcn4 by assessing β-galasctosidase activity (36). The *GCN4-lacZ* reporter plasmid was introduced into wild type and *maf1*Δ cells and resulting transformants were grown under standard conditions with a shift to glycerol medium and incubation at elevated temperatures. There was greater than 2-fold increased expression of *GCN4-lacZ* in *maf1*Δ cells under restrictive conditions compared to wild type cells (Fig. 9A). The induction of Gcn4 expression in *maf1*Δ was confirmed by western blot analysis. Total proteins isolated from wild type and *maf1*Δ cells were separated on SDS polyacrylamide gel, followed by incubation with anti-Gcn4 antibodies. Elevated levels of the Gcn4 protein were detected in *maf1*Δ cells, after shift to restrictive conditions (YPGly 37°C) (Fig. 9B). Although effect of Maf1 inactivation on Gcn4 expression is relatively weak, increased Gcn4 translation correlates with *GCN4-lacZ* induction in *maf1*Δ cells and is linked to pre-tRNA nuclear accumulation observed in the same growth conditions. Moreover, accumulation of tRNA precursors in *maf1*Δ nuclei activate Gcn4 in a manner independent on phosphorylation of eIF2α. (Fig. S3). These observations are consistent with results of Qiu et al., 2000 (48) and support the existence of a system communicating immature tRNA in the nucleus to the translation machinery.

DISCUSSION

tRNAs are essential components of the translation machinery and therefore their biosynthesis is tightly coupled to cell growth and metabolism. A mechanism for this coordination is provided by Maf1, a general negative regulator of RNA polymerase III transcription. Maf1 is essential for down-regulation of tRNA transcription during a transition from

fermentative to glycerol-based medium. Previously we showed that tRNAs encoded by intron-containing genes are the most sensitive to Maf1-mediated regulation (33). Those results led us to examine the potential effect of Maf1-mediated regulation on tRNA processing. Since the 5' and 3' ends and splicing junctions in the intron-containing tRNAs are not conserved and the introns themselves vary in sequence and length, we probed two intron-containing tRNAs to verify the ubiquity of the Maf1 effects. Both primary transcripts and end-processed intron-containing tRNA precursors were abnormally abundant in the absence of Maf1. It is noteworthy that *maf1*Δ cells have also elevated primary transcript of intron-less $tRNA_i^{Met}$ indicating an effect on the end-processing of this particular pre-tRNA. Initial transcripts of other tRNAs encoded by genes lacking introns are hardly detectable but the mature forms are more abundant in *maf1*Δ cells than wild-type cells. These data are consistent with our previously published results which showed increased levels of mature $tRNA^{His}$ in *maf1*Δ cells grown in medium with a nonfermentable carbon source (6). Since 80% of the tRNAs in yeast are encoded by intron-less genes, they account for the global increase of tRNA in *maf1*Δ observed earlier on ethidium bromide stained gels (6). Additionally, accumulation of unprocessed intron-containing transcript accounts for relatively high increase of intron-containing tRNAs (33).

In response to changing growth conditions, Maf1 shuttles between nucleus and cytoplasm (14). The cytoplasmic location provides a mechanism to prevent Maf1 from interaction with the transcription machinery under favorable growth circumstances. One tempting hypothesis, excluded here, was that Maf1 might be involved in tRNA splicing in the cytoplasm independent from its known nuclear role in negatively regulating Pol III transcription. Instead we have shown that, Maf1-mediated repression of Pol III transcription in the nucleus indirectly influences pre-tRNA maturation.

Further we sought to identify the step in tRNA maturation which is saturated by the high levels of primary transcripts produced when Maf1 is missing. Our data argue against saturation of *RPR1*, RNA component of RNase P machinery in *maf1*Δ nuclei, found before as the limiting for RNase P-mediated processing (42, 43, 47, 48). In addition we excluded saturation of cytoplasmic splicing machinery in the absence of Maf1 and detected an increased amount of intron-containing tRNA in *maf1*Δ nuclei (Fig. 6). We found that *maf1*Δ cells are defective in nuclear export of intron-containing tRNAs.

We then addressed the issue of why mainly processing of tRNAs encoded by intron-containing tRNA genes is altered in *maf1*Δ cells. First, *maf1*Δ may preferentially transcribe intron-containing tRNA genes. Second, end-maturation could be more efficient in tRNAs encoded by intron-less genes. Finally, there are more pathways for nuclear export of intron-less tRNA than for intron-containing tRNA. So far, intron-containing tRNAs can be exported to the cytoplasm by only exportin, Los1. In contrast tRNAs lacking introns can be exported by two exportins, Los1 and Msn5 (50). Thus, two different categories of tRNAs may be differently regulated at the nuclear export step.

It is quite possible that in addition to Los1-limited tRNA nuclear export in *maf1*Δ cells also other processes may be limited for efficient pre-tRNA processing, especially under unfavorable conditions. Systematic regulation of tRNA processing by environmental growth conditions has not been experimentally addressed so far. Possibly growth of cells in glycerol-medium at 37°C negatively affects early steps of tRNA processing. Nevertheless, among the various steps of tRNA maturation, Los1-mediated tRNA trafficking seems to be a main target for selective control. It was recently shown that inactivation of Xpo-t, the Los1 homologue in mammals, resulted in elevated autophagy and reduced activity of mTORC1 pathway. The authors suggested that the subcellular localization of tRNA plays a role in the signaling a nutritional stress in mammalian cells (51). In yeast, regulation of tRNA export contributes to the proper execution of G1 checkpoint (47). This response is mediated via differential cellular localization of Los1. Normally Los1 shuttles between the nucleus and the cytoplasm whereas after DNA damage Los1 is mainly found in the cytoplasm. Although the mechanism is not clear, localization of Los1 is controlled by the cell cycle checkpoint proteins Rad53 and Mec1. Los1 intracellular distribution is also dependent on nutrient supply and Snf1-mediated signaling (52). As a consequence of the Los1 mislocalization, intron-containing pre-tRNAs accumulate in the nucleus (47).

According to our model (Fig. 10) transcription and nuclear export are two major controls of tRNA biosynthesis. Both Maf1-

mediated Pol III regulation and Los1-dependent nuclear export are regulated by environment circumstances in coordinated manner. Under favorable growth conditions in glucose medium Maf1 is phosphorylated thus preventing Pol III repression and Los1 is localized in nuclear membrane providing active tRNA export. Following a shift to repressive conditions, tRNA transcription is inhibited due to Maf1 dephosphorylation and concurrently tRNA export is lowered by redistribution of Los1 to the cytoplasm. By mutual regulation of transcription and export, differences in growth conditions have a substantial effect on the relative levels of pre-tRNA and mature tRNA species. In contrast, transcription and export are uncoupled in strain lacking Maf1 resulting in abnormal high levels of pre-tRNA. It will be interesting to assess the joint regulation of Maf1 and Los1 by cellular signaling.

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FOOTNOTES

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FIGURES LEGENDS

Fig. 1. ***maf1*Δ cells accumulate tRNA precursors.** *maf1*Δ and wild type (wt) control strain BY4742 were grown to mid-log phase in rich glucose medium (YPD) and one half of each culture was shifted to rich medium containing glycerol (YPGly) and cultivated for 2h at 37°C. Northern analysis of intron-containing $tRNA^{Phe}$(GAA) and $tRNA^{Leu}$(CAA) using probes that recognize both, pre-tRNA and mature tRNA. (A); intron-containing $tRNA^{Leu}$(CAA) using distinct probes, specific to precursors or mature tRNA. (B); intron-less $tRNA_i^{Met}$(CAU) and $tRNA^{His}$(GUG)) (C). The sequences of tRNA-specific oligonucleotide probes are specified in Experimental Procedures. Positions of initial transcripts, end-processed intron-containing pre-tRNA and mature tRNA are indicated. pre-tRNAs and

mature tRNAs were quantified. Bars represent levels for primary transcript retaining 5' and 3' termini, intron-containing precursor and mature form normalized to loading control. The fold of change in *maf1*Δ cells cultivated under indicated conditions was calculated relative to expression in control strain (wt) at standard conditions (YPD). Standard deviation was calculated on the basis of three independent experiments.

Fig. 2. **pre-tRNA levels depend on nuclear Maf1.** (A) Wild type BY4742 (wt), *maf1*Δ, *msn5*Δ and *maf1*Δ *msn5*Δ cells were grown to mid-log phase in rich glucose medium (YPD) and one half of each culture was shifted to rich medium containing glycerol (YPGly) and cultivated for 2h at 37°C. Northern hybridization was performed with probes specific to pre-tRNA^{Leu} and mature tRNA^{Leu} (CAA). 5.8S rRNA served as a loading control. (B) Quantification of primary transcripts (—▭-▭—) and end-matured, intron-containing pre-tRNA^{Leu} (CAA) (▭-▭) levels. The fold of change in each strain cultivated under indicated conditions was calculated relative to expression in (wt) control strain at the same growth conditions. Standard deviation was calculated on the basis of three independent experiments.

Fig. 3. **Transcription inhibition prevents accumulation of pre-tRNA in *maf1*Δ cells.** *maf1*Δ and wt cells were grown to mid-log phase in rich glucose medium (YPD) and then were treated with 5 μg/ml thiolutin. Cells were harvested just before (t0) and after thiolutin treatment at the indicated time points. (A) Northern analysis was performed with probe recognizing pre-tRNA^{Leu}(CAA). The positions of primary transcript and end-matured, intron-containing precursor were indicated. U3 snoRNA served as a loading control. (B) Quantification of pre-tRNA^{Leu}(CAA) levels in wt (solid lines and closed squares) and *maf1*Δ (dashed lines and open squares). Curves show the ratio of primary transcripts (—▭-▭—) and end-matured, intron-containing pre-tRNA^{Leu}(CAA) (▭-▭) at each time point relative to its levels in the wild type strain immediately before thiolutin addition (t0). Each value itself was first normalized to U3 snoRNA. Standard deviation was calculated on the basis of three independent experiments.

Fig. 4. **Overexpression of *RPR1* gene has no effect on pre-tRNA accumulation in *maf1*Δ cells.** Wild type BY4742 (wt) and *maf1*Δ cells transformed with control plasmid pFL44L (designated []) or overexpressing *RPR1* ([*RPR1*]) were grown in SC-ura medium containing 2% glucose, shifted to SC-ura medium containing 2% glycerol and cultivated for 2h at 37°C. (A) Northern hybridization was performed with probes specific to RNA *RPR1*, pre-tRNA^{Leu} (CAA) or U3 snoRNA (used as a control). (B) Bars indicate the ratio of initial transcript and end-matured, intron-containing pre-tRNA^{Leu} (CAA) levels relative to their levels in wild-type cells bearing control plasmid pFL44L (designated wt/[]). The levels of *RPR1*precursor and its mature form were quantified. Bars represent the ratio of both *RPR1* transcripts relative to their levels in wild-type cells bearing control plasmid pFL44L (designated wt/[]). Each value itself was first normalized to U3 snoRNA. Standard deviation was calculated on the basis of three independent experiments.

Fig. 5. **Cytoplasmic splicing endonuclease is not the limiting for processing of intron-containing pre-tRNA that accumulate in *maf1*Δ cells**. Wild type W303 (wt), W303 *maf1*Δ (*maf1*Δ) or control W303 *sen2-24* (*sen2-24*) strains were transformed with plasmids bearing cytoplasmic tRNA splicing endonuclease genes under control of galactose-inducible promoter. As a control used the same strains transformed with the empty vectors. Transformants were grown in minimal glucose medium supplemented as required. + indicates induction by galactose addition to final concentration 2% for 2h; - indicates no induction; 23°C and 37°C are the temperature conditions. Northern hybridization was performed with probes specific to tRNA^{Ile}(UAU). Lanes: 1: *maf1*Δ with induced plasmids bearing cytoplasmic tRNA splicing endonuclease genes; 2: *maf1*Δ with induced empty vectors; 3: *maf1*Δ with un-induced plasmids bearing cytoplasmic tRNA splicing endonuclease genes; 4: *maf1*Δ with un-induced empty vectors; 5: wt with induced plasmids bearing cytoplasmic tRNA splicing endonuclease genes; 6: wt with induced empty vectors; 7: wt with un-induced plasmids bearing cytoplasmic tRNA splicing endonuclease genes 8: wt with un-induced empty vectors; 9: *sen2-42* with un-induced plasmids bearing cytoplasmic tRNA splicing endonuclease genes at 23°C; 10: *sen2-42* with un-induced plasmids bearing cytoplasmic tRNA splicing endonucleases genes at 37°C; 11: *sen2-42* with

induced plasmids bearing cytoplasmic tRNA splicing endonuclease genes at 23°C; 12: *sen2-42* with induced plasmids bearing cytoplasmic tRNA splicing endonuclease genes at 37°C. RNAs for lanes 9-12 were extracted and probed at the same times as those in lanes 1-8 but were run on a separate gel).

Fig. 6. ***maf1*Δ cells accumulate pre-tRNA^{Leu}(CAA) in the nucleus.** *maf1*Δ and control wild type cells were grown in glucose medium (YPD) at 30°C to early-log phase. Part of the culture was shifted to glycerol medium (YPGly) for 2 h at 37°C. Cultures were harvested and subjected to fluorescence in situ hybridization (FISH). The Cy3 labeled probe specific to sequence of pre-tRNA^{Leu}(CAA) intron and DAPI staining were applied.

Fig. 7**. Los1 is limiting for intron–containing tRNA export and processing in *maf1*Δ cells.** Wild type (wt) BY4742, *los1*Δ and *maf1*Δ cells, transformed with control plasmid Yep24 (designated []) or overexpressing *LOS1* ([*LOS1*]) were grown in SC-ura glucose medium. Part of the culture was shifted to minimal glycerol medium (Sc-ura + glycerol) for 2 h at 37ºC. (A) Northern hybridization was performed with probes specific to pre-tRNA^{Leu}(CAA)or U3 snoRNA (used as a control). (B) Quantification of the pre-tRNA^{Leu} (CAA) levels. Bars represent levels for primary transcript retaining 5' and 3' termini and intron-containing precursor of tRNA^{Leu}(CAA) normalized to U3 snoRNA transcript. The fold of change in each strain was calculated relative to expression in wild-type strain bearing control plasmid YEp24 (designated as wt/[]). Standard deviation was calculated on the basis of three independent experiments.

Fig. 8. **Localization of Los1 exportin is affected by growth conditions but not by Maf1.** Strain BY4742 *LOS1-myc* (wt) and BY4742 *maf1*Δ *LOS1-myc* (*maf1*Δ) were grown to early logarithmic phase in YPD. Part of the culture was shifted to glycerol medium (YPGly) for 2 h at 37ºC. Localization of Los1 was examined by indirect immunofluorescence microscopy using anti-myc antibodies. Nuclear DNA was stained with DAPI.

Fig. 9. **Gcn4 induction in *maf1*Δ cells**. A *GCN4-lacZ* (*CEN*, *URA*) reporter plasmid was introduced into wild type and *maf1*Δ mutant cells. Transformants were grown in YPD grown to early log phase in rich glucose medium (YPD) at 30°C, then transferred to glycerol medium (YPGly) and incubated at 37°C for 2 h. (A) β-galactosidase activity was measured in yeast extracts and correlated to protein concentration (arbitrary units). (B) total proteins were isolated as described in Experimental Procedures. Equal amounts of proteins were resolved on 10% SDS–polyacrylamide gel and subjected to immunoblot analysis using specific rabbit anti-Gcn4 or mouse anti-Act1 antibodies (used as a loading control).

Fig. 10. **Maf1-mediated transcription control and nuclear export by Los1 are regulated by environmental conditions in coordinated manner**. (A) Under favorable growth conditions in glucose medium Maf1 is phosphorylated thus preventing Pol III repression and Los1 is localized in nuclear membrane providing active export of intron-containing tRNA precursors (*wt* panel). tRNA synthesis is increased in the absence of Maf1 but export is saturated (*maf1*Δ panel). (B) Under repressive conditions on glycerol medium both tRNA synthesis and tRNA export are decreased, respectively, by Maf1-Pol III interaction and Los1 redistribution to the cytoplasm (*wt* panel). This coupling is lost in the absence of Maf1 resulting in nuclear accumulation of intron containing pre-tRNAs (*maf1*Δ panel).

Fig. 1

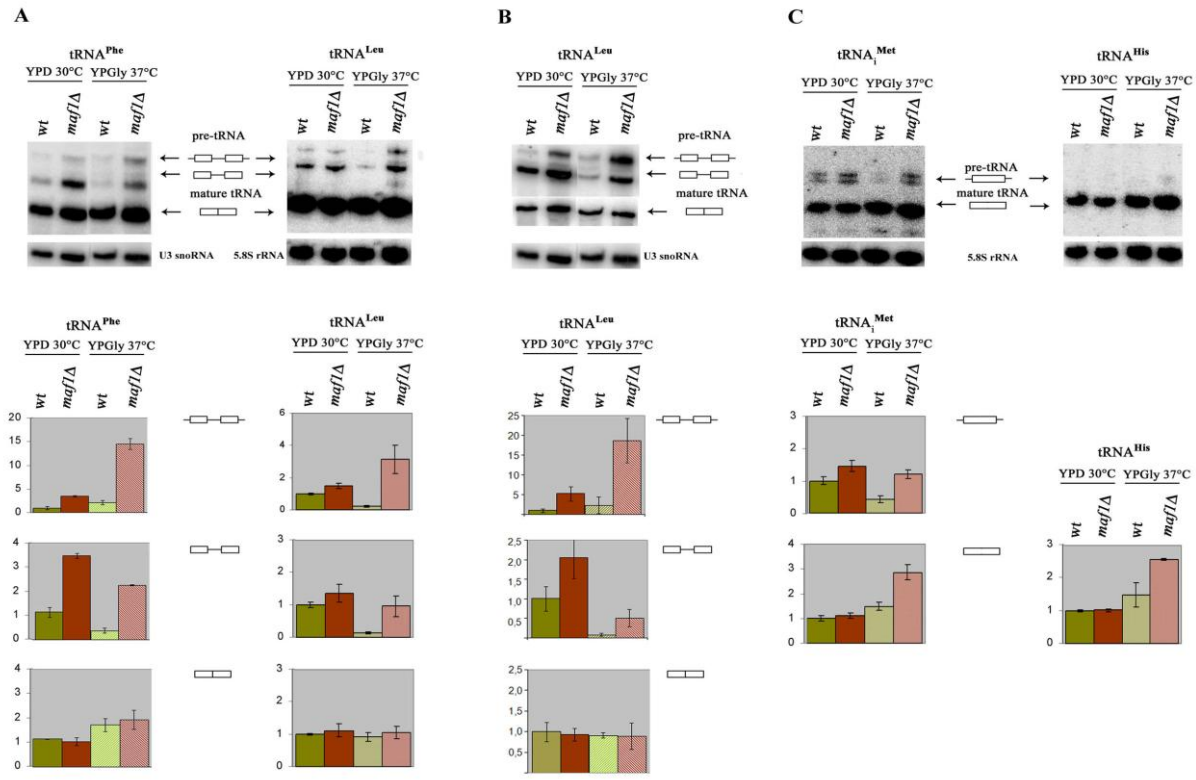

A
B
C
tRNA^Phe
tRNA^Leu
tRNA^Met_i
tRNA^His
YPD 30°C
YPGly 37°C
wt
maf1Δ
pre-tRNA
mature tRNA
U3 snoRNA
5.8S rRNA

Fig. 2

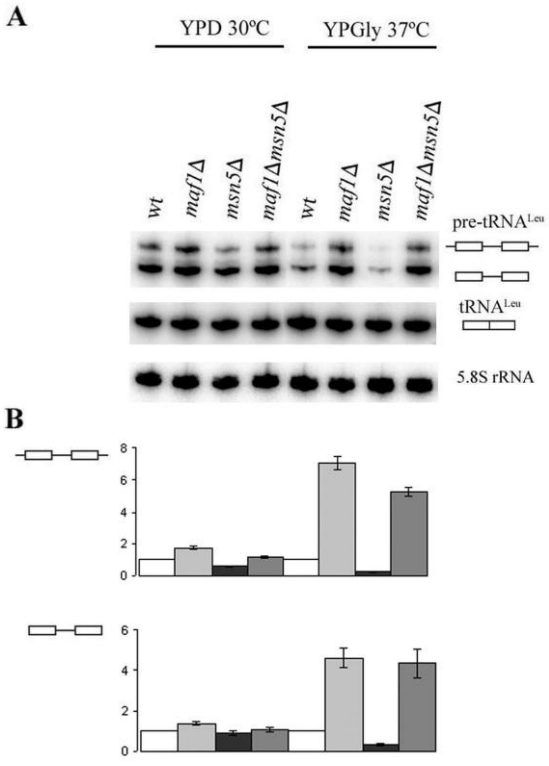

A
YPD 30°C
YPGly 37°C
wt
maf1Δ
msn5Δ
maf1Δmsn5Δ
pre-tRNA^Leu
tRNA^Leu
5.8S rRNA
B

Fig. 3

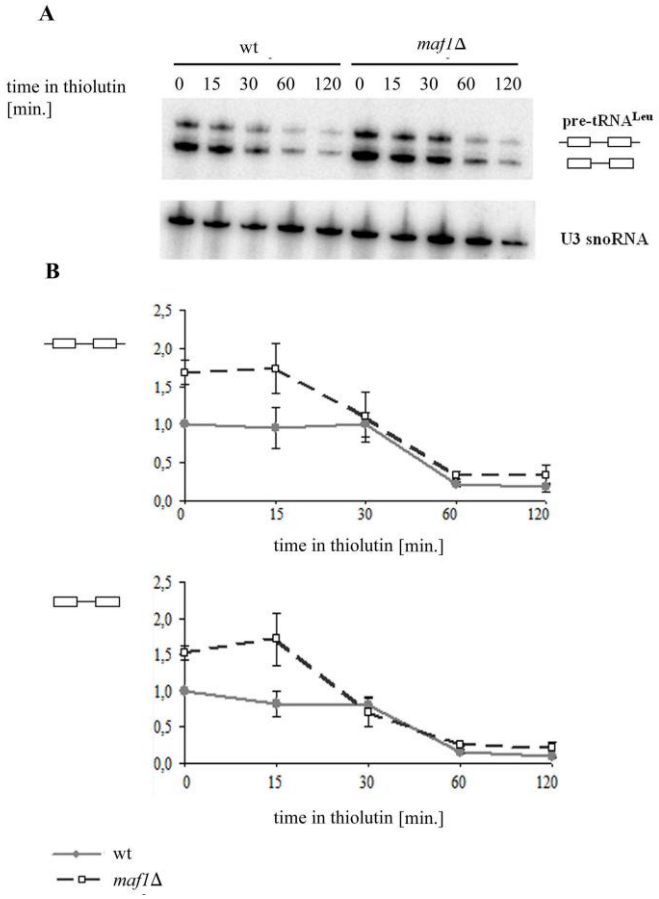

A
wt
maf1Δ
time in thiolutin [min.]
0 15 30 60 120 0 15 30 60 120
pre-tRNALeu
U3 snoRNA
B
2,5
2,0
1,5
1,0
0,5
0,0
0 15 30 60 120
time in thiolutin [min.]
time in thiolutin [min.]
wt
maf1Δ

Fig. 4

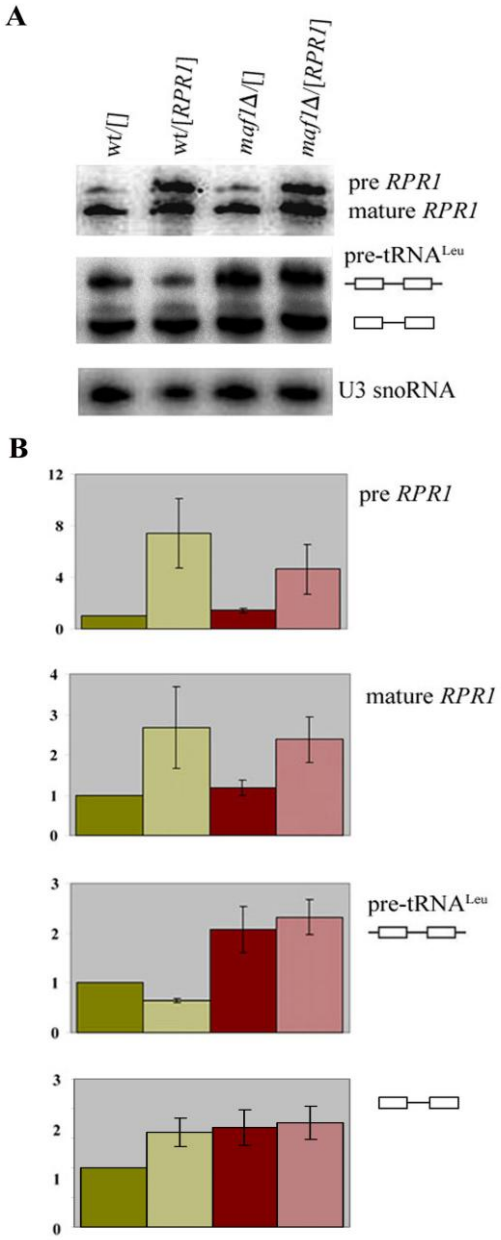

A
wt/[]
wt/[RPR1]
maf1Δ/[]
maf1Δ/[RPR1]
pre RPR1
mature RPR1
pre-tRNALeu
U3 snoRNA
B
pre RPR1
mature RPR1
pre-tRNALeu

Fig. 5

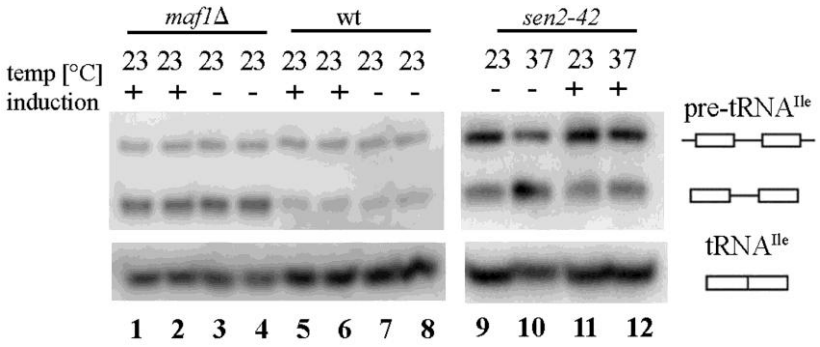

maf1Δ
wt
sen2-42
temp [°C]
induction
23 23 23 23 23 23 23 23
+ + - - + + - -
23 37 23 37
- - + +
pre-tRNAIle
tRNAIle
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 6

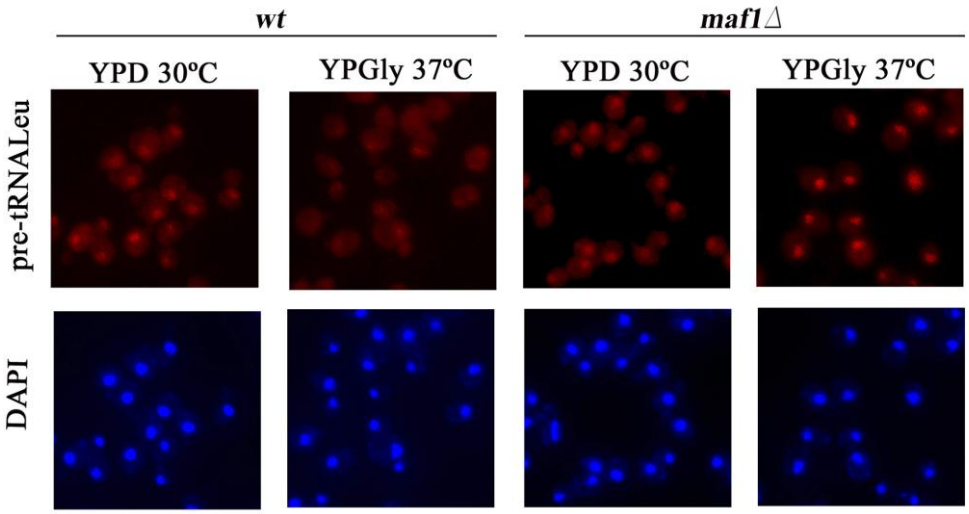

wt
maf1Δ
YPD 30ºC
YPGly 37ºC
YPD 30ºC
YPGly 37ºC
pre-tRNALeu
DAPI

Fig. 7

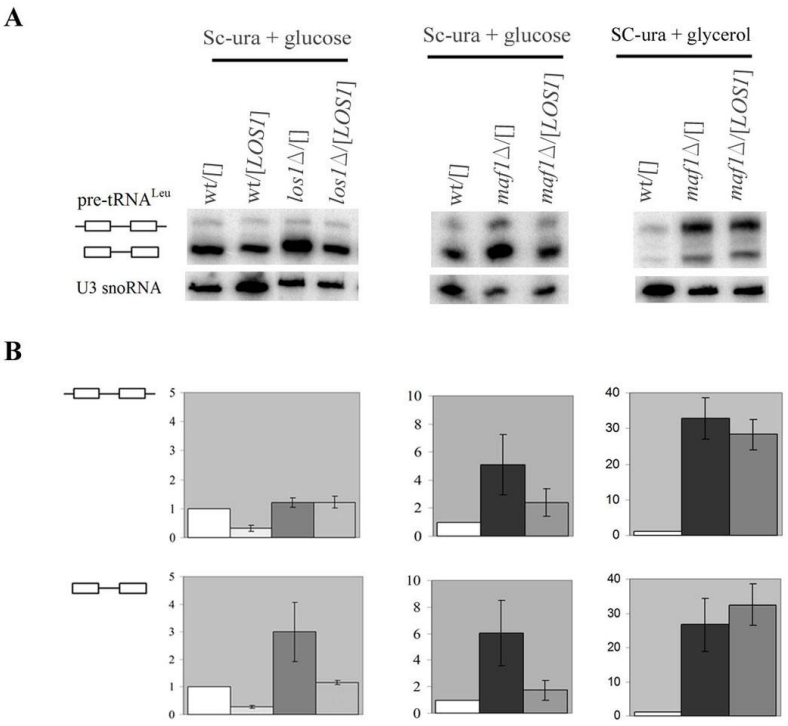

A
Sc-ura + glucose
Sc-ura + glucose
SC-ura + glycerol
wt/[]
wt/[LOSI]
los1Δ/[]
los1Δ/[LOSI]
wt/[]
maf1Δ/[]
maf1Δ/[LOSI]
wt/[]
maf1Δ/[]
maf1Δ/[LOSI]
pre-tRNALeu
U3 snoRNA
B

Fig. 8

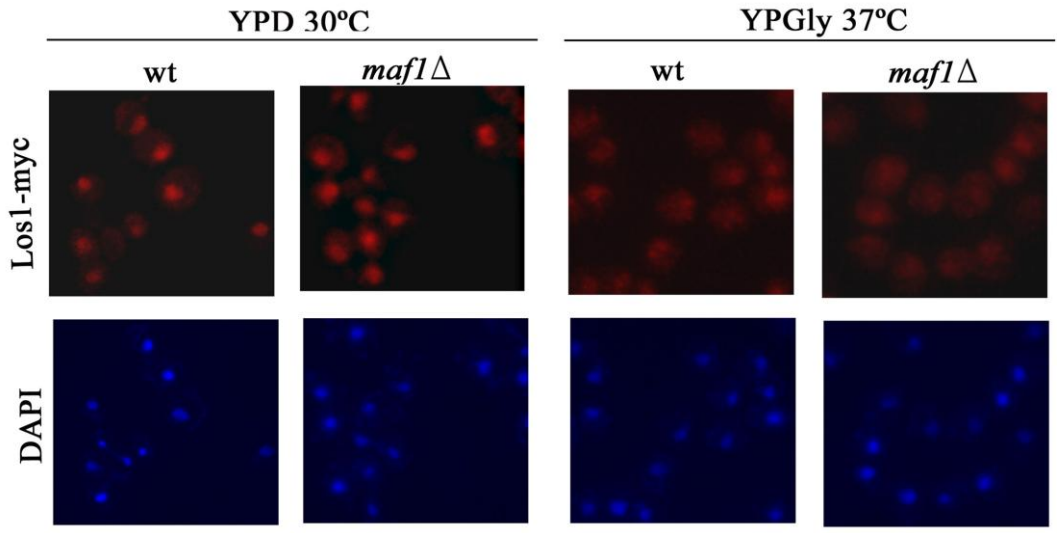

YPD 30ºC
YPGly 37ºC
wt
maf1Δ
wt
maf1Δ
Los1-myc
DAPI

Fig. 9

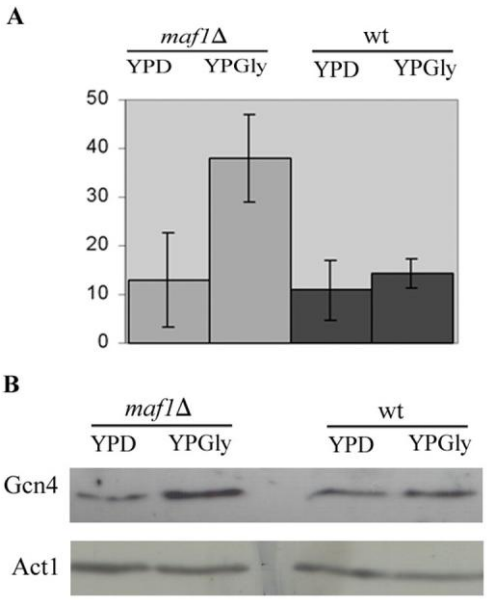
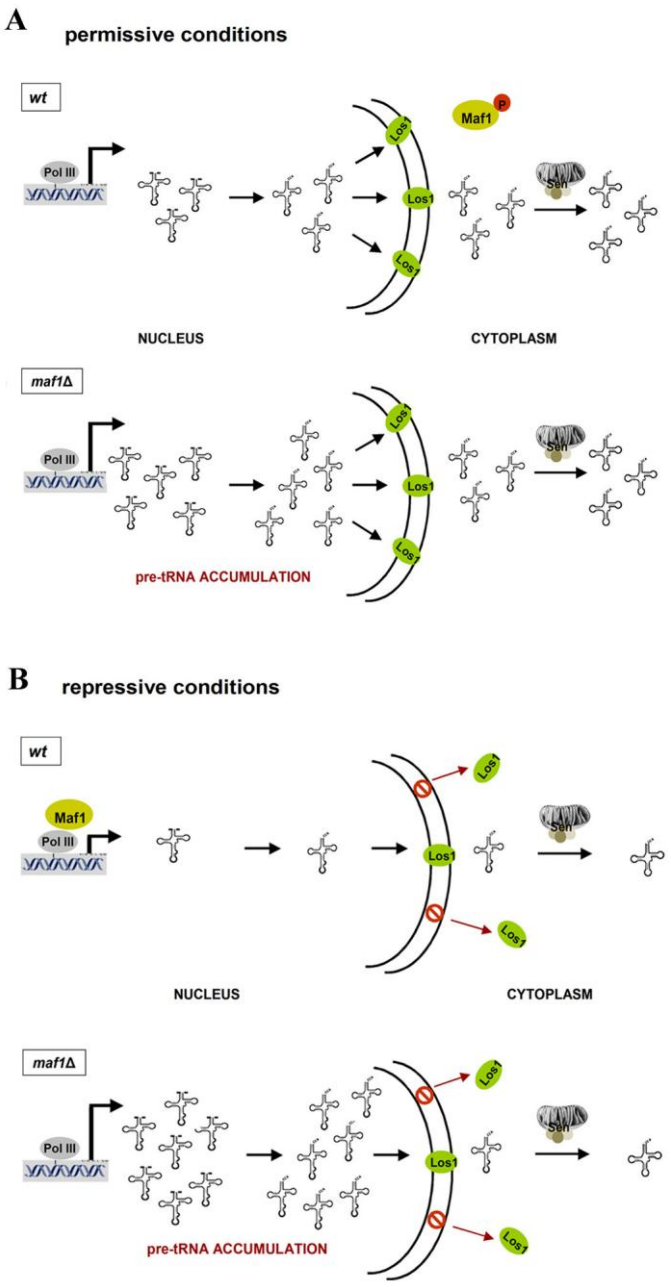

A
maf1Δ
wt
YPD
YPGly
YPD
YPGly
50
40
30
20
10
0
B
maf1Δ
wt
YPD
YPGly
YPD
YPGly
Gcn4
Act1

Fig. 10


A
permissive conditions
wt
Pol III
Maf1
P
Los1
Los1
Los1
Sen1
NUCLEUS
CYTOPLASM
maf1Δ
Pol III
Los1
Los1
Los1
Sen1
pre-tRNA ACCUMULATION
B
repressive conditions
wt
Maf1
Pol III
Los1
Los1
Los1
Sen1
NUCLEUS
CYTOPLASM
maf1Δ
Pol III
Los1
Los1
Los1
Sen1
pre-tRNA ACCUMULATION