Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Identification of factors that promote biogenesis of tRNA$_{CGA}^{Ser}$

Fu Xu, Yang Zhou, Anders S. Byström, and Marcus J.O. Johansson

Department of Molecular Biology, Umeå University, Umeå, Sweden

ABSTRACT

A wide variety of factors are required for the conversion of pre-tRNA molecules into the mature tRNAs that function in translation. To identify factors influencing tRNA biogenesis, we previously performed a screen for strains carrying mutations that induce lethality when combined with a *sup61-T47:2C* allele, encoding a mutant form of tRNA$_{CGA}^{Ser}$. Analyzes of two complementation groups led to the identification of Tan1 as a protein involved in formation of the modified nucleoside N^4-acetylcytidine (ac^{4}C) in tRNA and Bud13 as a factor controlling the levels of ac^{4}C by promoting *TAN1* pre-mRNA splicing. Here, we describe the remaining complementation groups and show that they include strains with mutations in genes for known tRNA biogenesis factors that modify (*DUS2, MOD5* and *TRM1*), transport (*LOS1*), or aminoacylate (*SES1*) tRNA$_{CGA}^{Ser}$. Other strains carried mutations in genes for factors involved in rRNA/mRNA synthesis (*RPA49, RRN3* and *MOT1*) or magnesium uptake (*ALR1*). We show that mutations in not only *DUS2, LOS1* and *SES1* but also in *RPA49, RRN3* and *MOT1* cause a reduction in the levels of the altered tRNA$_{CGA}^{Ser}$. These results indicate that Rpa49, Rrn3 and Mot1 directly or indirectly influence tRNA$_{CGA}^{Ser}$ biogenesis.

Introduction

Eukaryotic transfer RNA genes are transcribed by RNA polymerase III generating precursor tRNAs (pre-tRNAs) that undergo a series of processing steps to generate mature functional tRNAs [1–3]. These steps include the removal of 5′ leader and 3′ trailer sequences, posttranscriptional addition of CCA to the 3′ termini, and the removal of intervening sequences in pre-tRNAs transcribed from intron-containing genes. All pre-tRNAs also undergo post-transcriptional modification of a subset of their nucleosides [1–3]. Some modified nucleosides are found in essentially all tRNA species while others are present in a specific or a subclass of tRNAs. Many pre-tRNA processing steps occur in the nucleus, including end-maturation and numerous nucleoside modifications [1–3]. However, some nucleoside modifications only occur after nuclear export. Moreover, the subcellular location of pre-tRNA splicing varies between organisms and in *Saccharomyces cerevisiae* splicing occurs after the pre-tRNA has been exported to the cytoplasm.

The essential and intron-containing *sup61+* (*tS(CGA)C*) gene encodes the only serine isoacceptor, tRNA$_{CGA}^{Ser}$, that efficiently decodes UCG codons in *S. cerevisiae* [4]. We previously identified four mutant *sup61* alleles in a screen for strains that require the *TRM2* gene, encoding the tRNA (m^{5}U$_{54}$) methyltransferase, for growth [5]. The *sup61 trm2* double mutants are viable at 25°C but inviable or very slow growing at 30°C. The m^{5}U$_{54}$ residue is present in tRNA$_{CGA}^{Ser}$ and the lack of Trm2 causes decreased abundance of the altered tRNA$_{CGA}^{Ser}$ species. In addition to the requirement for *TRM2*, direct tests showed that the growth of the *sup61* mutants is dependent on other

enzymes that catalyze nucleoside modifications in tRNA$_{CGA}^{Ser}$ [5]. These enzymes (Pus4, Trm1, and Trm3) are required for the formation of pseudouridine (Ψ) at position 55, N^2,N^2-dimethylguanosine (m$_2^2$G) at position 26 and 2′-O-methylguanosine (Gm) at position 18 in tRNAs [6–8]. Similar to the absence of Trm2, the lack of Pus4, Trm1, or Trm3 causes a reduction in the levels of the altered tRNA$_{CGA}^{Ser}$. Moreover, introduction of a null allele of *LHP1* into the *sup61* mutants was found to inhibit growth and reduce the levels of the altered tRNA$_{CGA}^{Ser}$ [5]. The Lhp1 protein promotes tRNA folding and protects the 3′ end of pre-tRNAs from exonucleolytic digestion [9,10]. Collectively, these findings suggest that the tRNA$_{CGA}^{Ser}$ species in the *sup61* mutants is sensitized to perturbations of the tRNA maturation pathway.

To identify novel factors promoting tRNA maturation, we performed a screen for mutations lethal in combination with one of the four *sup61* alleles [11]. The screen identified mutants representing 12 different complementation groups and detailed analyzes of one group led to the identification of *TAN1* as a gene required for the formation of N^4-acetylcytidine (ac^{4}C) in tRNA [11]. The ac^{4}C nucleoside is present at position 12 in leucine and serine isoacceptors, and the inactivation of *TAN1* causes a lack of ac^{4}C in tRNA and a reduction in the abundance of the altered tRNA$_{CGA}^{Ser}$ [11]. Another complementation group consisted of a strain with a mutation in the *BUD13* gene [12]. *BUD13* encodes a subunit of the heterotrimeric pre-mRNA retention and splicing (RES) complex, which promotes splicing and nuclear retention of a subset of intron-containing pre-mRNAs [13–18]. The requirement for

Bud13 in the *sup61* mutant is caused by an important role of the RES complex in splicing and nuclear retention of *TAN1* pre-mRNA and consequently in the formation of ac^{4}C [12]. In this study, we describe the remaining mutants identified in the screen and show that they do not only define strains with mutations in genes for known tRNA biogenesis factors but also those involved in RNA polymerase I and II transcription or Mg^{2+} uptake.

Results

Several different factors that promote modification of tRNA$_{CGA}^{Ser}$ are required for growth of sup61-T47:2C cells

The screen for mutations lethal in combination with the *sup61-T47:2C* allele, encoding a tRNA$_{CGA}^{Ser}$ species with an alteration in the variable arm (Figure 1(a)), identified mutants representing 12 complementation groups [11]. One group consisted of strains in which the mutation was genetically linked to the *sup61* locus and this group was excluded from further analysis. The mutant gene in each of the 11 remaining groups (Table 1) was identified by complementing the phenotype with a yeast genomic library and subsequent confirmation that the original mutation was genetically linked to the locus for the complementing gene. As the *sup61-T47:2C* allele generates a requirement for several different tRNA modifying enzymes [5,11], we expected that the screen would identify factors involved in the modification of tRNA$_{CGA}^{Ser}$. In addition to *trm1, tan1* and *bud13* mutants, the screen identified strains with mutations in the *DUS2* and *MOD5* genes (Table 1) [11,12,19]. These genes encode the tRNA modifying enzymes that catalyze the formation of dihydrouridine (D) at position 20 and N^6-isopentenyladenosine (i^{6}A) at position 37, respectively (Figure 1(a)) [20–23]. To demonstrate unambiguously that *DUS2* and *MOD5* are required for growth of cells with the altered tRNA$_{CGA}^{Ser}$, we independently combined, in the presence of a rescuing *sup61$^+$*-containing plasmid, the *sup61-T47:2C* mutation with *dus2Δ* and *mod5Δ* alleles. The resulting *sup61-T47:2C dus2Δ* and *sup61-T47:2C mod5Δ* strains were dependent on the plasmid for growth at both 30°C and 25°C (Figures 1(b) and S1), confirming that the alteration in tRNA$_{CGA}^{Ser}$ causes a requirement for Dus2 and Mod5.

As the inactivation of any of several tRNA modifying factors in *sup61-T47:2C* cells causes a decreased abundance of the altered tRNA$_{CGA}^{Ser}$ [5,11], it seemed likely that mutations in *DUS2* or *MOD5* would induce a similar defect. Even though the *sup61$^+$* gene is essential, we previously showed that the inviability of *sup61Δ* cells is suppressed by increased dosage of a gene (*tS(UGA)P*) coding for tRNA$_{ncm5UGA}^{Ser}$ [24]. This finding suggested that the introduction of a high-copy *tS (UGA)P* plasmid into the double mutants may suppress the dependence on the *sup61$^+$* plasmid and allow analyzes of tRNA$_{CGA}^{Ser}$ levels. Increased dosage of the *tS(UGA)P* gene counteracted the requirement for the *sup61$^+$* plasmid in the *sup61-T47:2C dus2Δ* strain both at 30°C and 25°C (Figures 1 (c) and S1). However, the requirement for the *sup61$^+$* plasmid was not suppressed by increased *tS(UGA)P* dosage in *sup61-*

T47:2C mod5Δ cells, preventing further analyzes of the strain (Figures 1(c) and S1). An i^{6}A$_{37}$ residue is also found in tRNA$_{ncm5UGA}^{Ser}$ [25] and the inability of the high-copy *tS (UGA)P* plasmid to suppress the *sup61-T47:2C mod5Δ* strain suggests that the isopentenyl group at A$_{37}$ enhances the ability of tRNA$_{ncm5UGA}^{Ser}$ to read the near-cognate UCG codon.

To investigate the influence of Dus2 on tRNA$_{CGA}^{Ser}$ abundance, we used northern blotting to analyze tRNA$_{CGA}^{Ser}$ transcripts in wild-type, *sup61-T47:2C, dus2Δ*, and *sup61-T47:2C dus2Δ* strains carrying the high-copy *tS(UGA)P* plasmid. The blots were also probed for tRNA$_i^{Met}$, which served as the loading control. The analyzes revealed a decreased abundance of mature tRNA$_{CGA}^{Ser}$ in the *dus2Δ sup61-T47:2C* strain compared to the *dus2Δ* and *sup61-T47:2C* single mutants (Figure 1(d,e)). Thus, the effect of the *dus2Δ* allele on the abundance of the altered tRNA$_{CGA}^{Ser}$ is similar to that observed for the inactivation of other tRNA modifying factors [5,11].

The abundance of the sup61-T47:2C-encoded tRNA$_{CGA}^{Ser}$ is reduced in cells with mutant ses1 or los1 alleles

In addition to tRNA modification mutants, we expected that the screen would define strains with reduced function in other steps of tRNA biogenesis. Accordingly, two of the remaining complementation groups consisted of strains carrying mutations in the *SES1* or *LOS1* gene (Table 1). The essential *SES1* gene codes for the seryl-tRNA synthetase that aminoacylates serine tRNA isoacceptors [26] whereas *LOS1* encodes a nonessential factor that mediates nuclear export of pre-tRNAs [3,27–29]. One of the two isolated *sup61-T47:2C ses1* double mutants *(ses1-40)* was able to lose the rescuing *sup61$^+$* vector at 25°C, generating a strain that is slow-growing at 25°C and inviable at 30°C (Figure 2(a)). Similarly, a *sup61-T47:2C los1Δ* double mutant is slow-growing at 25°C and inviable at 30°C (Figure 2(a)). Northern blot analyzes of wild-type, *sup61-T47:2C, ses1-40, sup61-T47:2C ses1-40, los1Δ*, and *sup61-T47:2C los1Δ* cells grown at 25°C revealed that the abundance of the mature tRNA$_{CGA}^{Ser}$ is reduced in the double mutants compared to the respective single mutants (Figure 2(b,c)). Further, the *ses1-40 sup61-T47:2C* mutant shows reduced levels of partially processed pre-tRNA$_{CGA}^{Ser}$ (Figure 2(b) and Table S1), indicating that the stability and/or processing of the mutant pre-tRNA$_{CGA}^{Ser}$ is affected by the *ses1-40* allele. Similarly, the accumulation of unspliced pre-tRNA$_{CGA}^{Ser}$ in *los1Δ* cells [27,30] is less pronounced in the *sup61-T47:2C los1Δ* mutant (Figure 2(b) and Table S1).

In addition to its essential role in aminoacylation, Ses1 was recently shown to stimulate the formation of 3-methylcytidine (m^{3}C) at position 32 in tRNA$_{CGA}^{Ser}$ and tRNA$_{ncm5UGA}^{Ser}$ [31]. The methylation of C$_{32}$ is catalyzed by the Trm140 protein and the modification is known to be present in three out of four tRNA^{Ser} and all three tRNA^{Thr} species [32,33]. Ses1 was found to co-purify with Trm140 and its stimulatory role in the formation of m^{3}C was restricted to the serine isoacceptors [31]. To investigate the possibility that the *ses1-40* allele influences the viability of *sup61-T47:2C* cells by inducing a tRNA modification defect, we
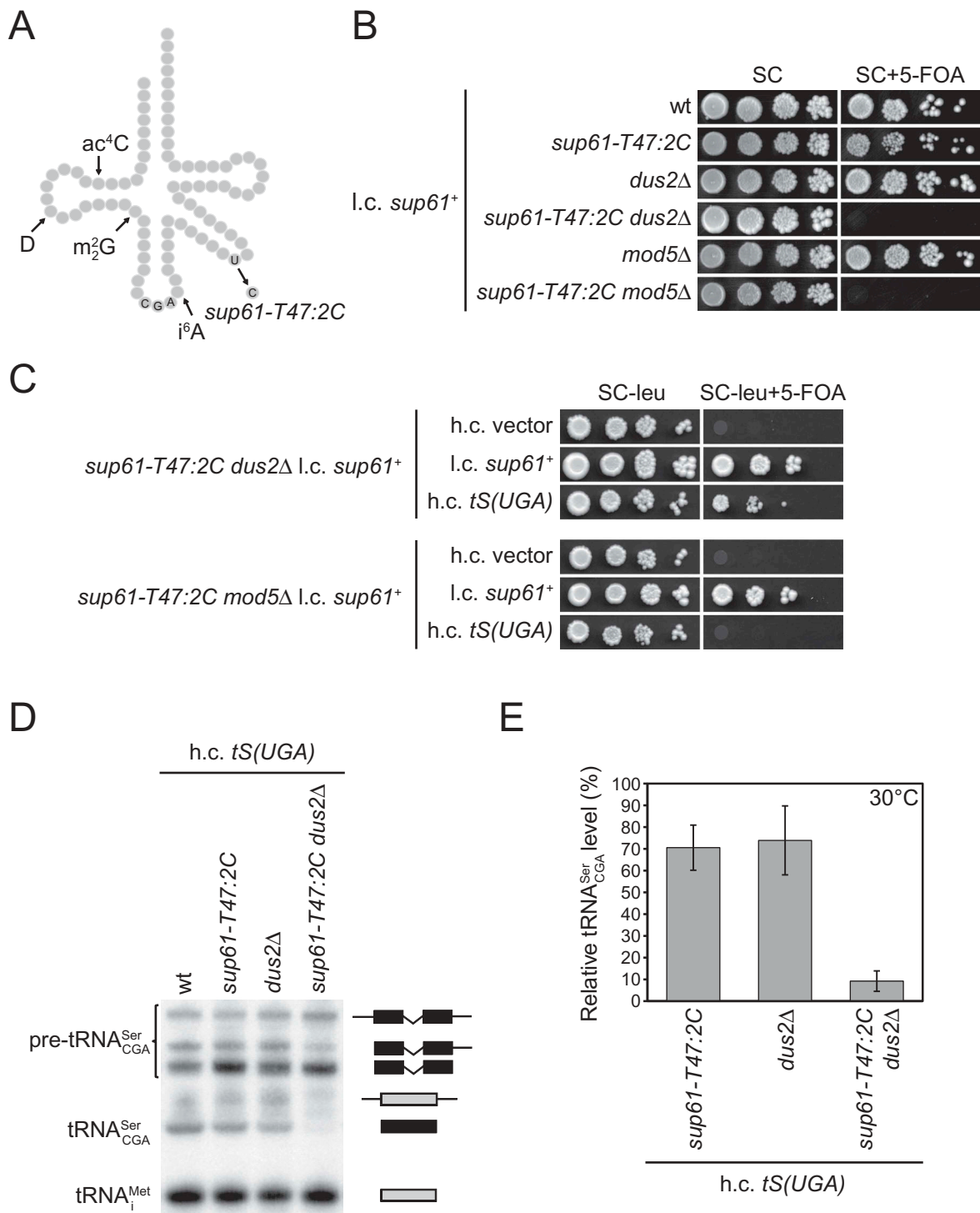
Figure 1. The *DUS2* and *MOD5* genes are required for growth of *sup61-T47:2C* cells. (a) Schematic secondary structure of tRNA$_{CGA}^{Ser}$. The alteration caused by the *sup61-T47:2C* allele and the positions of ac^{4}C$_{12}$, D$_{20}$, m$_2^2$G$_{26}$ and i^{6}A$_{37}$ are indicated. (b) Effects of *dus2Δ* and *mod5Δ* alleles on growth of *sup61-T47:2C* cells. The wild-type (UMY2220), *sup61-T47:2C* (MJY926), *dus2Δ* (UMY2872), *sup61-T47:2C dus2Δ* (UMY4286), *mod5Δ* (UMY2565), and *sup61-T47:2C mod5Δ* (UMY4285) strains carrying the low-copy (l.c.) *URA3* plasmid pRS316-*sup61*⁺ were grown over-night at 30°C in liquid synthetic complete medium (SC). The cells were serially diluted, spotted onto SC plates and SC plates containing 5-fluoroorotic acid (SC+ 5-FOA), and incubated at 30°C for 3 days. Only cells that have lost the *URA3* plasmid are able to grow on 5-FOA-containing medium [64]. (c) Effects of increased *tS(UGA)P* dosage on growth of *sup61-T47:2C dus2Δ* and *sup61-T47:2C mod5Δ* cells. The *sup61-T47:2C dus2Δ* and *sup61-T47:2C mod5Δ* strains from (b) were transformed with the empty high copy (h.c) *LEU2* plasmid pRS425 [65], the l.c. *LEU2* plasmid pRS315-*sup61*⁺, and the h.c. *LEU2* plasmid pRS425-*tS(UGA)P*. After purifications by single cell streaks on SC plates lacking uracil and leucine (SC-ura-leu), the transformants were grown over-night at 30°C in liquid SC medium lacking leucine (SC-leu). Cells were serially diluted, spotted onto SC-leu and SC-leu+ 5-FOA plates, and incubated at 30°C for 3 days. (d) Northern analysis of total RNA isolated from the wild-type, *sup61-T47:2C, dus2Δ*, and *sup61-T47:2C dus2Δ* strains carrying the h.c. *LEU2* plasmid pRS425-*tS(UGA)P*. The cells were grown in SC-leu medium at 30°C. The blot was probed for pre-tRNA$_{CGA}^{Ser}$, tRNA$_{CGA}^{Ser}$, and tRNA$_i^{Met}$ using radiolabeled oligonucleotides. tRNA$_i^{Met}$ serves as the loading control. The position and identity of the tRNA$_{CGA}^{Ser}$ (black) and tRNA$_i^{Met}$ (gray) species are indicated on the right. (e) Influence of a *dus2Δ* allele on the abundance of the mature tRNA$_{CGA}^{Ser}$. The mature tRNA$_{CGA}^{Ser}$ signal in (d) and two additional independent experiments was normalized to the corresponding tRNA$_i^{Met}$ signal and the value for each strain expressed relative to that for the wild-type, which was set to 100%. The standard deviation is indicated.

Table 1. The mutants define twelve complementation groups.

Complementation group	Number of mutants	Gene
I	3	sup61-T47:2C
II	2	TRM1
III	3	TAN1
IV	1	BUD13
V	2	DUS2
VI	2	MOD5
VII	2	SES1
VIII	1	LOS1
IX	1	MOT1
X	2	RPA49
XI	1	RRN3
XII	1	ALR1

used HPLC to analyze the nucleoside composition of total tRNA from wild-type and *ses1-40* cells grown at 30°C. We also analyzed the nucleoside composition of total tRNA from the *los1Δ* mutant grown at 30°C. The analyzes showed that the levels of the modified nucleosides normally found in tRNA$_{CGA}^{Ser}$, including m^{3}C, are unaffected in total tRNA from the *ses1-40* and *los1Δ* mutants (Table S2 and Figure S2). However, any effect of the *ses1-40* allele on the m^{3}C levels in the serine isoacceptors may be masked by the m^{3}C residues in the tRNA^{Thr} species. To directly test if the absence of m^{3}C affects growth of *sup61-T47:2C* cells, we combined a *trm140Δ* allele with the *sup61-T47:2C* mutation. The *sup61-T47:2C trm140Δ* strain was viable with only a minor growth defect compared to the *sup61-T47:2C* single mutant (Figure S2). This finding suggests that any effect of the *ses1-40* allele on the formation of m^{3}C in tRNA$_{CGA}^{Ser}$ is not the cause of the growth defect of *sup61-T47:2C ses1-40* cells. Collectively, our results show that Ses1 and Los1 are important to maintain the levels of the altered tRNA$_{CGA}^{Ser}$.

Factors involved in RNA polymerase I and II transcription influence the growth of sup61-T47:2C cells

Three complementation groups defined strains with mutations in genes, *RPA49*, *RRN3* or *MOT1*, encoding factors involved in RNA polymerase I and/or II transcription (Table 1). *RPA49* encodes a nonessential subunit of RNA polymerase I (Pol I) [34] whereas *RRN3* codes for an essential Pol I transcription factor [35]. The *MOT1* gene codes for an essential protein that has global effects on gene expression by controlling the distribution and activity of the TATA-binding protein (TBP) at promoters of RNA polymerase II (Pol II) transcribed genes [36–40]. In addition to its role in Pol II transcription, Mot1 has also been suggested to regulate Pol I transcription [41].

Cells deleted for the *RPA49* gene are viable, but they show a slow-growth phenotype that is more pronounced at 25°C than at 30°C (Figure S3) [34,42]. However, the growth defect caused by one of the two *rpa49* alleles (*rpa49-27*) identified from the screen was less severe than that induced by the deletion (Figure S3). The *sup61-T47:2C rpa49-27* strain was able to lose the rescuing *sup61$^+$* plasmid at 25°C generating a strain that is viable but very slow growing at 30°C (Figure 3(a)). Similarly, the *sup61-T47:2C*
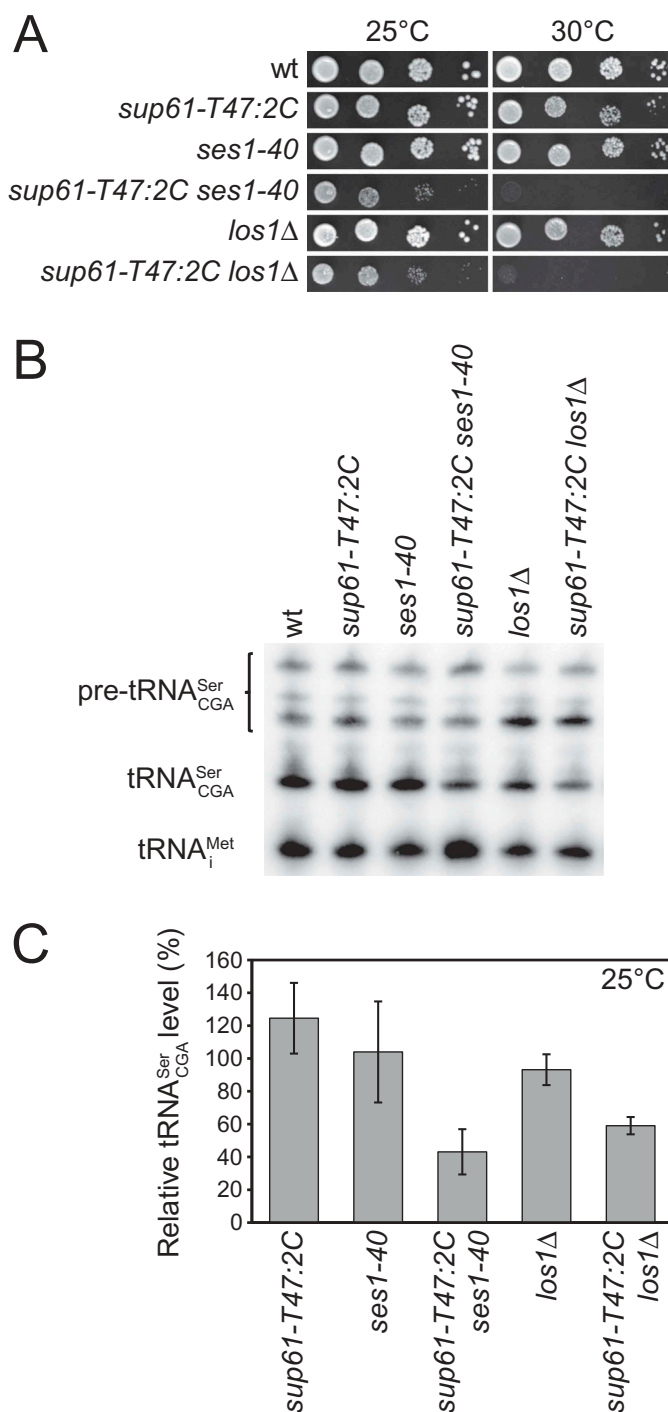

Figure 2. Influence of the *ses1-40 and los1Δ* alleles on growth and tRNA$_{CGA}^{Ser}$ abundance. (a) Effects of the *ses1-40 and los1Δ* alleles on growth of *sup61-T47:2C* cells. The wild-type (UMY2220), *sup61-T47:2C* (MJY926), *ses1-40* (MJY924), *sup61-T47:2C ses1-40* (MJY925), *los1Δ* (UMY2441), and *sup61-T47:2C los1Δ* (UMY2861) strains were grown over-night at 25°C in liquid SC medium. The cells were serially diluted, spotted onto SC plates, and incubated at 25°C for 3 or at 30°C for 2 days. (b) Northern analysis of total RNA isolated from the strains described in (a) grown in SC medium at 25°C. The blot was probed for pre-tRNA$_{CGA}^{Ser}$, tRNA$_{CGA}^{Ser}$, and tRNA$^{Met}_i$ using radiolabeled oligonucleotides. tRNA$^{Met}_i$ serves as the loading control. (c) Influence of *ses1-40 and los1Δ* alleles on the levels of mature tRNA$_{CGA}^{Ser}$. The tRNA$_{CGA}^{Ser}$ signal in (b) and two additional independent experiments was normalized to the corresponding tRNA$^{Met}_i$ signal and the value for each strain expressed relative to that for the wild-type, which was set to 100%. The standard deviation is indicated.
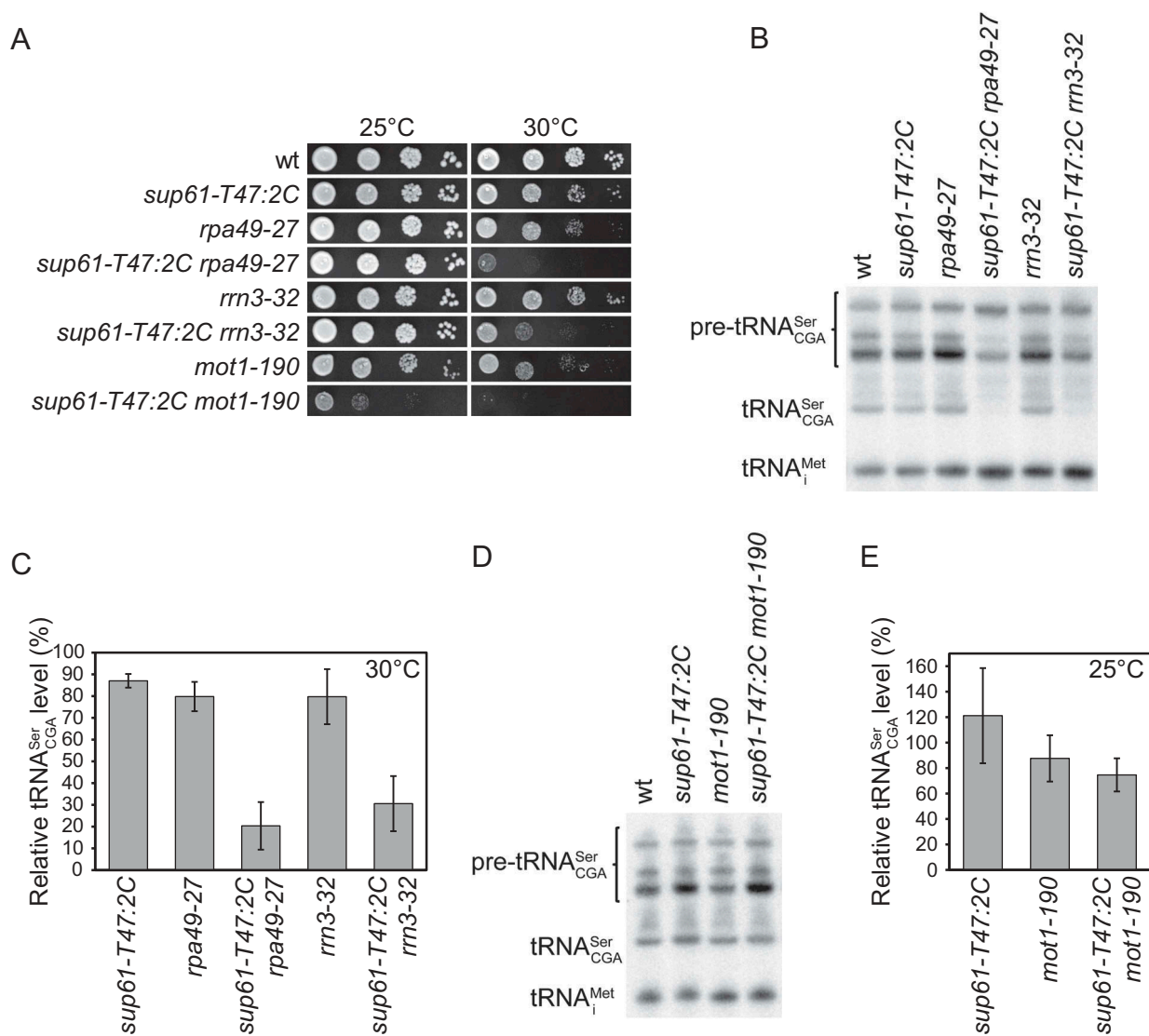
Figure 3. Mutations in the *MOT1, RPA49* and *RRN3* genes are detrimental to cells with a *sup61-T47:2C* allele. (a) Effects of the *mot1-190, rpa49-27* and *rrn3-32* alleles on growth of *sup61-T47:2C* cells. The wild-type (UMY2220), *sup61-T47:2C* (MJY926), *rpa49-27* (MJY890), *sup61-T47:2C rpa49-27* (MJY891), *rrn3-32* (MJY894), *sup61-T47:2C rrn3-32* (MJY895), *mot1-190* (MJY892), and *sup61-T47:2C mot1-190* (MJY893) strains were grown over-night at 25°C in liquid SC medium. Cells were serially diluted, spotted onto SC plates, and incubated at 25°C for 3 or at 30°C for 2 days. (b) Northern blot analysis of total RNA isolated from wild-type, *sup61-T47:2C, rpa49-27, sup61-T47:2C rpa49-27, rrn3-32* and *sup61-T47:2C rrn3-32* strains grown in SC medium at 30°C. The blot was probed for pre-tRNA$_{CGA}^{Ser}$, tRNA$_{CGA}^{Ser}$, and tRNA$_i^{Met}$ using radiolabeled oligonucleotides.tRNA$_i^{Met}$ serves as the loading control. (c) Influence of *rpa49-27* and *rrn3-32* alleles on the levels of mature tRNA$_{CGA}^{Ser}$. The tRNA$_{CGA}^{Ser}$ signal in (b) and two additional independent experiments was normalized to the corresponding tRNA$_i^{Met}$ signal and the value for each strain expressed relative to that for the wild-type, which was set to 100%. The standard deviation is indicated. (d) Northern blot analysis of total RNA isolated from wild-type, *sup61-T47:2C, mot1-190*, and *sup61-T47:2C mot1-190* strains grown in SC medium at 25°C. The blot was probed as described in (b). (e) Influence of the *mot1-190* allele on tRNA$_{CGA}^{Ser}$ abundance. The tRNA$_{CGA}^{Ser}$ signal in (d) and three additional independent experiments was normalized to the corresponding tRNA$_i^{Met}$ signal and the value for each strain expressed relative to that for the wild-type, which was set to 100%. The standard deviation is indicated.

rrn3-32 and *sup61-T47:2C mot1-190* strains were able to lose the rescuing *sup61⁺* plasmid at 25°C. The *sup61-T47:2C rrn3-32* strain is viable but slow-growing at 30°C whereas the *sup61-T47:2C mot1-190* strain is inviable at 30°C and very slow-growing at 25°C (Figure 3(a)).

The effect of the *rpa49-27, rrn3-32* and *mot1-190* alleles on tRNA$_{CGA}^{Ser}$ abundance was examined in cells grown at either 30°C (*rrn3-32* and *rpa49-27*) or 25°C (*mot1-190*). These analyzes revealed that the level of the mature tRNA$_{CGA}^{Ser}$ is reduced in the *sup61-T47:2C rrn3-32* and *sup61-T47:2C rpa49-27* strains compared to the respective single mutants (Figure 3(b,c)).

Moreover, the *sup61-T47:2C rrn3-32* and *sup61-T47:2C rpa49-27* strains show reduced levels of partially processed pre-tRNA$_{CGA}^{Ser}$ (Figure 3(b)) and Table S1). Only a slight decrease in tRNA$_{CGA}^{Ser}$ levels was observed in the *sup61-T47:2C mot1-190* strain (Figure 3(d,e)), suggesting that the effect on tRNA$_{CGA}^{Ser}$ abundance may not, at least by itself, explain the severe growth defect of the double mutant. Analyzes of the nucleoside composition of total tRNA from the *rpa49-27, rrn3-32* and *mot1-190* single mutants revealed no apparent tRNA modification defect (Table S2), indicating that Rpa49, Rrn3 and Mot1 influences tRNA$_{CGA}^{Ser}$ levels by another mechanism.

Mutations in the ALR1 gene are detrimental for growth of sup61-T47:2C cells

The final complementation group consisted of a strain with a mutation in the *ALR1* gene, which encodes the main Mg^{2+} importer of *S. cerevisiae* [43,44]. In addition to the importance of Mg^{2+} as a cofactor/counterion, it plays an important role in establishing and maintaining structures of nucleic acids, proteins and membranes. Strains with mutations in *ALR1* show reduced intracellular Mg^{2+} levels and an *alr1Δ* mutant is, depending on genetic background, either inviable or very slow growing on standard media [43–45]. These phenotypes are partially suppressed by supplementation of the media with Mg^{2+} [43,44]. We found that supplementation of the medium with Mg^{2+} does not suppress the *sup61*+-plasmid dependence of *sup61-T47:2C alr1-11* cells at either 30°C or 25°C (Figures 4 (a, b) and S4). Moreover, analyzes of the effects of the *alr1* allele on $tRNA_{CGA}^{Ser}$ levels was prevented by the finding that increased $tRNA_{ncm5UGA}^{Ser}$ expression does not suppress the plasmid dependence of the *sup61-T47:2C alr1-11* strain even on Mg^{2+}-supplemented medium (Figures 4(c) and S4). As no tRNA modification defect is apparent in *alr1-11* cells (Table S2), the cause of the synergistic interaction remains unclear. Nevertheless, our findings suggest that cells with the altered $tRNA_{CGA}^{Ser}$ are sensitive to defects in Mg^{2+} uptake.

Discussion

We previously showed that the viability of *sup61-T47:2C* cells is dependent on several different factors promoting maturation of $tRNA_{CGA}^{Ser}$ [5,11,12]. In this study, we describe additional factors required for growth of strains with the altered form of $tRNA_{CGA}^{Ser}$. We show that mutations in genes required for modification (*DUS2* and *MOD5*), transport (*LOS1*), and aminoacylation (*SES1*) of $tRNA_{CGA}^{Ser}$ are deleterious to cells with the *sup61-T47:2C* allele. Similar to the inactivation of other factors influencing tRNA biogenesis, the *dus2, los1,* and *ses1* alleles cause a reduced abundance of the altered $tRNA_{CGA}^{Ser}$. These findings provide further support for the notion that *sup61-T47:2C* cells are sensitized for perturbations in tRNA biogenesis.

In addition to mutations in genes for known tRNA biogenesis factors, we found that mutations in the *MOT1, RPA49,* or *RRN3* gene inhibit growth of *sup61-T47:2C* cells and cause a reduction in the levels of the altered $tRNA_{CGA}^{Ser}$. The Mot1 protein is thought to influence both Pol I and Pol II transcription [36–41,46] whereas the function of Rpa49 and Rrn3 appears to be restricted to Pol I transcription [34,35,42]. The mechanisms by which Mot1, Rpa49 and Rrn3 influence tRNA biogenesis is unclear, but it is possible that they indirectly influence the process through their involvement in Pol I transcription. Transcription of tRNA
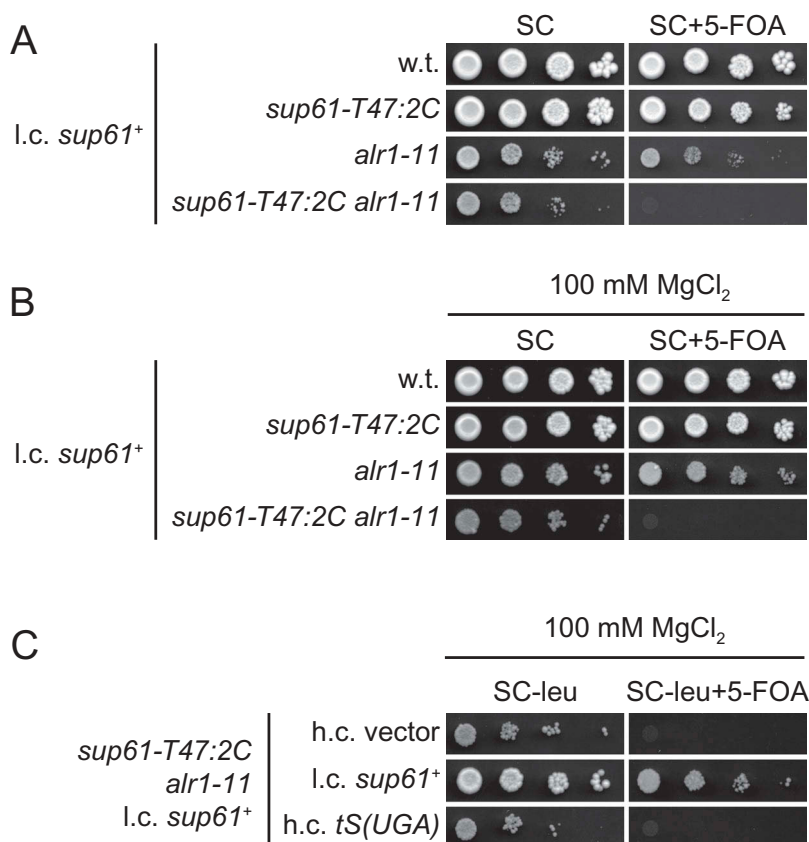

Figure 4. Genetic interactions between the *alr1-11* and *sup61-T47:2C* alleles. (a and b) Growth phenotypes of strains with an *alr1-11* and/or *sup61-T47:2C* allele. The wild-type (UMY2220), *sup61-T47:2C* (MJY926), *alr1-11* (UMY3002), *sup61-T47:2C alr1-11* (UMY2967) strains carrying the l.c. *URA3* plasmid pMJ1421 (*sup61*+) were grown overnight at 30°C in liquid SC medium (A) or in liquid SC medium supplemented with 100 mM $MgCl_2$ (b). Cells were serially diluted, spotted onto SC and SC + 5-FOA (A) or SC and SC+ 5-FOA plates containing 100 mM $MgCl_2$ (b), and incubated at 30°C for 3 days. (c) Effect of increased *tS(UGA)P* dosage on growth of *sup61-T47:2C alr1-11* cells. The *sup61-T47:2C alr1-11* strain described in (a) was transformed with the indicated h.c. or l.c. *LEU2* plasmids. Transformants were grown at 30°C in liquid SC-leu medium supplemented with 100 mM $MgCl_2$. Cells were serially diluted, spotted onto SC-leu and SC-leu+ 5-FOA plates containing 100 mM $MgCl_2$, and incubated at 30°C for 3 days.

genes as well as 5′-end processing of pre-tRNAs has been reported to localize to the nucleolus [47–49]. Moreover, dissociation of pre-tRNA from the nucleolus and defects in 5′ end processing have been observed in cells defective in Pol I transcription or rRNA processing [50,51]. As mutations in *MOT1, RPA49*, and *RRN3* affect nucleolar morphology [52,53], the *mot1-190, rpa49-27*, and *rrn3-32* alleles may influence tRNA$_{CGA}^{Ser}$ biogenesis by disrupting the balance and/or order of pre-tRNA processing. The *rpa49-27* and *rrn3-32* alleles induce reduced levels of not only the mature but also the partially processed forms of the altered tRNA$_{CGA}^{Ser}$ (Figure 3(b) and Table S1), indicating that pre-tRNA processing and/or stability is affected in the mutants. The abundance of the primary unprocessed pre-tRNA$_{CGA}^{Ser}$ is, however, largely unaffected by the *mot1-190, rpa49-27* and *rrn3-32* alleles (Figure 3(b,d) and Table S1), suggesting that the mutations do not cause a defect in 5′-end processing.

The finding that a mutation in the *ALR1* gene is lethal in combination with the *sup61-T47:2C* allele indicates that a reduction in intracellular Mg^{2+} levels may cause inefficient folding and/or decreased stability of the altered tRNA$_{CGA}^{Ser}$. Alternatively, the genetic interaction could be a consequence of the effect of reduced Mg^{2+} on translational fidelity [54–56], i.e. decreased intracellular Mg^{2+} levels may reduce the ability of the altered tRNA$_{CGA}^{Ser}$ to read the UCG codon. It should, however, be noted that Mg^{2+} is important for a vast number of cellular processes and it cannot be excluded that the genetic interaction is caused by a more indirect mechanism.

Cells with mutations in the single-copy and essential *sup61+* are sensitized for defects in tRNA biogenesis. The screen for mutations lethal when combined with a *sup61-T47:2C* allele is an unbiased genetic approach to identify factors promoting tRNA biogenesis. As our screen was far from being saturated, additional factors would likely be identified in an expanded screen.

Materials and methods

Yeast strains, media, and genetic procedures

Strains used in this study are listed in Table S3. Yeast media were prepared essentially as described [57] using a slightly different composition of the drop-out mix [58]. Difco yeast nitrogen base without amino acids was purchased from Becton Dickinson (291940).

The screen for mutations lethal in combination with the *sup61-T47:2C* mutation and the subsequent identification of plasmids that complement the phenotype has been described [11]. To investigate linkage between the complementing gene and the original mutation, we cloned the gene or a DNA fragment close to the gene into an integrative vector and targeted the plasmid marker (*TRP1* or *URA3*) to the corresponding locus in strain UMY2256. This strain was crossed to the relevant mutant (s), carrying pMJ1421 (*URA3, ADE3, sup61+*) or pMJ1422 (*TRP1, ADE3, sup61+*) [11], and tetrad analysis revealed that the ability to lose the *sup61+* plasmid always co-segregated with

the integrated *TRP1/URA3* marker, i.e., the mutation was in all cases linked to the locus for the complementing gene.

One copy of the *DUS2, MOD5, RPA49*, and *TRM140* genes was independently deleted in the diploid strain UMY2366 by a PCR-mediated strategy [59,60]. The generated heterozygous diploids were allowed to sporulate and the *dus2Δ* (UMY2872), *mod5Δ* (UMY2565), *rpa49Δ* (MJY1111) and *trm140Δ* (MJY1110) strains obtained from tetrads. The *dus2Δ* and *mod5Δ* strains were crossed to a *sup61-T47:2C* strain, carrying a *sup61+* gene on a plasmid, and rescued double mutants obtained from tetrads. The *sup61-T47:2C trm140Δ* double mutant was obtained from a cross between UMY2256 and MJY1110.

The *sup61-T47:2C alr1-11, sup61-T47:2C ses1-40, sup61-T47:2C rrn3-32, sup61-T47:2C mot1-190* and *sup61-T47:2C rpa49-27* mutants identified in the screen, all carrying a rescuing *sup61+*-containing plasmid, were backcrossed at least three times to UMY2256. The backcrossed strains were crossed to UMY2219 and *alr1-11, ses1-40, rrn3-32, mot1-190*, and *rpa49-27* single mutants obtained from tetrads.

Plasmid constructions

A low copy *LEU2* plasmid harboring the *sup61+* gene (pRS315-*sup61+*) was constructed by cloning a BamHI/HindIII DNA fragment from pRS316-*sup61+* [5] into the corresponding sites of pRS315 [61]. Plasmids pMJ1421, pMJ1422, and pRS425-*tS(UGA)P* have been described [5,24].

RNA methods

Total RNA for northern blotting was isolated [58] from exponentially growing cells at an optical density at 600 nm (OD_{600}) of ≈ 0.5. An aliquot of slow-growing double mutants was, during harvesting, streaked on a SC plate and incubated at the appropriate temperature. Only cell pellets from cultures that contained no or negligible amounts of faster growing revertants were processed further. The procedures for northern blotting have been described [5,11]. The oligonucleotides used to detect precursor and mature tRNA$_{CGA}^{Ser}$ were 5′- AGCCGAACTTTTTATTCCATTCG-3′ and 5′-GC CCAAGAGATTTCGAGTCTCT-3′, respectively. To detect tRNA$_i^{Met}$, the oligonucleotide 5′-GGACATCAGGGTTATGA GCC-3′ was used.

For HPLC analyzes of modified nucleosides, the strains were grown in SC medium at 30°C to OD_{600} ≈ 0.8. Cells were harvested and total tRNA isolated as previously described [62]. Typically, 50μg of total tRNA was digested to nucleosides [63] using nuclease P1 (Sigma-Aldrich, N8630) and bacterial alkaline phosphatase (Sigma-Aldrich, P4252). The hydrolysate was analyzed by HPLC [63] using a Develosil C30 reversed phase column (Phenomenex, CH0-5690) and buffer A (0.01M $NH_4H_2PO_4$, 2.5% methanol (v/v) at pH 5.3), buffer B (0.01M $NH_4H_2PO_4$, 20% methanol (v/v) at pH 5.1), and buffer C (0.01M $NH_4H_2PO_4$, 35% acetonitrile (v/v)) as the elution buffers. To be able to separate m^3C from U, the methanol concentration in buffer A was changed to 6% (v/v).

Acknowledgments

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

ORCID

Marcus J.O. Johansson http://orcid.org/0000-0001-7117-7426

References

[1] Phizicky EM, Hopper AK. tRNA biology charges to the front. Genes Dev. 2010;24(17):1832–1860.

[2] Hopper AK. Transfer RNA post-transcriptional processing, turnover, and subcellular dynamics in the yeast *Saccharomyces cerevisiae*. Genetics. 2013;194(1):43–67.

[3] Chatterjee K, Nostramo RT, Wan Y, et al. tRNA dynamics between the nucleus, cytoplasm and mitochondrial surface: location, location, location. Biochim Biophys Acta. 2018;1861(4):373–386.

[4] Etcheverry T, Salvato M, Guthrie C. Recessive lethality of yeast strains carrying the *SUP61* suppressor results from loss of a transfer RNA with a unique decoding function. J Mol Biol. 1982;158(4):599–618.

[5] Johansson MJO, Byström AS. Dual function of the tRNA(m(5)U54) methyltransferase in tRNA maturation. RNA. 2002;8(3):324–335.

[6] Becker HF, Motorin Y, Planta RJ, et al. The yeast gene *YNL292w* encodes a pseudouridine synthase (Pus4) catalyzing the formation of psi55 in both mitochondrial and cytoplasmic tRNAs. Nucleic Acids Res. 1997;25(22):4493–4499.

[7] Ellis SR, Morales MJ, Li JM, et al. Isolation and characterization of the *TRM1* locus, a gene essential for the N2,N2-dimethylguanosine modification of both mitochondrial and cytoplasmic tRNA in *Saccharomyces cerevisiae*. J Biol Chem. 1986;261(21):9703–9709.

[8] Cavaille J, Chetouani F, Bachellerie JP. The yeast *Saccharomyces cerevisiae YDL112w* ORF encodes the putative 2′- O-ribose methyltransferase catalyzing the formation of Gm18 in tRNAs. RNA. 1999;5(1):66–81.

[9] Yoo CJ, Wolin SL. The yeast la protein is required for the 3′ endonucleolytic cleavage that matures tRNA precursors. Cell. 1997;89(3):393–402.

[10] Chakshusmathi G, Kim SD, Rubinson DA, et al. A La protein requirement for efficient pre-tRNA folding. Embo J. 2003;22 (24):6562–6572.

[11] Johansson MJO, Byström AS. The *Saccharomyces cerevisiae TAN1* gene is required for *N4*-acetylcytidine formation in tRNA. RNA. 2004;10(4):712–719.

[12] Zhou Y, Chen C, Johansson MJO. The pre-mRNA retention and splicing complex controls tRNA maturation by promoting *TAN1* expression. Nucleic Acids Res. 2013;41(11):5669–5678.

[13] Dziembowski A, Ventura AP, Rutz B, et al. Proteomic analysis identifies a new complex required for nuclear pre-mRNA retention and splicing. Embo J. 2004;23(24):4847–4856.

[14] Zhou Y, Johansson MJO. The pre-mRNA retention and splicing complex controls expression of the Mediator subunit Med20. RNA Biol. 2017;14(10):1411–1417.

[15] Tuo S, Nakashima K, Pringle JR. Apparent defect in yeast bud-site selection due to a specific failure to splice the pre-mRNA of a regulator of cell-type-specific transcription. PLoS One. 2012;7(10):e47621.

[16] Spingola M, Armisen J, Ares M Jr. Mer1p is a modular splicing factor whose function depends on the conserved U2 snRNP protein Snu17p. Nucleic Acids Res. 2004;32(3):1242–1250.

[17] Schmidlin T, Kaeberlein M, Kudlow BA, et al. Single-gene deletions that restore mating competence to diploid yeast. FEMS Yeast Res. 2008;8(2):276–286.

[18] Scherrer FW Jr., Spingola M. A subset of Mer1p-dependent introns requires Bud13p for splicing activation and nuclear retention. RNA. 2006;12(7):1361–1372.

[19] Johansson MJO, Byström AS. Transfer RNA modifications and modifying enzymes in *Saccharomyces cerevisiae*. In: editor, Grosjean H. Fine-tuning of RNA functions by modification and editing. Topics in Curr Genetics. Vol. 12. Berlin (Heidelberg): Springer-Verlag; 2005. p. 87–120.

[20] Xing F, Martzen MR, Phizicky EM. A conserved family of *Saccharomyces cerevisiae* synthases effects dihydrouridine modification of tRNA. RNA. 2002;8(3):370–381.

[21] Xing F, Hiley SL, Hughes TR, et al. The specificities of four yeast dihydrouridine synthases for cytoplasmic tRNAs. J Biol Chem. 2004;279(17):17850–17860.

[22] Dihanich ME, Najarian D, Clark R, et al. Isolation and characterization of *MOD5*, a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of *Saccharomyces cerevisiae*. Mol Cell Biol. 1987;7(1):177–184.

[23] Laten H, Gorman J, Bock RM. Isopentenyladenosine deficient tRNA from an antisuppressor mutant of *Saccharomyces cerevisiae*. Nucleic Acids Res. 1978;5(11):4329–4342.

[24] Johansson MJO, Esberg A, Huang B, et al. Eukaryotic wobble uridine modifications promote a functionally redundant decoding system. Mol Cell Biol. 2008;28(10):3301–3312.

[25] Piper PW. A correlation between a recessive lethal amber suppressor mutation in Saccharomyces cerevisiae and an anticodon change in a minor serine transfer RNA. J Mol Biol. 1978;122(2):217–235.

[26] Weygand-Durasevic I, Johnson-Burke D, Soll D. Cloning and characterization of the gene coding for cytoplasmic seryl- tRNA synthetase from *Saccharomyces cerevisiae*. Nucleic Acids Res. 1987;15(5):1887–1904.

[27] Hopper AK, Schultz LD, Shapiro RA. Processing of intervening sequences: a new yeast mutant which fails to excise intervening sequences from precursor tRNAs. Cell. 1980;19(3):741–751.

[28] Hellmuth K, Lau DM, Bischoff FR, et al. Yeast Los1p has properties of an exportin-like nucleocytoplasmic transport factor for tRNA. Mol Cell Biol. 1998;18(11):6374–6386.

[29] Sarkar S, Hopper AK. tRNA nuclear export in *Saccharomyces cerevisiae*: in situ hybridization analysis. Mol Biol Cell. 1998;9 (11):3041–3055.

[30] Hurt DJ, Wang SS, Lin YH, et al. Cloning and characterization of *LOS1*, a *Saccharomyces cerevisiae* gene that affects tRNA splicing. Mol Cell Biol. 1987;7(3):1208–1216.

[31] Han L, Marcus E, D'Silva S, et al. S. cerevisiae Trm140 has two recognition modes for 3-methylcytidine modification of the anticodon loop of tRNA substrates. RNA. 2017;23(3):406–419.

[32] D'Silva S, Haider SJ, Phizicky EM. A domain of the actin binding protein Abp140 is the yeast methyltransferase responsible for 3-methylcytidine modification in the tRNA anti-codon loop. RNA. 2011;17(6):1100–1110.

[33] Noma A, Yi S, Katoh T, et al. Actin-binding protein ABP140 is a methyltransferase for 3-methylcytidine at position 32 of tRNAs in *Saccharomyces cerevisiae*. RNA. 2011;17(6):1111–1119.

[34] Liljelund P, Mariotte S, Buhler JM, et al. Characterization and mutagenesis of the gene encoding the A49 subunit of RNA polymerase A in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA. 1992;89(19):9302–9305.

[35] Yamamoto RT, Nogi Y, Dodd JA, et al. *RRN3* gene of *Saccharomyces cerevisiae* encodes an essential RNA polymerase I transcription factor which interacts with the polymerase independently of DNA template. Embo J. 1996;15(15):3964–3973.

[36] Davis JL, Kunisawa R, Thorner J, et al. (Mot1 gene-product) Affects gene-expression and is required for viability in the yeast saccharomyces-cerevisiae. Mol Cell Biol. 1992;12(4):1879–1892.

[37] Auble DT, Hansen KE, Mueller CG, et al. Mot1, a global repressor of RNA polymerase II transcription, inhibits TBP binding to DNA by an ATP-dependent mechanism. Genes Dev. 1994;8(16):1920–1934.

[38] Geisberg JV, Moqtaderi Z, Kuras L, et al. Mot1 associates with transcriptionally active promoters and inhibits association of NC2 in *Saccharomyces cerevisiae*. Mol Cell Biol. 2002;22(23):8122–8134.

[39] Andrau JC, Van Oevelen CJC, Van Teeffelen HAAM, et al. Mot1p is essential for TBP recruitment to selected promoters during in vivo gene activation. Embo J. 2002;21(19):5173–5183.

[40] Viswanathan R, Auble DT. One small step for Mot1; one giant leap for other Swi2/Snf2 enzymes? Biochim Biophys Acta. 2011;1809(9):488–496.

[41] Dasgupta A, Sprouse RO, French S, et al. Regulation of rRNA synthesis by TATA-binding protein-associated factor Mot1. Mol Cell Biol. 2007;27(8):2886–2896.

[42] Gadal O, Mariotte-Labarre S, Chedin S, et al. A34.5, a nonessential component of yeast RNA polymerase I, cooperates with subunit A14 and DNA topoisomerase I to produce a functional rRNA synthesis machine. Mol Cell Biol. 1997;17(4):1787–1795.

[43] Graschopf A, Stadler JA, Hoellerer MK, et al. The yeast plasma membrane protein Alr1 controls Mg2+ homeostasis and is subject to Mg2+-dependent control of its synthesis and degradation. J Biol Chem. 2001;276(19):16216–16222.

[44] MacDiarmid CW, Gardner RC. Overexpression of the *Saccharomyces cerevisiae* magnesium transport system confers resistance to aluminum ion. J Biol Chem. 1998;273(3):1727–1732.

[45] Tran HG, Steger DJ, Iyer VR, et al. The chromo domain protein chd1p from budding yeast is an ATP-dependent chromatin-modifying factor. Embo J. 2000;19(10):2323–2331.

[46] Sikorski TW, Buratowski S. The basal initiation machinery: beyond the general transcription factors. Curr Opin Cell Biol. 2009;21(3):344–351.

[47] Thompson M, Haeusler RA, Good PD, et al. Nucleolar clustering of dispersed tRNA genes. Science. 2003;302(5649):1399–1401.

[48] Duan Z, Andronescu M, Schutz K, et al. A three-dimensional model of the yeast genome. Nature. 2010;465(7296):363–367.

[49] Bertrand E, Houser-Scott F, Kendall A, et al. Nucleolar localization of early tRNA processing. Genes Dev. 1998;12(16):2463–2468.

[50] Briand JF, Navarro F, Gadal O, et al. Cross Talk between tRNA and rRNA synthesis in *Saccharomyces cerevisiae*. Mol Cell Biol. 2001;21(1):189–195.

[51] Kendall A, Hull MW, Bertrand E, et al. A *CBF5* mutation that disrupts nucleolar localization of early tRNA biosynthesis in yeast also suppresses tRNA gene-mediated transcriptional silencing. Proc Natl Acad Sci USA. 2000;97(24):13108–13113.

[52] Albert B, Leger-Silvestre I, Normand C, et al. RNA polymerase I-specific subunits promote polymerase clustering to enhance the rRNA gene transcription cycle. J Cell Biol. 2011;192(2):277–293.

[53] Neumuller RA, Gross T, Samsonova AA, et al. Conserved regulators of nucleolar size revealed by global phenotypic analyses. Sci Signal. 2013;6(289):ra70.

[54] Szer W, Ochoa S. Complexing ability and coding properties of synthetic polynucleotides. J Mol Biol. 1964;8:823–834.

[55] Schlanger G, Friedman SM. Ambiguity in a polypeptide-synthesizing extract from *Saccharomyces cerevisiae*. J Bacteriol. 1973;115(1):129–138.

[56] Johansson MJO, Jacobson A. Nonsense-mediated mRNA decay maintains translational fidelity by limiting magnesium uptake. Genes Dev. 2010;24(14):1491–1495.

[57] Amberg DC, Burke DJ, Strathern JN. Methods in yeast genetics. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2005.

[58] Johansson MJO. Determining if an mRNA is a substrate of nonsense-mediated mRNA decay in *Saccharomyces cerevisiae*. In: Wajapeyee N, Gupta R, editors. Eukaryotic transcriptional and post-transcriptional gene expression regulation. Methods Mol Biol. Vol. 1507. New York (NY): Humana Press; 2017. p. 169–177.

[59] Brachmann CB, Davies A, Cost GJ, et al. Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast. 1998;14(2):115–132.

[60] Longtine MS, McKenzie A 3rd, Demarini DJ, et al. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast. 1998;14(10):953–961.

[61] Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics. 1989;122(1):19–27.

[62] Chen C, Huang B, Eliasson M, et al. Elongator complex influences telomeric gene silencing and DNA damage response by its role in wobble uridine tRNA modification. PLoS Genet. 2011;7(9):e1002258.

[63] Gehrke CW, Kuo KC. Ribonucleoside analysis by reversed-phase high performance liquid chromatography. In: Gehrke CW, Kuo KC, editors. Journal of chromatography library. Vol. Chromatography and modification of nucleosides analytical methods for major and modified nucleosides: HPLC, GC, MS, NMR, UV and FT-IR. Amsterdam: Elsevier; 1990. p. A3–A71.

[64] Boeke JD, LaCroute F, Fink GR. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol Gen Genet. 1984;197(2):345–346.

[65] Christianson TW, Sikorski RS, Dante M, et al. Multifunctional yeast high-copy-number shuttle vectors. Gene. 1992;110(1):119–122.