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Identification of factors that promote biogenesis of tRNA\textsubscript{Ser}^{CGA}

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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\textsubscript{Ser}^{CGA}. Analyzes of two complementation groups led to the identification of Tan1 as a protein involved in formation of the modified nucleoside \(N^\beta\)-acetylcytidine (ac\(^C\)) in tRNA and Bud13 as a factor controlling the levels of ac\(^C\) 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\textsubscript{Ser}^{CGA}. 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\textsubscript{Ser}^{CGA}. These results indicate that Rpa49, Rm3 and Mot1 directly or indirectly influence tRNA\textsubscript{Ser}^{CGA} 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\textsubscript{Ser}^{CGA}, 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\(^4\)U\(_{54}\)) methyltransferase, for growth [5]. The sup61 trm2 double mutants are viable at 25°C but inviable or very slowly growing at 30°C. The m\(^4\)U\(_{54}\) residue is present in tRNA\textsubscript{Ser}^{CGA} and the lack of Trm2 causes decreased abundance of the altered tRNA\textsubscript{Ser}^{CGA} 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\textsubscript{Ser}^{CGA} [5]. These enzymes (Pus4, Trm1, and Trm3) are required for the formation of pseudouridine (Ψ) at position 55, N\(^2\)N\(^\beta\)-dimethylguanosine (m\(_2\)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\textsubscript{Ser}^{CGA}. 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\textsubscript{Ser}^{CGA}. The Lhp1 protein promotes tRNA folding and protects the 3’ end of pre-tRNAs from exonuclease digestion [9,10]. Collectively, these findings suggest that the tRNA\textsubscript{Ser}\textsubscript{CGA} 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\(^\beta\)-acetylcytidine (ac\(^C\)) in tRNA [11]. The ac\(^C\) nucleoside is present at position 12 in leucine and serine isoacceptors, and the inactivation of TAN1 causes a lack of ac\(^C\) in tRNA and a reduction in the abundance of the altered tRNA\textsubscript{Ser}^{CGA} [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^3C [12]. In this study, we investigated 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^{Ser}_{CGA} 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^{Ser}_{CGA} 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^{Ser}_{CGA}. In addition to trm1, tan1 and bud13 mutants, the screen identified strains in 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^6A) 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^{Ser}_{CGA}, 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^{Ser}_{CGA} 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^{Ser}_{CGA} [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^{Ser}_{CGA} [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^{Ser}_{CGA} 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^6A_{37} residue is also found in tRNA^{Ser}_{CGA} [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^{Ser}_{CGA} to read the near-cognate UCG codon.

To investigate the influence of Dus2 on tRNA^{Ser}_{CGA} abundance, we used northern blotting to analyze tRNA^{Ser}_{CGA} 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^{Thr}_{CGA}, which served as the loading control. The analyzes revealed a decreased abundance of mature tRNA^{Ser}_{CGA} 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^{Ser}_{CGA} is similar to that observed for the inactivation of other tRNA modifying factors [5,11].

The abundance of the sup61-T47:2C-encoded tRNA^{Ser}_{CGA} 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 non-essential 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^{Ser}_{CGA} 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^{Ser}_{CGA} (Figure 2(b) and Table S1), indicating that the stability and/or processing of the mutant pre-tRNA^{Ser}_{CGA} is affected by the ses1-40 allele. Similarly, the accumulation of unspliced pre-tRNA^{Ser}_{CGA} 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^3C) at position 32 in tRNA^{Ser}_{CGA} and tRNA^{Thr}_{CGA} [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^3C 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\textsubscript{Ser\textsubscript{CGA}}. The alteration caused by the sup61-T47:2C allele and the positions of ac\textsubscript{C12}, D\textsubscript{32}, m\textsubscript{3}G\textsubscript{24}, and i\textsubscript{A37} are indicated. (b) Effects of dus2\textDelta and mod5\textDelta alleles on growth of sup61-T47:2C cells. The wild-type (UMY2220), sup61-T47:2C (UMY2926), dus2\textDelta (UMY2872), sup61-T47:2C dus2\textDelta (UMY4286), mod5\textDelta (UMY2565), and sup61-T47:2C mod5\textDelta (UMY4285) strains carrying the low-copy (l.c.) URA3 plasmid pRS316-sup61\textsuperscript{+} 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) dosage on growth of sup61-T47:2C dus2\textDelta and sup61-T47:2C mod5\textDelta strains from (b) were transformed with the empty high copy (h.c) LEU2 plasmid pRS425 [65], the l.c. LEU2 plasmid pRS315-sup61\textsuperscript{+}, and the h.c. LEU2 plasmid pRS425-ts(UGA). 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, dus2\textDelta, and sup61-T47:2C dus2\textDelta strains carrying the h.c. LEU2 plasmid pRS425-ts(UGA). The cells were grown in SC-leu medium at 30°C. The blot was probed for pre-tRNA\textsubscript{Ser\textsubscript{CGA}}, tRNA\textsubscript{Ser\textsubscript{CGA}}, and tRNA\textsubscript{Met} using radiolabeled oligonucleotides. tRNA\textsubscript{Met} serves as the loading control. The position and identity of the tRNA\textsubscript{Ser\textsubscript{CGA}} (black) and tRNA\textsubscript{Met} (gray) species are indicated on the right. (e) Influence of a dus2\textDelta allele on the abundance of the mature tRNA\textsubscript{Ser\textsubscript{CGA}}. The mature tRNA\textsubscript{Ser\textsubscript{CGA}} signal in (d) and two additional independent experiments was normalized to the corresponding tRNA\textsubscript{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.
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^{Ser}_{CGA}, including m^3C, 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^3C levels in the serine isoacceptors may be masked by the m^1C residues in the tRNA^{Thr} species. To directly test if the absence of m^3C 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^3C in tRNA^{Ser}_{CGA} 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^{Ser}_{CGA}.

**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

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Table 1. The mutants define twelve complementation groups.

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<th>Complementation group</th>
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<th>Gene</th>
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<td>sup61-T47:2C</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>TRM1</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>TAN1</td>
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<td>1</td>
<td>BUD13</td>
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<tr>
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<td>2</td>
<td>MOD5</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>VIII</td>
<td>1</td>
<td>MOT1</td>
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</tr>
<tr>
<td>X</td>
<td>2</td>
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<td>ALR1</td>
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Figure 2. Influence of the ses1-40 and los1Δ alleles on growth and tRNA^{Ser}_{CGA} 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^{Ser}_{CGA}, tRNA^{Ser}_{CGA}, 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^{Ser}_{CGA}. The tRNA^{Ser}_{CGA} 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.
The effect of the \textit{rpa49-27}, \textit{rrn3-32} and \textit{mot1-190} alleles on tRNA\textsubscript{Ser}CGA abundance was examined in cells grown at either 30°C (\textit{rrn3-32} and \textit{rpa49-27}) or 25°C (\textit{mot1-190}). These analyzes revealed that the level of the mature tRNA\textsubscript{Ser}CGA is reduced in the \textit{sup61-T47:2C rrn3-32} and \textit{sup61-T47:2C rpa49-27} strains compared to the respective single mutants (Figure 3(b,c)). Moreover, the \textit{sup61-T47:2C rrn3-32} and \textit{sup61-T47:2C rpa49-27} strains show reduced levels of partially processed pre-tRNA\textsubscript{Ser}CGA (Figure 3(b) and Table S1). Only a slight decrease in tRNA\textsubscript{Ser}CGA levels was observed in the \textit{sup61-T47:2C mot1-190} strain (Figure 3(d,e)), suggesting that the effect on tRNA\textsubscript{Ser}CGA 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 \textit{rpa49-27}, \textit{rrn3-32} and \textit{mot1-190} single mutants revealed no apparent tRNA\textsubscript{Ser}CGA modification defect (Table S2), indicating that Rpa49, Rrn3 and Mot1 influences tRNA\textsubscript{Ser}CGA 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 ALRI 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 ALRI 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, analyses of the effects of the alr1 allele on tRNA\(^{\text{Ser}}\)\(_{\text{CGA}}\) levels was prevented by the finding that increased tRNA\(^{\text{Ser}}\)\(_{\text{CGA}}\) 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\(^{\text{Ser}}\)\(_{\text{CGA}}\) 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\(^{\text{Ser}}\)\(_{\text{CGA}}\) [5,11,12]. In this study, we describe additional factors required for growth of strains with the altered form of tRNA\(^{\text{Ser}}\)\(_{\text{CGA}}\). We show that mutations in genes required for modification (DUS2 and MOD5), transport (LOS1), and aminoacetylation (SES1) of tRNA\(^{\text{Ser}}\)\(_{\text{CGA}}\) are deleterious to cells with the sup61-T47:2C allele. Similar to the inactivation of other factors affecting tRNA biogenesis, the dus2, los1, and ses1 alleles cause a reduced abundance of the altered tRNA\(^{\text{Ser}}\)\(_{\text{CGA}}\). 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\(^{\text{Ser}}\)\(_{\text{CGA}}\). 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

\[
\text{Mot1 protein} \quad \text{Rpa49 protein} \quad \text{Rrn3 protein}
\]

**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 (MIY926), 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) 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\(_2\), and incubated at 30°C for 3 days.

\[
\text{Mot1 protein} \quad \text{Rpa49 protein} \quad \text{Rrn3 protein}
\]
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\textsubscript{Ser\textsuperscript{CGA}} 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\textsubscript{Ser\textsuperscript{CGA}} (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\textsubscript{Ser\textsuperscript{CGA}} 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 ALRI gene is lethal in combination with the sup61-T47:2C allele indicates that a reduction in intracellular Mg\textsuperscript{2+} levels may cause inefficient folding and/or decreased stability of the altered tRNA\textsubscript{Ser\textsuperscript{CGA}}. Alternatively, the genetic interaction could be a consequence of the effect of reduced Mg\textsuperscript{2+} on translational fidelity [54–56], i.e. decreased intracellular Mg\textsuperscript{2+} levels may reduce the ability of the altered tRNA\textsubscript{Ser\textsuperscript{CGA}} to read the UCG codon. It should, however, be noted that Mg\textsuperscript{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\textsuperscript{+} 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\textsuperscript{+}) or pMJ1422 (TRP1, ADE3, sup61\textsuperscript{+}) [11], and tetrad analysis revealed that the ability to lose the sup61\textsuperscript{+} 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 (MY1111) and trm140Δ (MY1110) strains obtained from tetrads. The dus2Δ and mod5Δ strains were crossed to a sup61-T47:2C strain, carrying a sup61\textsuperscript{+} 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 MY1110.

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\textsuperscript{+}-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\textsuperscript{+} gene (pRS315-sup61\textsuperscript{+}) was constructed by cloning a BamHI/HindIII DNA fragment from pRS316-sup61\textsuperscript{+} [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\textsubscript{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\textsubscript{CGA} were 5’- AGCCGAACCTTTATTCATCG-3’ and 5’-GC CCAAGAGATTCGATCTCT-3’, respectively. To detect tRNA\textsubscript{CGA}\textsubscript{Net}, the oligonucleotide 5’-GGACATCGGGTTATGA GCC-3’ was used.

For HPLC analyses of modified nucleosides, the strains were grown in SC medium at 30°C to OD\textsubscript{660} = 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\textsubscript{4}H\textsubscript{2}PO\textsubscript{4}, 2.5% methanol (v/v) at pH 5.3), buffer B (0.01M NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4}, 20% methanol (v/v) at pH 5.1), and buffer C (0.01M NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4}, 35% acetonitrile (v/v)) as the elution buffers. To be able to separate m\textsuperscript{34}C from U, the methanol concentration in buffer A was changed to 6% (v/v).
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Disclosure statement

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