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Molecular reconstruction of a fungal genetic code alteration

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Abbreviations: tRNA, transfer RNA; SerRS, Seryl-tRNA synthetase; LeuRS, Leucyl-tRNA synthetase; Leu, leucine; Ser, serine; SDM, site-directed mutagenesis; β-gal, β-galactosidase; GST-β-gal, glutathione S-transferase (GST) and β-galactosidase genes; ONPG, o-nitrophenyl-β-D-galactopyranoside; γ-^{32}P-ATP, ATP labelled with phosphor 32

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Fungi of the CTG clade translate the Leu CUG codon as Ser. This genetic code alteration is the only eukaryotic sense-to-sense codon reassignment known to date, is mediated by an ambiguous serine tRNA ($tRNA_{CAG}^{Ser}$), exposes unanticipated flexibility of the genetic code and raises major questions about its selection and fixation in this fungal lineage. In particular, the origin of the $tRNA_{CAG}^{Ser}$ and the evolutionary mechanism of CUG reassignment from Leu to Ser remain poorly understood. In this study, we have traced the origin of the $tDNA_{CAG}^{Ser}$ gene and studied critical mutations in the $tRNA_{CAG}^{Ser}$ anticodon-loop that modulated CUG reassignment. Our data show that the $tRNA_{CAG}^{Ser}$ emerged from insertion of an adenosine in the middle position of the 5'-CGA-3' anticodon of a $tRNA_{CGA}^{Ser}$ ancestor, producing the 5'-CAG-3' anticodon of the $tRNA_{CAG}^{Ser}$, without altering its aminoacylation properties. This mutation initiated CUG reassignment while two additional mutations in the anticodon-loop resolved a structural conflict produced by incorporation of the Leu 5'-CAG-3' anticodon in the anticodon-arm of a $tRNA^{Ser}$. Expression of the mutant $tRNA_{CAG}^{Ser}$ in yeast showed that it cannot be expressed at physiological levels and we postulate that such downregulation was essential to maintain Ser misincorporation at sub-lethal levels during the initial stages of CUG reassignment. We demonstrate here that such low level CUG ambiguity is advantageous in specific ecological niches and we propose that misreading tRNAs are targeted for degradation by an unidentified tRNA quality control pathway.

Introduction

The genetic code is viewed as frozen and universal.[1] However 20 codon reassignments have been discovered in diverse bacterial and eukaryotic species, indicating that the code evolves.[2] These alterations involve both nonsense and sense codons, but, with exception of the fungal CTG clade, eukaryotic nuclear sense codons are not reassigned.[2,3] Mitochondria are particularly interesting because they reassigned both nonsense and sense codons in multiple phylogenetic lineages and the reassignment patterns indicate that codons starting with A or U are more prone to reassignment than those starting with C or G. In other words, the strength of the first codon-anticodon base pair is fundamental to the evolution of genetic code alterations.[2] Beyond this, the molecular pathways of codon reassignment are poorly understood and difficult to rationalize due to proteome disruption and negative impact on fitness.

A remarkable genetic code alteration occurs in the fungal CTG clade where the CUG codon is reassigned from Leu to Ser.[4-6] Surprisingly, proteome-wide incorporation of both Ser (97%) and Leu (3%) at CUG sites still occurs in most CTG clade species due to dual recognition of the $tRNA_{CAG}^{Ser}$ by the Seryl- and the Leucyl-tRNA synthetases (SerRS and LeuRS).[7,8] The distinct chemical properties of Leu (hydrophobic, located in the hydrophobic core of proteins) and Ser (polar, located on protein surfaces), suggest that this genetic code alteration should have generated massive disruption of the proteome of the CTG clade ancestor, raising the question of how did these fungi survive such extreme genetic chaos. An important finding from comparative genome analysis of fungal species is that the negative pressure produced by incorporation of both Ser and Leu at CUGs erased all CUGs from the genome of the CTG clade ancestor, eliminating the toxicity of CUG ambiguity altogether.[4,9] Indeed, the CUGs present in extant CTG clade species re-emerged recently from single and double simultaneous mutations of codons coding for Ser or amino acids with similar chemical properties. In other words, the CUGs re-emerged in presence of ambiguity and only those CUG protein sites that tolerate insertion of both Ser and Leu were selected,[4,9] as demonstrated by X-Ray chrystalography and molecular modeling of *C. albicans* proteins.[10] We are left, therefore, with three major evolutionary questions, namely how did the $tRNA_{CAG}^{Ser}$ appear,

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which is its evolutionary pathway and why was it not eliminated by natural selection?

Previous in silico studies suggested that the mutant $tRNA_{CAG}^{Ser}$ that decodes the CUG codon as Ser emerged 272 ± 25 million y ago, prior to the *Saccharomyces* and *Candida* split (170 ± 27 million y ago) from a $tRNA^{Ser}$.[9] Therefore, the $tRNA_{CAG}^{Ser}$ is a typical $tRNA^{Ser}$, but contains a Leu 5'-CAG-3' anticodon. It also has m^1G at position 37 (m^1G_{37}) in the anticodon-loop, which is typical of $tRNA^{Leu}$ and is not present in $tRNA^{Ser}$. This methylated guanosine is functionally very important as it improves decoding accuracy by preventing frameshifting and is also a leucylation identity determinant.[11] Another remarkable structural feature of the $tRNA_{CAG}^{Ser}$ is the presence of guanosine at position 33 (G_{33}). In general, tRNAs have a highly conserved uridine at position 33 (U_{33}), which is critical for the correct U-turn of the anticodon-loop and for stacking of the anticodon bases.[12] It is still unclear how the phosphate backbone of the $tRNA_{CAG}^{Ser}$ turns and maintains correct stacking of the anticodon bases with G_{33}, however structural probing data show that G_{33} distorts the top of the anticodon-stem of the $tRNA_{CAG}^{Ser}$ and prevents efficient leucylation of the tRNA by the LeuRS.[11,13] These data suggest that a minimal set of mutations in the anticodon-loop of the $tRNA_{CAG}^{Ser}$ played critical roles in the evolution of CUG reassignment.[14]

To better understand the role of those anticodon-loop mutations, we have reconstructed in vivo the early steps of the evolutionary pathway of the $tRNA_{CAG}^{Ser}$. For this, we have used molecular phylogeny of a large set of tRNA sequences produced by fungal genome sequencing initiatives and traced the origin of the $tRNA_{CAG}^{Ser}$ at the isoacceptor level. We then engineered yeast strains that recapitulate the critical steps of CUG reassignment.[9,15] Our data show that expression of the mutant $tRNA_{CAG}^{Ser}$ is strongly repressed by an unidentified tRNA quality control pathway and that the G_{33} and m^1G_{37} mutations fine tune the decoding efficiency of the $tRNA_{CAG}^{Ser}$. We postulate that such $tRNA_{CAG}^{Ser}$ downregulation was essential to ensure cell viability during the initial stages of CUG reassignment. We further show that selection of the $tRNA_{CAG}^{Ser}$ was possible because low level Ser misincorporation into proteins produces adaptive phenotypes in specific ecological niches.

Results

Model system of CUG reassignment. To reconstruct the $tRNA_{CAG}^{Ser}$ evolutionary pathway, we have traced the origin of the ancestral $tRNA^{Ser}$ by aligning the sequences of the Leu and Ser tRNAs of the fungal CTG clade species. This clade includes *Candida albicans* (ca), *Candida dubliniensis* (cd), *Candida tropicalis* (ct), *Candida parapsilosis* (cp), *Lloderomyces elongisporus* (le), *Candida guilliermondii* (cg), *Debaryomyces hansenii* (dh) and *Candida lusitaniae* (cl), whose genomes have been sequenced recently.[4] A phylogenetic tree built using the maximum likelihood method (**Fig. 1A**) showed that the $tRNA_{CAG}^{Ser}$ forms a branch of $tRNA^{Ser}$ rather than $tRNA^{Leu}$ and is more closely related with the $tRNA_{CGA}^{Ser}$ than with the other $tRNA^{Ser}$ (**Fig. 1B**). The multiple sequence alignment of $tRNA_{CAG}^{Ser}$ sequences of the CTG clade species showed high variability in the $tRNA_{CAG}^{Ser}$ anticodon-stem domain (**Fig. 2A**), which was surprising considering the semi-conserved structural match between the anticodon-loop and the anticodon-stem in tRNA isodecoders. This match modulates the efficiency of codon decoding.[16] This atypical nucleotide variability of the anticodon-arm of the $tRNA_{CAG}^{Ser}$ therefore provides evidence for rapid evolution of the tRNA anticodon-arm to accommodate the instability produced by the 5'-CAG-3' sequence in the anticodon-loop of the $tRNA_{CGA}^{Ser}$ ancestor. Interestingly, anticodon-arm sequence variability was also observed in $tRNA_{CGA}^{Ser}$ and $tRNA_{GCU}^{Ser}$, suggesting that these tRNAs are also evolving faster than the other $tRNA^{Ser}$ (**Fig. 2B**).

Beyond the nucleotides N_{33}, N_{35} and N_{37} in the anticodon-loop and the nucleotide variability identified in the anticodon-stem, the $tRNA_{CAG}^{Ser}$ has a typical structure of $tRNA^{Ser}$ isoacceptors, suggesting that most of the evolutionary changes of the $tRNA_{CAG}^{Ser}$ occurred in its anticodon-arm, with mutations G_{33}, A_{35} and G_{37} playing the major roles in CUG reassignment. To clarify this hypothesis, we have re-introduced these mutations in the *C. albicans* $tRNA_{CGA}^{Ser}$ (**Fig. 3**) by site-directed mutagenesis (SDM) and reconstructed each step of the CUG reassignment pathway in yeast. The serylation efficiency of these mutant tRNAs was determined in vitro and their decoding efficiency was determined in vivo by evaluating the impact of Ser misincorporation at Leu-CUG codons on yeast fitness.

Serylation of the $tRNA_{CAG}^{Ser}$ played a neutral role in CUG reassignment. Since the *C. albicans* SerRS recognizes the extra-arm and the acceptor stem of the $tRNA^{Ser}$ and does not contact the anticodon-arm,[17-21] significant alterations in serylation efficiency of the mutant $tRNA_{CGA}^{Ser}$ were not expected (**Fig. 3**). However, small effects on serylation could not be excluded due to the distortion induced by G_{33} in the anticodon-stem.[11,13] To clarify this issue, the mutant tRNAs were transcribed in vitro using T7 RNA polymerase and were aminoacylated using recombinant *C. albicans* SerRS expressed and purified from *E. coli*. The aminoacylation efficiency of the mutant tRNAs (A_{35}, A_{35}+G_{37} and A_{35}+G_{37}+G_{33}) was similar to the wild-type control tRNA (WT) (**Fig. 4**), confirming that mutations in the anticodon loop of $tRNA_{CGA}^{Ser}$ did not affect SerRS recognition and aminoacylation, at least in vitro.

We have further confirmed the neutrality of the anticodon-arm mutations on serylation of the $tRNA_{CAG}^{Ser}$ using an in vivo-forced evolution strategy. We have hypothesized that the destabilization of the proteome of haploid *S. cerevisiae* cells by Ser misincorporation at CUGs would decrease fitness, allowing for selection of mutations that abolished serylation of the $tRNA_{CAG}^{Ser}$ or increased its turnover rate. Mapping such mutations on the structure of the $tRNA_{CAG}^{Ser}$ should permit identifying serylation identity determinants. To test this hypothesis, the $tDNA_{CAG}^{Ser}$ gene was integrated into the genome of diploid yeast cells using the KanMx4 gene integration cassette (**Fig. 5A**). The recombinant diploid cells were then sporulated, tetrads were dissected and spores were allowed to grow in media containing geneticin to select viable haploid spores containing the KanMx4 cassette and the $tRNA_{CAG}^{Ser}$ gene (**Fig. 5A**). Since Ser misincorporation is lethal in certain haploid backgrounds,[14] spore viability in selective media was entirely dependent on mutations that inactivated the $tRNA_{CAG}^{Ser}$ (**Fig. 5A**). Such inactivating mutations were identified by PCR amplification

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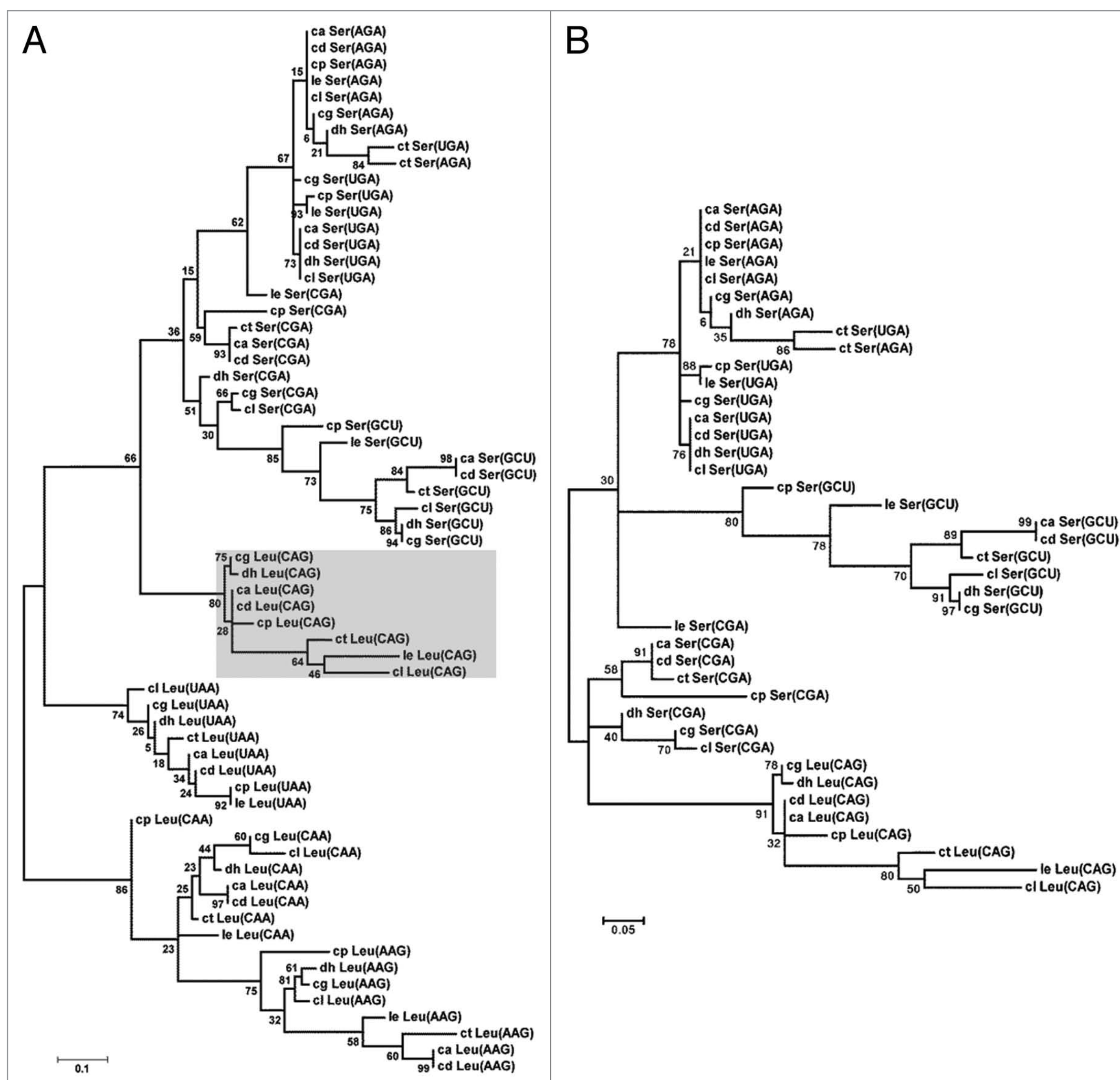

Figure 1. Phylogeny of Ser and Leu tRNAs of the CTG clade species. (**A**) The phylogenetic analysis of Ser and Leu tRNAs from the CTG clade species was constructed using a maximum likelihood method, which separated the tRNAs in Leu and Ser groups. (**B**) The $tRNA_{CAG}^{Ser}$ aligned closer to the $tRNA_{CGA}^{Ser}$, suggesting that it evolved from the $tRNA_{CGA}^{Ser}$ ancestor. The tRNAs analyzed belong to *Candida albicans* (ca), *Candida dubliniensis* (cd), *Candida tropicalis* (ct), *Candida parapsilosis* (cp), *Lloderomyces elongisporus* (le), *Candida guilliermondii* (cg), *Debaryomyces hansenii* (dh) and *Candida lusitaniae* (cl). Bootstrap values for all nodes are displayed. The phylogenetic analysis was performed with Molecular Evolutionary Genetic Analysis (MEGA5) software and tRNA sequences were retrieved from the Candida Gene Order Browser.

of the $tRNA_{CAG}^{Ser}$ gene, followed by Sanger sequencing. Each of the viable spores screened contained at least one mutation in the $tDNA_{CAG}^{Ser}$ gene, which accumulated in the extra-arm and acceptor-stem of the $tRNA_{CAG}^{Ser}$ (**Fig. 5B**). Since these structural domains contain the known identity determinants of eukaryotic $tRNA^{Ser}$,[17,18,20,22] the data support the hypothesis that viability was sustained by inhibition of $tRNA_{CAG}^{Ser}$ serylation. However, some mutations accumulated in the TΨC-arm and one was detected in the anticodon-stem, which are not known to interact with the SerRS. But, these mutant $tRNA^{Ser}$ were not detected by northern blot (**Fig. S1A**) and cells expressing them had identical growth rate to control cells (not expressing $tRNA_{CAG}^{Ser}$) (**Fig. S1B**). In vitro aminoacylation of these mutant tRNAs with purified SerRS showed sharp decrease in serylation levels (**Fig. S2A and B**). Altogether, these data suggested that the mutations affected both tRNA stability and serylation. More importantly, the lack of inactivating mutations in the anticodon-loop supported our hypothesis that mutational alteration of the anticodon-loop of the $tRNA_{CAG}^{Ser}$ did not impact on the serylation of the $tRNA_{CAG}^{Ser}$. In other words, the CUG reassignment was driven by a small number of mutations in the anticodon-arm of the $tRNA_{CAG}^{Ser}$ ancestor that had minor impact on its serylation efficiency.

Expression and in vivo charging of the misreading $tRNA_{CAG}^{Ser}$. To determine the effect of the anticodon-loop mutations (A_{35}, A_{35}+G_{37} and A_{35}+G_{37}+G_{33}) on the cellular activity of the $tRNA_{CGA}^{Ser}$, the WT $tDNA_{CGA}^{Ser}$ gene with its flanking regions

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Figure 2. Variability of anticodon sequences of tRNAs from CTG clade. (**A**) The aligned $tRNA_{CAG}^{Ser}$ and $tRNA_{CGA}^{Ser}$ sequences from the CTG clade species showed high variability in the anticodon region. (**B**) Sequence of anticodon arm of $tRNA^{Ser}$ from CTG clade species showing higher variability in the anticodon-stem of $tRNA_{CAG}^{Ser}$ and $tRNA_{CGA}^{Ser}$, the other tRNAs have more conserved anticodon-stems. The indicated nucleotides are conserved while filled circles correspond to sequence variation. CTG clade species analyzed include *Candida albicans* (ca), *Candida dubliniensis* (cd), *Candida tropicalis* (ct), *Candida parapsilosis* (cp), *Lloderomyces elongisporus* (le), *Candida guilliermondii* (cg), *Debaryomyces hansenii* (dh) and *Candida lusitaniae* (cl). Multiple tRNA sequence alignments were performed with CLC sequence viewer 6 software and tRNA sequences from CTG clade species were obtained with Candida Gene Order Browser.

(150 bp fragment) was amplified by PCR from *C. albicans* gDNA and was cloned into the yeast pRS315 single-copy vector. This allowed for single-copy expression of the $tRNA_{CGA}^{Ser}$ in yeast. The same construct was then used to introduce the single A_{35}, double A_{35}+G_{37} and triple A_{35}+G_{37}+G_{33} mutations into the $tDNA_{CGA}^{Ser}$ for single-copy expression. Since the WT and mutant tRNAs were expressed from the same vector and the same tDNA fragment, the effect of each mutation on the cellular abundance of the $tRNA_{CAG}^{Ser}$ could be determined using comparative northern blot analysis. The electrophoretic mobility of the WT $tRNA_{CGA}^{Ser}$ and mutant $tRNA_{CAG}^{Ser}$ expressed in yeast was similar to that of the WT $tRNA_{CGA}^{Ser}$ expressed in *C. albicans*, indicating the mutant $tRNA_{CAG}^{Ser}$ were correctly processed and expressed in yeast (**Fig. 6A**). Similar expression levels of the WT $tRNA_{CGA}^{Ser}$ were observed in *C. albicans* and yeast, showing that yeast expresses *C. albicans* tRNAs as expected (**Fig. 6A**). However, the single A_{35}, double A_{35}+G_{37} and triple A_{35}+G_{37}+G_{33} mutations reduced the cellular abundance of the mutant tRNAs by 57%, 65% and 55%, respectively (**Fig. 6A and B**), indicating that the anticodon-loop mutations destabilized and likely targeted the mutant $tRNA_{CAG}^{Ser}$ for degradation. Similar results were observed for multi-copy expression of the mutant tRNAs, indicating that increased gene copy number did not increase expression (**Fig. S3**). We cannot exclude the possibility that transcriptional downregulation may have occurred; however, this is unlikely because the tDNA gene promoters are intragenic[23] and the expression of the WT tRNA was not affected, nor was the expression of the control $tRNA_{CCC}^{Gly}$ that was used as internal control for data normalization (**Fig. 6A**).

To test whether the various mutant $tRNA_{CGA}^{Ser}$ were charged in vivo, total tRNA samples were extracted from yeast under acidic conditions (see Materials and Methods) and were fractionated on an acidic 6.5% polyacrylamyde gel containing 8M urea, which separates charged from uncharged tRNA.[24] These tRNAs were then transferred to a hybond-N membrane and were subjected to northern blot analysis. In vitro deacylated tRNAs were used as internal controls. As expected, the WT *C. albicans* $tRNA_{CGA}^{Ser}$ was fully charged in *C. albicans* CaI4 cells; however, ~45% of this WT tRNA was not charged in yeast (**Fig. 6C**), suggesting that the serylation systems of both fungi are not identical. Similar results were obtained for all three mutant $tRNA_{CAG}^{Ser}$ (**Fig. 6C**). These gels further confirmed the low expression levels of the mutant tRNAs, showing that the anticodon-loop mutations affected mainly their expression with little impact on relative levels of serylation.

The impact of CUG reassignment on protein stability and yeast fitness. Proteome-wide Ser misincorporation at Leu-CUG

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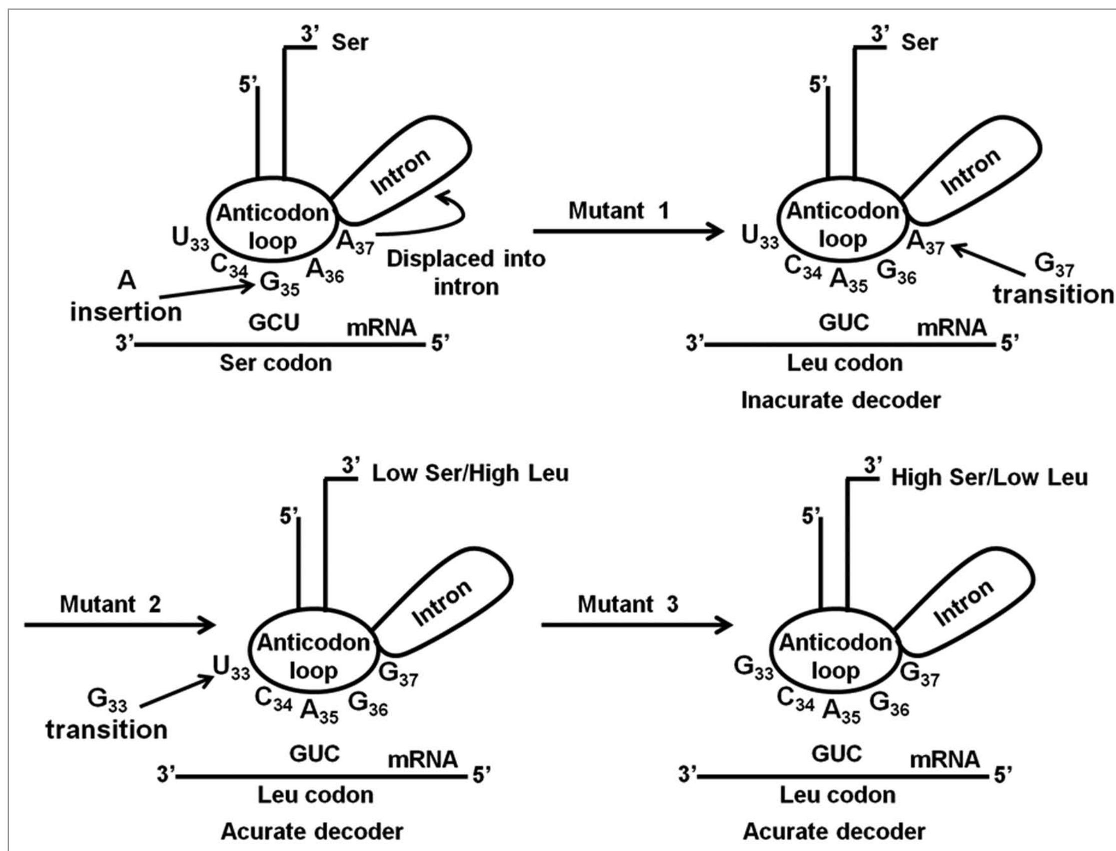

Figure 3. Putative evolutionary pathway of the *Candida albicans* $tRNA_{CAG}^{Ser}$. In silico studies suggested that insertion of an adenosine in the middle position of the anticodon of a $tRNA_{CGA}^{Ser}$ gene transformed the 5'-CGA-3' into a 5'-CAG-3' anticodon, originating the Ser tRNA mutant 1, which is capable of decoding Leu CUG as Ser. Decoding accuracy and efficiency requires two other mutations, namely G_{37} (mutant 2) and G_{33} (mutant 3). The second mutation permitted the recognition of the $tRNA_{CAG}^{Ser}$ by the LeuRS in addition to its cognate SerRS, which introduced ambiguity at CUG codons, while the third mutation lowered leucylation efficiency of the $tRNA_{CAG}^{Ser}$.

sites by the WT *C. albicans* $tRNA_{CAG}^{Ser}$ is toxic and even lethal in certain yeast genetic backgrounds.[14,25] We have used the fitness cost of this toxicity as an in vivo proxy of the decoding efficiency of the mutant $tRNA_{CAG}^{Ser}$. Expression of the A_{35} mutant decreased growth rate by 30% while the double mutant decreased it by 23% (**Fig. 7A and B**). The slightly smaller relative growth rate decrease of the A_{35}+G_{37} is likely associated with the fact that G_{37} is a leucylation identity determinant, i.e., the LeuRS recognizes A_{35} and m^1G_{37},[26] competes with the SerRS for the $tRNA_{CAG}^{Ser}$ and produces Leu-$tRNA_{CAG}^{Ser}$ that incorporates Leu at CUG codons,[11] reducing slightly Ser misincorporation levels and toxicity. The G_{33} mutation (triple mutant A_{35}+G_{37}+G_{33}) decreased growth rate by 46% (**Fig. 7B**), suggesting that this mutation improves decoding efficiency of the tRNA (higher level of Ser misincorporation). This is due to the combined increase in expression of the triple mutant relative the double mutant (**Fig. 6B**) and the negative effect of the G_{33} mutation on leucylation of the $tRNA_{CGA}^{Ser}$ (G_{33} is a leucylation antideterminant).[11]

The above data indicate that the A_{35} initial mutation produced a functional $tRNA^{Ser}$ that could misincorporate Ser at Leu-CUG codons. This tRNA destabilized the proteome and decreased yeast fitness. It also showed that the G_{37} mutation had a secondary impact on fitness relative to the critical A_{35} mutation, while the G_{33} mutation likely improved Ser misincorporation levels significantly. To better clarify the role of the latter mutations on CUG reassignment, we have used a β-galactosidase (β-gal) thermostability assay[14] as readout for Ser misincorporation efficiency. The *E. coli LacZ* gene contains 54 CUG codons, making β-gal a sensitive reporter of Ser misincorporation at CUGs in vivo.[14] Yeast cells expressing the empty plasmid (pRS315), the WT $tRNA_{CGA}^{Ser}$ or one of the three mutant $tRNA_{CAG}^{Ser}$, were co-transformed with the pGL-C1 plasmid, which encodes a fusion between glutathione S-transferase (GST) and β-galactosidase genes (GST-β-gal).[14] The thermal inactivation profiles of β-gal produced in presence of both controls ($tRNA_{CGA}^{Ser}$ and empty pRS315 vector) were similar (**Fig. 7C**), indicating that the *C. albicans* WT $tRNA_{CGA}^{Ser}$ is an authentic Ser decoder in yeast. However, thermal stability of β-gal produced in presence of the mutant $tRNA_{CAG}^{Ser}$ decreased by 40% (**Fig. 7C**). Total β-gal activity was in line with β-gal thermal stability (**Fig. 7D**) as cells expressing A_{35}, A_{35}+G_{37} and A_{35}+G_{37}+G_{33} tRNAs showed relative decrease of 65%, 60% and 58% of β-gal activity. There was a small difference between the single, double and triple mutants, suggesting that decoding efficiency of the three tRNAs was similar (**Fig. 7D**).

Selective advantages introduced by proteome-wide Ser misincorporation. The negative impact of the anticodon mutations

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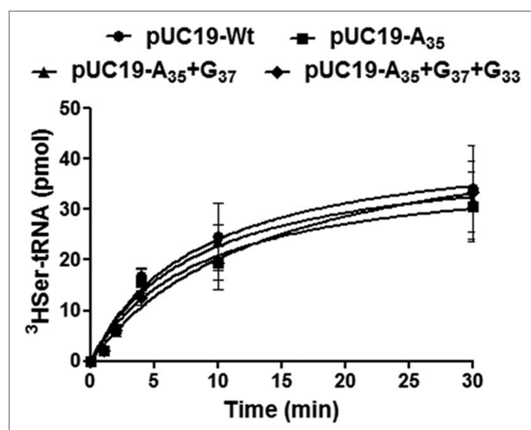

Figure 4. Mutations in the anticodon loop of the $tRNA_{CAG}^{Ser}$ do not affect serylation. In vitro aminoacylation assays were performed with in vitro synthesized wild-type (pUC19-WT) and the mutant $tRNA_{CGA}^{Ser}$, A_{35} (pUC19-A_{35}), $A_{35}+G_{37}$ (pUC19-$A_{35}+G_{37}$) and $A_{35}+G_{37}+G_{33}$ (pUC19-$A_{35}+G_{37}+G_{33}$). Aminoacylation reactions were performed using purified *C. albicans* SerRS overexpressed in *E. coli*. Data represent the mean ± SD of three independent experiments.

on yeast fitness is a strong hurdle to explaining the evolution of the CTG clade genetic code alteration since such evolution needs to be rationalized under negative selective pressure. It is simply not possible to explain how the $tRNA_{CAG}^{Ser}$ was selected. We have shown previously that mistranslation can have mild positive outcomes under specific growth conditions and we postulated that CUG reassignment may have evolved under specific conditions were mistranslation produces adaptive phenotypes.[27] Here we extend those initial studies using a phenotypic array probing 18 growth conditions, which included different nutrients and stressors, namely medium without glucose, 2% galactose, 2% glycerol, acidity, caffeine, ethanol, sorbitol, SDS, cadmium, copper, chromium, lithium, geneticin, paramomycin, menadione, EDTA, calcium and sodium chloride. The data showed that Ser misincorporation at CUG codons had mainly negative outcomes; however, near-neutral and positive outcomes were visible in some cases (**Fig. 8**). For example, there was a strong growth advantage (500%) of the cells expressing the $tRNA_{CAG}^{Ser}$ in presence of copper sulfate (2.5 mM) and also in presence of cadmium chloride (50 μM) (90%) relative to the control cells. Sorbitol (1.5 M), pH 6 and menadione (150 μM) had near neutral effects, but growth was very poor in presence of caffeine (10 mM), calcium chloride (0.75 M), chromium trioxide (0.5 mM) and EDTA (0.5 mM). Therefore, mistranslation produces antagonistic pleiotropic phenotypes that CUG reassignment expanded the adaptive ecological landscape of the CTG clade ancestor by allowing it to proliferate in ecological niches where WT cells could not survive or competed poorly with the mistranslating cells.

Discussion

The discovery of codon reassignments raises a series of new questions about the evolution of the genetic code and the biological relevance of altering codon identity. Two theories have been proposed to explain the evolution of codon reassignments, namely the near-neutral "codon capture theory"[28-30] and the non-neutral "ambiguous intermediate theory."[31] The former postulates that biased genome G+C pressure erases codons from the genome and that such codons can be re-introduced through mutation, acquiring a new meaning without altering protein structure.[30,32] This theory is strongly supported by the disappearance of the A+T rich AUA (Ile) and AGA (Arg) and the CGG (Arg) codons in *Micrococcus* sp and *Mycoplasma* sp, whose genome G+C is 75% and 25%, respectively.[33-35] However, this is a controversial theory because mitochondria have very high A+T content, use the full set of 61 sense codons of the genetic code and some of the reassigned codons violate the A+T biases, for example, the reassignment of the UAA stop codon to Trp and AAA (Lys) to Asn.[2] The ambiguous intermediate theory postulates that mutant misreading tRNAs introduce codon ambiguities whose selection led to codon reassignment through disappearance of the cognate tRNAs.[36] This theory implies that codon misreading produces phenotypic advantages that allow for selection of the mutant misreading tRNAs. However, the theory does not explain what kind of selective advantages may arise from codon misreading, creating the difficulty of explaining how codon misreading is sustained and fixed. We have previously demonstrated that codon ambiguity and biased G+C pressure work in similar ways since CUG ambiguity erased almost all CUGs from the genome of the CTG clade ancestor,[9] suggesting that over time the toxicity of codon ambiguity can be eliminated. But, it has been remarkably difficult to explain how the ambiguous $tRNA_{CAG}^{Ser}$ was maintained over the time frame of CUG elimination. The discovery that codon ambiguity can be advantageous under specific ecological conditions[37-41] is, therefore, of paramount importance to explain the evolution of genetic code alterations. One may not find the specific ecological condition of CUG reassignment, but the selective advantages produced by codon ambiguity raise the possibility that ambiguous tRNAs can be selected. The other main issue raised by the ambiguous intermediate theory is the molecular mechanism of codon ambiguity. Clearly, tRNAs are not the only molecules that influence codon decoding fidelity and other translational factors are likely involved in codon reassignment. This has been demonstrated by the multiple reassignments of stop codons in many phylogenetic groups, which are associated with mutations in both release factors[42,43] and tRNAs/aaRS pairs,[44,45] and also by the expansion of the genetic code to incorporate selenoscysteine[46] and pyrrolysine[47] where a series of novel translational factors are involved.

The near-neutral role of the SerRS in CUG reassignment. Our data demonstrate that the evolutionary pathway of CUG reassignment in the fungal CTG clade is linked to the evolution of the $tRNA_{CAG}^{Ser}$. Indeed, no relevant roles have yet been found for the SerRS and LeuRS or any other translational factor in this reassignment pathway.[9] Therefore, the study of the phylogeny, structure and function of the $tRNA_{CAG}^{Ser}$ is likely to elucidate how the CTG clade ancestor reassigned the CUG codon. Our aminoacylation data show similar serylation efficiencies for the WT and mutant $tRNA^{Ser}$, confirming that the SerRS did not play an active role in CUG reassignment.[14] This can be explained by the yeast serylation determinants. The yeast SerRS does not interact

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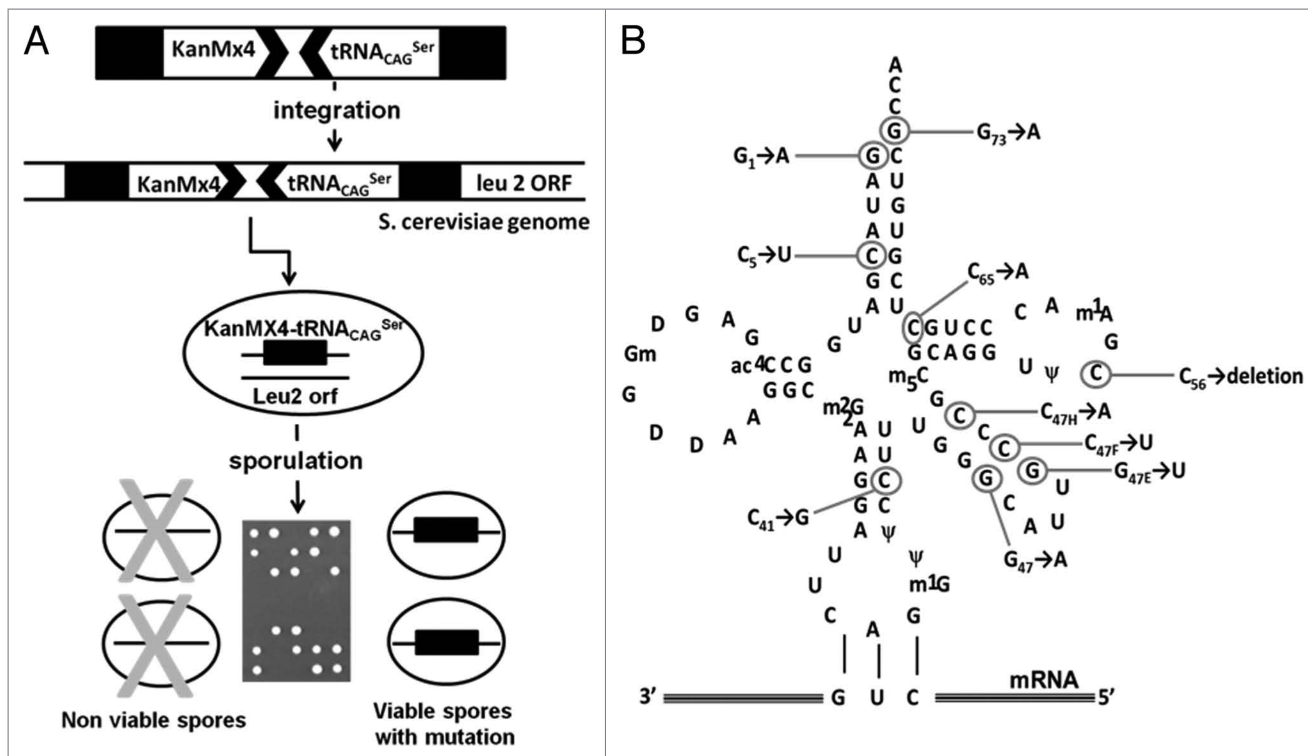

Figure 5. In vivo forced evolution methodology used to identify mutations that inactivate the *C. albicans* $tRNA_{CAG}^{Ser}$. (**A**) In vivo forced evolution was used to screen for mutations that affect the *C. albicans* $tRNA_{CAG}^{Ser}$ stability and identity. This method was based on PCR amplification of the DNA cassette containing the $tRNA_{CAG}^{Ser}$ fused to the KanMx4 gene and subsequent integration by homologous recombination into the LEU2 locus of diploid yeast cells. Yeast containing the $tRNA_{CAG}^{Ser}$ gene integrated in the genome were sporulated and tetrades were dissected using a micromanipulator. The $tRNA_{CAG}^{Ser}$ gene was amplified from viable spores by PCR and the amplified DNA was sequenced. (**B**) The in vivo forced evolution strategy using the $tRNA_{CAG}^{Ser}$ gene expressed in yeast cells identified 10 different mutations. Most mutations localized in the acceptor-stem and variable-arm.

with the anticodon of yeast $tRNA^{Ser}$ and serylation is dependent on the discriminator base G_{73} and 3 GC base pairs located in the extra-arm, away from the anticodon-arm.[21,48,49] Our in vivo forced evolution data supports this hypothesis as inactivating mutations accumulated in the extra-arm and acceptor-stem of the $tRNA_{CAG}^{Ser}$, rather than in the anticodon-arm (**Fig. 5B**). We have identified mutations in tRNA structural domains that are not known to interact with the SerRS, namely the C_{65} to A_{65} mutation and deletion of C_{56} in the TΨC-arm, but these mutations likely disrupted the tRNA L-shape fold and targeted the tRNA for degradation (**Fig. S1**). Indeed, C_{56} forms a non-Watson-Crick tertiary interaction with G_{19} in the D-arm and is essential for the correct folding of the cloverleaf structure into the L-shape 3D structure, while C_{65} is critically located between the TΨC and acceptor stems[50] and its substitution may alter the structure of the TΨC loop. The G_{73} to A_{73} inactivating mutation identified in our forced evolution experiment is very exciting because A_{73} is a leucylation discriminator while G_{73} is a serylation discriminator.[20,48,51,52] Indeed, $tRNA^{Ser}$ are efficiently leucylated if the G_{73} discriminator (typical of Ser tRNAs) is mutated to A_{73}.[20] Moreover, the converse mutation of A_{73}→G_{73} changes human $tRNA^{Leu}$ into a Ser-isoacceptor, showing minimal structural requirements for conversion of $tRNA^{Leu}$ into $tRNA^{Ser}$ and vice-versa. These observations raise the intriguing question of how the CTG clade LeuRS recognizes the $tRNA_{CAG}^{Ser}$ containing G_{73}. Previous in vitro and in vivo data from our laboratory show that the *C. albicans* LeuRS recognizes and charges the WT $tRNA_{CAG}^{Ser}$, leading to approximately 3% incorporation of Leu at CUG sites,[8] despite the presence of G_{73} in the $tRNA_{CAG}^{Ser}$. It will be interesting to crystalize and determine the 3D-structure of the LeuRS-$tRNA_{CAG}^{Ser}$ complex in order to solve this puzzling question.

Apart from the mutations mentioned above, our forced evolution screen identified U_5 and A_1 mutations in the acceptor stem of the $tRNA_{CAG}^{Ser}$. The U_5 mutation destabilized and decreased sharply the serylation of the $tRNA_{CAG}^{Ser}$ (**Figs. S1 and S2**), likely due to disruption of the $tRNA_{CAG}^{Ser}$ interaction with the SerRS.[21] Finally, the mutations identified in the extra-arm hit the main serylation identity determinants (three GC base pairs) and prevented serylation of the $tRNA_{CAG}^{Ser}$ by the SerRS[18,19] (**Fig. S2**). Selection of these mutations by forced evolution is likely associated with lack of serylation and destabilization of the mutant tRNA, as shown in **Figures S1 and S2**.

Roles of the $tRNA_{CAG}^{Ser}$ in CUG reassignment. The phylogenetic data obtained to date strongly support the hypothesis that a minimal set of mutations in the anticodon-arm of the $tRNA_{CAG}^{Ser}$ are the key elements of CUG reassignment.[9] Our data showed that toxicity levels associated with Ser misincorporation were $A_{35}+G_{37}+G_{33} > A_{35} > A_{35}+G_{37}$, while β-gal activity and thermo stability data indicated that Ser misincorporation was similar in the three mutants. Anticodon context rules posit that 5'-CAG-3'

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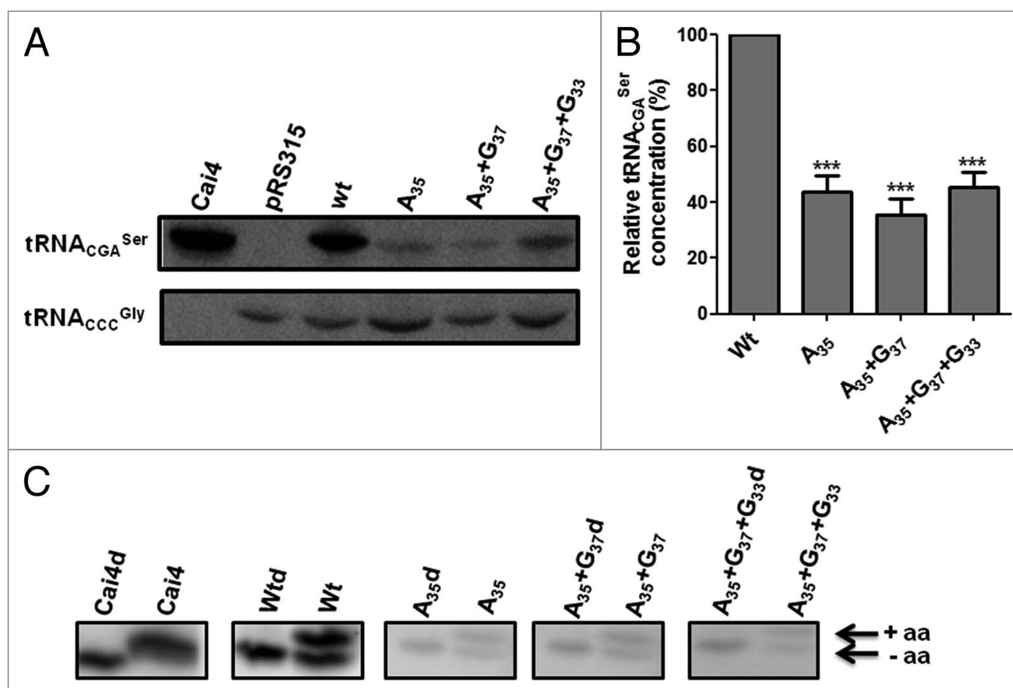

Figure 6. The mutants tRNA$_{CAG}^{Ser}$ are expressed at low level in yeast. (**A**) To check the in vivo expression of the WT tRNA$_{CGA}^{Ser}$ and mutant tRNA$_{CGA}^{Ser}$ in yeast, 50 µg of total tRNA were extracted and purified under acidic conditions and were fractionated on 15% polyacrylamide gels containing 8 M urea at room temperature. tRNA$_{CGA}^{Ser}$ and tRNA$_{CCC}^{Gly}$ were detected using γ-^{32}P-ATP-tDNA$_{CGA}^{Ser}$ and γ-^{32}P-ATP-tDNA$_{CCC}^{Gly}$ probes. Cai4 corresponds to total tRNA purified from *C. albicans*. Membranes were exposed for 24 h to a K-screen and were visualized using a Bio-Rad Molecular Imager FX. (**B**) Quantification of expression of the mutant tRNAs relative to the WT tRNA. Data represent the mean ± s.e.m. of three independent experiments (***$p < 0.001$, one-way Anova post Bonferroni's test with CI 95% relative to Wt tRNA$_{CGA}^{Ser}$). (**C**) In vivo aminoacylation of the WT and mutant tRNA$_{CGA}^{Ser}$ in yeast. In vivo aminoacylation of WT and mutant tRNA$_{CGA}^{Ser}$ was evaluated using acidic northern blot analysis. In vitro deacylated and in vivo acylated tRNAs were fractionated on 6.5% polyacrylamide gels containing 8 M urea at 4°C, using 10 mM sodium acetate (pH = 4.5) buffer. The tRNA$_{CGA}^{Ser}$ was detected using γ-^{32}P-ATP-tRNA$_{CGA}^{Ser}$ probe. Cai4 corresponds to tRNA extracted from *C. albicans*. Membranes were exposed for 24 h to a K-screen and were visualized using the Bio-Rad Molecular Imager FX.

anticodons require a methyl group at G_{37} (m^1G_{37}) instead of A_{37} to prevent frameshifting during mRNA decoding.[11] Therefore, translational fidelity pressure may have led to selection of the G_{37} mutation. This raises the possibility that the lower toxicity of the A_{35}+G_{37} mutant is due to both increased decoding accuracy and increased leucylation efficiency, as m^1G_{37} is a leucylation determinant.[15] The G_{33} mutation is interesting and surprising. Available tRNA crystal structures show that the U_{33} is required for the turn of the anticodon-loop (U-turn). G_{33} distorts the turn of the phosphate backbone in the anticodon-loop and alters the structure of the RNA helix of the anticodon-stem.[12,13,53] Therefore, the selective pressure that drove selection of the G_{33} mutation is still unclear, but it is likely that G_{33} keeps leucylation of the tRNA$_{CAG}^{Ser}$ within tolerable limits, as G_{33} is a leucylation antideterminant.[11]

Our most surprising result was the strong negative impact of the anticodon-loop mutations on the expression of the mutant tRNAs. We were not able to identify the molecular mechanism that mediates that tRNA downregulation, but possible explanations are repression of Pol III transcription of the tDNA$_{CGA}^{Ser}$ gene, or increased turnover of the expressed mutant tRNA$_{CGA}^{Ser}$. The former hypothesis is unlikely because the mutant and control tDNA genes were cloned in the same plasmid with identical upstream and downstream sequences. Since Pol III promoters are intragenic and the flanking sequences have little influence on transcription, one is tempted to assume that repression of Pol III transcription may not explain the downregulation of the mutant tRNA$_{CGA}^{Ser}$. However, the mutations introduced in the anticodon-loop of the tRNA$_{CGA}^{Ser}$ may have altered the pattern of modification of the anticodon-arm or distorted the RNA-helix of the anticodon-stem, targeting the tRNA for degradation. The previous finding that G_{33} distorts the anticodon-stem helix of the tRNA$_{CAG}^{Ser}$[13] supports this hypothesis. More interestingly, our data show that the mutant tRNA$_{CAG}^{Ser}$ are not degraded by known tRNA degradation pathways since their expression in yeast cells harboring knockouts in the genes involved in tRNA degradation, namely *RNY1, XRN1, TRF4, TRF5, MET22, AIR2* genes, did not increase tRNA$_{CGA}^{Ser}$ expression (**Fig. S4**). However, one cannot exclude compensatory effects due to functional redundancy of the tRNA metabolism pathways, i.e., knocking out one pathway may not produce a visible phenotype due to compensatory upregulation of the other pathways.

Putative pathway of CUG reassignment. The non-neutral evolution of the CTG clade genetic code alteration involving two chemically distinct amino acids (Ser is polar while Leu is hydrophobic) poses a significant biological problem whose solution is still incomplete. Indeed, our reconstruction model of the reassignment pathway shows that Ser misincorporation at CUG sites has major negative impact on fitness, raising the puzzling question of how the tRNA$_{CAG}^{Ser}$ was selected under negative

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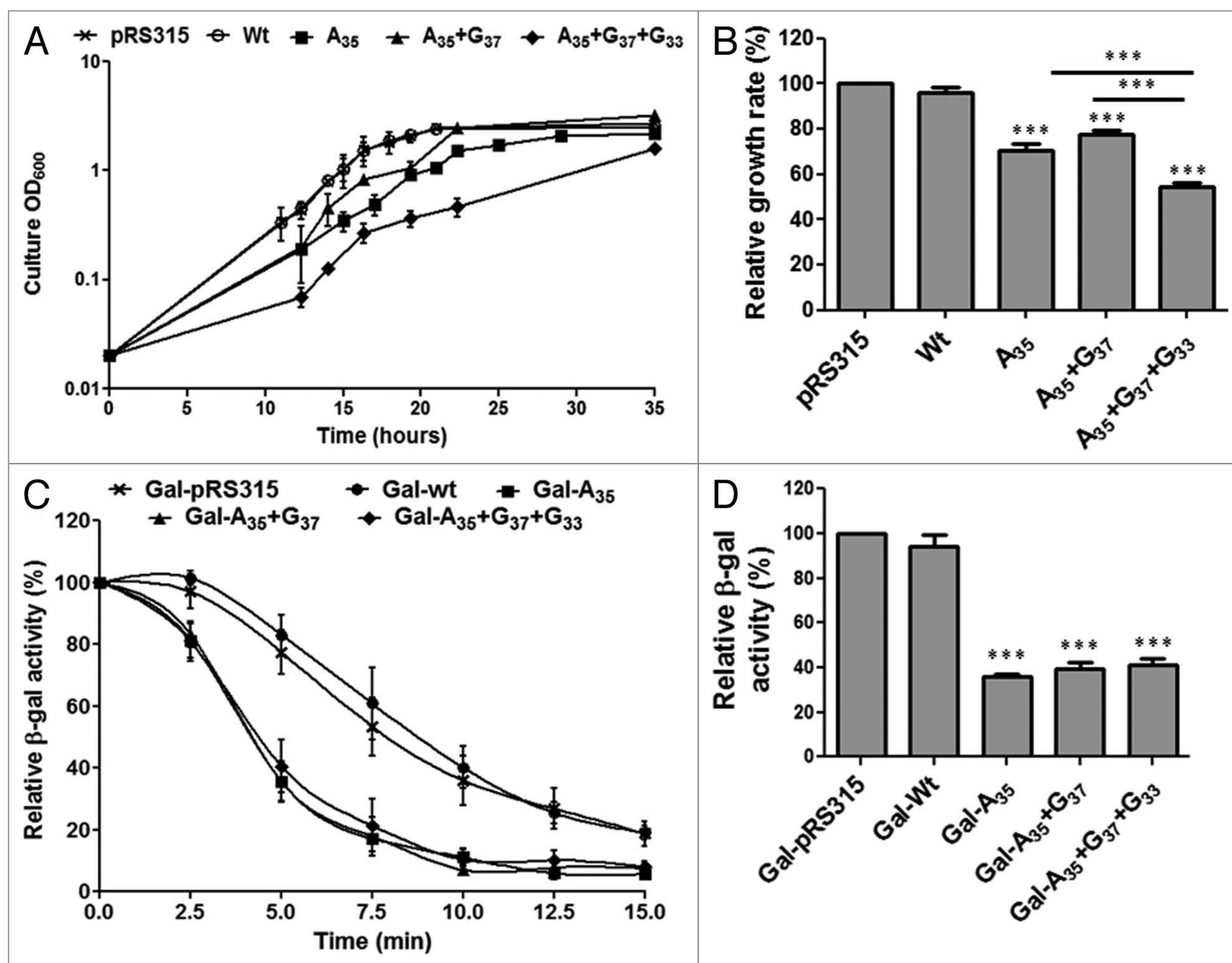

Figure 7. Effect of CUG reassignment on yeast fitness. (**A**) Partial CUG reassignment had a strong negative impact on yeast growth rate. Yeast cell cultures transformed with WT and mutant *C. albicans* $tRNA_{CGA}^{Ser}$ were inoculated at an initial OD_{600} of 0.02 and were grown in selective medium at 30°C, 180 rpm, until they reached stationary phase. (**B**) The relative growth rate of cells transformed with WT and mutant $tRNA_{CGA}^{Ser}$ was determined using exponential growth phase values, relative to the control cells. (**C**) To determine the effect of Ser misincorporation on protein structure *E. coli* β-gal was co-expressed with WT $tRNA_{CGA}^{Ser}$ and mutant $tRNA_{CGA}^{Ser}$ in yeast cells. Thermoinactivation profiles were determined by measuring β-gal activity after a denaturation step at 47°C. The β-gal activity that remained functional after thermal inactivation was measured by incubating cells at 30°C for 2 min with ONPG. β-gal activity at each time point corresponds to the % of activity relative to total activity measured in cells prior to denaturation. (**D**) β-gal activity was quantified after incubating total cell protein extracts at 30°C with ONPG until a pale yellow color appeared. Reactions were stopped with addition of 1 M sodium carbonate. Data represent the mean ± s.e.m. of triplicates of three independent clones (***p < 0.001 for one-way Anova post Bonferroni's multiple comparison test with CI 95%, relative to pRS315).

pressure. The results described here highlight a new feature of the evolution of this genetic code alteration, namely that low level expression of the misreading tRNAs may have minimized Ser misincorporation at CUGs, reducing initial proteome instability and the negative impact on fitness. The combination of the selective advantages produced by Ser misincorporation in specific environmental conditions and decreased toxicity associated with tRNA downregulation may have allowed Ser misincorporating cells to explore expanded ecological landscapes. In other words, we can envision an evolutionary scenario where the negative impact of Ser misincorporation was not an impediment to gradual reassignment of the CUG codon. We postulate that such scenario allowed time for accommodation of the Ser misincorporations through accumulation of compensatory mutations in the genome.

Materials and Methods

Strains and growth conditions. *Saccharomyces cerevisiae* BMA64 strain (EUROSCARF, acc. number 20000D, genotype MATa/MATa; ura3-52/ura3-52; trp1Δ2/trp1Δ2; leu2-3_112/leu2-3_112; his3-11/his3-11; ade2-1/ade2-1; can1-100/can1-100), CEN-PK2 (EUROSCARF, acc. number 30000D, genotype MATa/MATα ura3-52/ura3-52; trp1-289/trp1-289; leu2-3,112/leu2-3,112; his3Δ 1/his3Δ 1; MAL2-8^C/MAL2-8^C; SUC2/SUC2'), BY4743 (EUROSCARF, acc. number 20000D, genotype MATa/MATα his3Δ 0/his3Δ 0; leu2Δ/leu2Δ 0; met15Δ 0/MET15; LYS2/lys2Δ 0; ura3Δ 0/ura3Δ 0) and respective BY4743 knockouts for RNY1 (EUROSCARF, acc. number Y22129), XRN1 (EUROSCARF, acc. number Y34540), TRF4 (EUROSCARF, acc. number Y36265), TRF5 (EUROSCARF,

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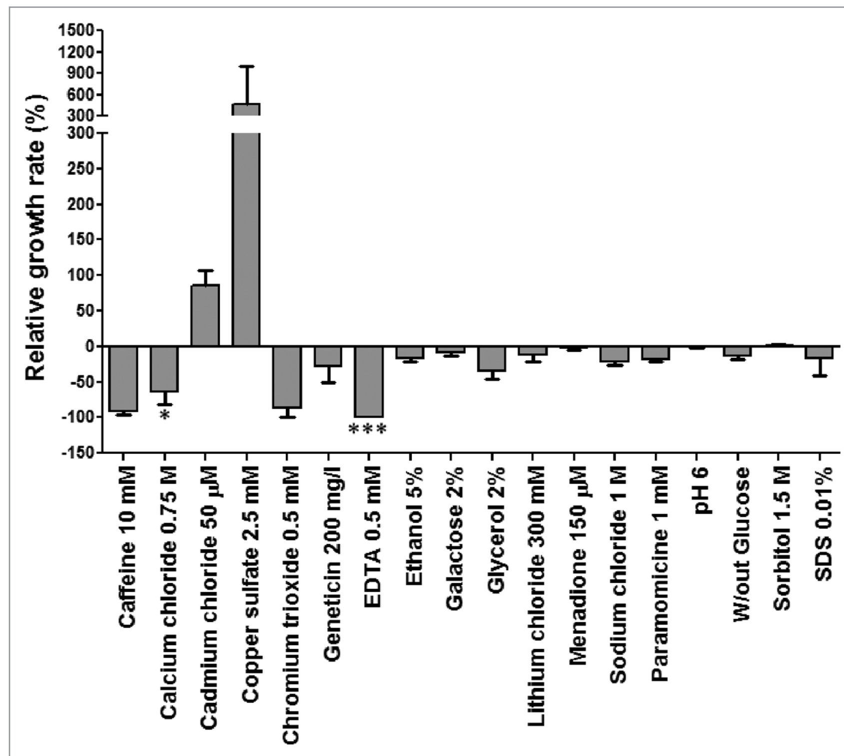

Figure 8. Partial CUG reassignment produces pleiotropic phenotypes in yeast. The growth rate of cells mistranslating the CUG codon as Leu/Ser relative to the control strain in presence of 18 different environmental conditions was measured. Results represent growth advantage (> 0) or disadvantage (< 0) relative to control strain (pRS315). Data represent the mean ± s.e.m. of duplicates of 3 independent clones (***$p < 0.001$, *$p < 0.05$ one-way Anova post Bonferroni's multiple comparison test with CI 95% relative to pRS315).

acc. number 31145), MET22 (EUROSCARF, acc. number Y31756) and AIR2 (EUROSCARF, acc. number Y33873) were grown at 30°C with or without agitation (180 rpm) in YPD + 2% agar (Formedium) or minimal medium (0.67% yeast nitrogen base without amino acids, 100 μg/ml of each of the required amino acids, 2% glucose, 2% agar) (Formedium). In the case of yeast expressing the integrative tRNA, YPD medium was supplemented with 200 mg/l of geneticin (Formedium, #G4185). *Candida albicans* CAI4 strain (ura3D::imm434/ura3::imm434) was grown at 30°C with or without agitation (180 rpm) in YPD medium. *Escherichia coli* strain DH5α (F-φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk–, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-) was grown at 37°C with or without agitation (180 rpm) in LB broth medium or in LB 2% agar (Formedium) supplemented with ampicilin 50 μg/ml (Sigma-Aldrich).

Plasmids construction and transformation. For heterologous expression of the *C. albicans* $tRNA_{CGA}^{Ser}$ gene in yeast, a genomic DNA fragment of 150 bp encoding the *C. albicans* $tRNA_{CGA}^{Ser}$ gene (AAATTTGACA GTGTGGCCGA GCGGTTAAGG CGTCTGACTC GAATCTTATT CGCGTTATCA GTTGGG CTTT GCCCGCGCAG GTTCGAATCC TGCTGCTGTC GTCATAAGTT ATTTTTTTGT TTCTTGAATA TTTTT TTCCC AC) retrieved from Candida Genome Database (www.candidagenome.org), previously amplified by PCR and digested with BamHI and SalI restriction enzymes (Fermentas) was ligated to single-copy expression vector pRS315 using T4 DNA ligase (Fermentas). Ligations were transformed in *E. coli* DH5α competent cells using standard transformation protocols. (Sambrook et al., 2008) Plasmid DNA was extracted using plasmid mini prep kit (QIAGEN) and sequenced. The mutations proposed for the evolutionary pathway, namely the A_{35} insertion, A_{37}→G_{37} transition and U_{33}→G_{33} transversion were introduced in sequential order in $tRNA_{CGA}^{Ser}$ gene previously cloned in plasmid pRS315 by site directed mutagenesis (SDM) using Pfu DNA polymerase (Fermentas). Briefly, in the pRS315 plasmid containing the $tRNA_{CGA}^{Ser}$ WT sequence (plasmid WT) was inserted by SDM an adenosine into the middle position of the anticodon (position 35) of the $tRNA_{CGA}^{Ser}$ gene, originating the A_{35} plasmid. The A_{37}→G_{37} transition was inserted into $tRNA_{CGA}^{Ser}$ gene containing the A_{35} mutation (A_{35} plasmid) by SDM originating the A_{35}+G_{37} plasmid. Finally, the transversion of U_{33}→G_{33} was introduced in the $tRNA_{CGA}^{Ser}$ gene containing the A_{35} and G_{37} mutations (A_{35}+G_{37} plasmid) by SDM originating the A_{35}+G_{37}+G_{33} plasmid. Details of the oligonucleotides used and plasmids constructed are listed in **Tables S1 and S2**. All SDM reactions were performed with 50 ng of plasmid template DNA in 1x Pfu DNA polymerase buffer, 2 mM $MgSO_4$, 20 pmol of each specific oligonucleotide (**Table S1**), 200 μM of each dNTP and 2.5 units of Pfu DNA polymerase (Fermentas) in a final volume of 50 μl. PCR reactions were performed in a thermal cycler (BioRad) using a standard program of 95°C 30 sec followed by 18 cycles (95°C 30 sec, 55°C 1 min, 68°C 7 min). The SDM products were digested for 2 h at 37°C with 20 unit of DpnI (Fermentas) and then transformed in DH5α competent cells. Plasmid DNA was

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extracted using minipreparation kits and sequenced by Sanger sequencing.

S. cerevisiae BMA64 cells were transformed using the lithium acetate method,[54] with pRS315 and the constructed plasmids (WT, A_{35}, A_{35}+G_{37} and A_{35}+G_{37}+G_{33}). Transformants were selected in minimal medium lacking leucine and the tDNA genes were amplified directly from yeast colonies and were sequenced using oligonucleotides listed in **Table S1**.

Phylogenetic analyses. Sequence of Ser and Leu tRNAs from CTG clade species were retrieved from the Candida Gene Order Browser version 2.0 (cgob.ucd.ie/). tRNA sequences were aligned using multiple sequence alignments with CLC Sequence Viewer 6 software. The phylogenetic tree was constructed using a maximum-likelihood (ML) method with Molecular Evolutionary Genetics Analysis (Mega 5) software.[55] Bootstraps were estimated from 1,000 nonparametric bootstrap runs.

Forced evolution methodology. The *Candida albicans* $tRNA_{CAG}^{Ser}$ gene fused with the KanMX4 gene and the KanMX4 gene were amplified by PCR from the plasmids pUA69 and pUA707,[25] using the oligonucleotides listed in **Table S1**. PCR products (500 ng) previously purified with QIAquick PCR purification kit (QIAGEN) were integrated into the genome of *S. cerevisiae* CEN-PK2 using the PCR-based gene disruption method.[56] Cells were selected in YPD+geneticin (200 mg/l) (Formedium). KanMX4 and tRNA gene integrations were confirmed by PCR and Sanger sequencing. Selected clones containing the $tRNA_{CAG}^{Ser}$ gene integrated into the genome were grown overnight in YPD+geneticin and then collected, washed and transferred to sporulation medium (1% potassium acetate, 0.1% yeast extract and 0.05% glucose) until sporulation occurred. Spores were dissected using a MSM System Series 300 micromanipulator (Singer) as described previously.[25] From grown spores, DNA was extracted using the Wizard genomic DNA purification kit (Promega) and $tRNA_{CAG}^{Ser}$ gene was amplified by PCR and sequenced using the oligonucleotides listed in **Table S1**.

In vitro tRNA synthesis. tRNA constructs for in vitro transcription containing a T7 promoter and the WT *C. albicans* $tRNA_{CGA}^{Ser}$ sequence were assembled using six DNA oligonucleotides (**Table S1**), which were first annealed and then ligated between HindIII and BamHI restriction sites of plasmid pUC19. In the resulting plasmid were inserted by SDM the single mutation A_{35}, the double mutation A_{35}+G_{37} and the triple mutation A_{35}+G_{37}+G_{33}. All the oligonucleotides used and details of the plasmids constructed are listed in **Tables S1 and S2**. In vitro transcription using T7 RNA polymerase was performed as previously described.[57] Transcripts were fractioned on 8% polyacrilamide-8 M urea gels and were eluted from the gels using a dialysis membrane (Dialysis tubing, high retention seamless cellulose tubing, Sigma), in 0.5x TBE, 150 V, 2 h. Prior to use, tRNAs were refolded as described previously.[58]

Aminoacylation assays. Aminoacylation of tRNA was performed at 30°C in 100 mM HEPES-KOH pH = 7.6, 20 μM of serine/L-[^{3}H(G)]-Serine (500 Ci/mol) (Perkin Elmer, #NET248001MC), 15 mM magnesium chloride, 4 mM DTT, 2 mM ATP, 15 mM potassium chloride, 0.1 mg/ml BSA and 5 μM tRNA transcripts. The reaction was initiated by the addition of 50 nM of purified SerRS[10,59] and aliquots (13 μl) of the reaction mixture were spotted onto Whatman 3MM discs at various time intervals. Discs were washed for 10 min in 5% TCA and once with ethanol for 10 sec, at room temperature. Radiolabelled aminoacyl-tRNA was quantified using a liquid scintillation counter (Beckman Coulter).

β-Galactosidase assays. Yeast cells expressing the empty plasmid (pRS315), WT and the mutant $tRNA_{CGA}^{Ser}$ were co-transformed with the multi-copy vector pGL-C1 which contains the β-Galactosidase (β-gal) gene under the control of the GPD promoter and is fused with the glutathione S-transferase (GST-β-gal). Yeast cells expressing both vectors were selected on minimal medium lacking Leu and Trp. The resulting double transformants were named Gal-pRS315, Gal-Wt, Gal-A_{35}, Gal-A_{35}+G_{37} and Gal-A_{35}+G_{37}+G_{33}. β-gal thermoinactivation was monitored directly in yeast cells as described previously.[14,60] Briefly, 500 μl of exponentially growing yeast cells transformed both with pGL-C1 and each one of the plasmids expressing the mutant tRNAs were harvested, washed and ressuspended in 800 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM $NaH_2PO_4 \cdot 2H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM 2-mercaptoethanol, pH 7.0), 20 μl of 0.1% SDS and 50 μl of chloroform. Cell suspension was vortex for 30 sec and incubated at 47°C from T0' up to T15' in triplicates. This β-gal unfolding step was followed by a refolding step, performed by incubating samples on ice for 30 min. To quantify the residual β-gal activity samples were incubated at 30°C for 5 min and 200 μl of o-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml) (Calbiochem, #48712) were then added to each tube. Reactions were allowed to proceed for 2 min and then stopped by addition of 400 μl of 1 M Na_2CO_3. Cell suspension was centrifuged at 12,000 × g for 10 min and OD_{420} was measured. Relative β-Gal thermoinactivation was calculated as the % of variation of β-gal activity at each time point, relative to cells that were not incubated at 47°C.

Total β-gal activity was determined as described previously[61] with small modifications. Briefly, 500 μl of yeast cells in mid-exponential phase were collected, washed, ressuspended in 250 μl of breaking buffer (100 mM Tris-Cl pH 8, 1 mM DTT and 20% Glycerol), 12.5 μl of PMSF (40 mM in 100% isopropanol) and 150 μl of glass beads. Cells were disrupted with three cycles (5,000 rpm 10 sec followed of 2 min on ice) in a Precellys system (Omni international). Cells suspensions were centrifuged at 2,300 × g for 15 min and 990 μl of Z buffer were added to 10 μl of cell extracts. Extracts were incubated at 30°C with 200 μl of ONPG (4 mg/ml) until a pale yellow color appeared. Reactions were stopped with 500 μl of 1 M Na_2CO_3 and OD_{420} was measured. Protein concentration of cell extracts was quantified using the BCA protein quantification kit (Pierce). Activity of β-gal was calculated as follows: Activity = (OD420 × 1.7)/[protein concentration (mg/ml) × volume extract (ml) × time (minutes)]. β-gal activity was normalized relative to the activity of control cells (Gal-pRS315).

tRNA isolation. RNA was isolated by hot-phenol method[62] with few modifications. Briefly, 250 ml cultures grown until early stationary phase were harvested and frozen overnight at –80°C. Cell pellets were ressuspended in 12 ml of lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA, 0.5% SDS) and 12 ml of acid phenol

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chloroform (5:1 pH 4.7, Sigma), vigorous mixed (vortex) and heated at 65°C for 1 h. The aqueous phase was separated from the phenolic phase by centrifugation at 8,000 × g for 30 min at 4°C. Aqueous phase was re-extracted with same volume of phenol by centrifugation at 7,000 × g for 20 min at 4°C and next with the same volume of Chloroform Isoamyl Alcohol 24:1 (Fluka). RNA was precipitated overnight at –30°C with 3 volumes of ethanol 100% and 0.1 volumes of 3 M sodium acetate pH 5.2. RNAs were harvest by centrifugation at 7,000 × g for 30 min at 4°C, ressuspended in 0.1 M sodium acetate pH 4.5 and applied to a 50 ml DEAE-cellulose (Sigma, #D3764) column equilibrated with the same buffer as previously described.[14] tRNAs were washed with 0.1 M sodium acetate/0.3 M sodium chloride, eluted with 0.1 M sodium acetate/1 M sodium chloride, precipitated with ethanol and ressuspended in 10 mM sodium acetate pH 4.5/1 mM EDTA. For deacylation, 200 μg of tRNAs were incubation at 37°C, 1 h in 1 M Tris pH 8.0/1 M EDTA buffer, followed by ethanol precipitation and were resuspended in 20 μl of 10 mM sodium acetate pH 4.5/1 mM EDTA.

Northern blot analysis. tRNAs were fractioned in polyacrylamide gels as previously described.[24] Briefly, 50 μg of total tRNAs were fractionated in 12–15% polyacrylamide (40% Acril:Bis) gels containing 8 M urea (30 cm long, 0.8 mm thick), buffered with 1x TBE pH 8.0 at room temperature, 550 V for 15 h. For acidic northern blot analysis 50 μg of total tRNAs acylated and deacylated were fractionated in 6.5% polyacrylamide (40% Acril:Bis) gels containing 8 M urea buffered with 0.01 M sodium acetate (pH = 4.5) at 4°C, 250 V for 36 h. Fractionated tRNAs were transferred to Hybond-N membranes (Amersham, #RPN203N) using a Semy-Dry Trans Blot (Bio-Rad) at 0.8 mA/cm^2 of membrane for 35–45 min. Immobilization of transferred RNAs on membranes was performed employing the STRATAGENE UV crosslinker (Stratagene). Membranes were pre-hybridized at 55°C during 2 h in hybridization solution (6x SSPE/0.05% SDS/5x Denhardt's solution; 50x Denhardt's solution = 0.02% bovine, serum albumin, 0.02% polyvinylpyrrolidone and 0.02% Ficoll). Probes were prepared by incubating at 37°C for 1 h, reactions containing 10 pmol of dephosphorilated oligonucleotides and 4 μl of γ-^{32}P-ATP (3000 Ci/mmol) (Perkin Elmer, #NEG002A250UC) in 1x T4 kinase buffer with 10 mM spermidine and 16 units of T4 kinase (Takara, #2021A). Labeled probes were extracted with phenol:chlorophom:isolamyl alcohol (Sigma). Membrane hybridization was performed overnight in 10 ml of hybridization solution with γ-^{32}P-ATP-labeled probe. Membranes were washed at hybridization temperature four times during 3 min in 2x SSPE/0.5% SDS, wrapped in saran wrap and exposed 24 h to a K-screen and visualized using a Molecular Imager FX (Biorad). Probes used for northern blot analysis are listed in **Table S1**.

Phenomics of mistranslating strains. Phenotyping analysis was performed as described previously[63] with few modifications. Briefly, yeast cells were grown until middle exponential phase and 1 × 10^8 cells were collected. Six 10-fold serial dilutions were spotted using a liquid handling station (Caliper LifeSciences) in plates with minimal medium lacking Leu, supplemented with the appropriate stressor. After 5 d of growth, the diameter of the colonies was measured using Image J software. The growth rate of each dilution was scored relative to the non-stress control plate. These growth rate values were normalized for the control strain (pRS315) and the final percentage of growth score was obtained by calculating the median growth rate of all dilutions in each stress condition.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Materials

Supplemental materials may be found here:
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