# Evolving Mistranslating tRNAs Through a Phenotypically Ambivalent Intermediate in *Saccharomyces cerevisiae*

**Matthew D. Berg,*,[1] Kyle S. Hoffman,* Julie Genereaux,* Safee Mian,* Ryan S. Trussler,* David B. Haniford,* Patrick O'Donoghue,*,[†] and Christopher J. Brandl*,[1]**

*Department of Biochemistry and [†]Department of Chemistry, University of Western Ontario, London, Ontario N6A 5C1, Canada

ORCID IDs: 0000-0002-7924-9241 (M.D.B.); 0000-0003-0170-2090 (K.S.H.); 0000-0002-9062-492X (S.M.); 0000-0001-8015-9668 (C.J.B.)

**ABSTRACT** The genetic code converts information from nucleic acid into protein. The genetic code was thought to be immutable, yet many examples in nature indicate that variations to the code provide a selective advantage. We used a sensitive selection system involving suppression of a deleterious allele (*tti2-L187P*) in *Saccharomyces cerevisiae* to detect mistranslation and identify mechanisms that allow genetic code evolution. Though $tRNA^{Ser}$ containing a proline anticodon (UGG) is toxic, using our selection system we identified four $tRNA^{Ser}_{UGG}$ variants, each with a single mutation, that mistranslate at a tolerable level. Mistranslating $tRNA^{Leu}_{UGG}$ variants were also obtained, demonstrating the generality of the approach. We characterized two of the $tRNA^{Ser}_{UGG}$ variants. One contained a G26A mutation, which reduced cell growth to 70% of the wild-type rate, induced a heat shock response, and was lost in the absence of selection. The reduced toxicity of $tRNA^{Ser}_{UGG}$-G26A is likely through increased turnover of the tRNA, as lack of methylation at G26 leads to degradation via the rapid tRNA decay pathway. The second $tRNA^{Ser}_{UGG}$ variant, with a G9A mutation, had minimal effect on cell growth, was relatively stable in cells, and gave rise to less of a heat shock response. *In vitro*, the G9A mutation decreases aminoacylation and affects folding of the tRNA. Notably, the G26A and G9A mutations were phenotypically neutral in the context of an otherwise wild-type $tRNA^{Ser}$. These experiments reveal a model for genetic code evolution in which tRNA anticodon mutations and mistranslation evolve through phenotypically ambivalent intermediates that reduce tRNA function.

**KEYWORDS** tRNA; mistranslation; anticodon; genetic selection

THE genetic code is often thought to be evolutionarily static, with each codon specifying a single amino acid as a result of an ancient "frozen accident" (Crick 1968). In fact, the genetic code has evolved and continues to evolve, as is evident in the deviations from the standard genetic code commonly seen in bacteria and archaea, as well as the nuclear and mitochondrial genomes of eukaryotes [reviewed in Santos *et al.* (2004) and Ling *et al.* (2015)]. Altered reading of the code can result in ambiguous decoding, where one codon is decoded as greater than one amino acid. Over the course of evolution, ambiguous decoding is thought to be a precursor to complete reassignment of the codon meaning from one amino acid to another.

The identification of missense suppressor tRNAs has demonstrated the adaptive advantage of altering the genetic code. Many of the first missense suppressors were single-nucleotide substitutions in the anticodon of glycine tRNAs in *Escherichia coli* that suppressed mutations in the *trpA* gene [reviewed by Murgola (1985)]. Another example of a genetic code alteration is the codon reassignment of CUG from leucine to serine in *Candida albicans*, which Santos *et al.* (1996) propose provides cells with greater thermotolerance by inducing the heat shock response. Variation to the genetic code also has a beneficial effect in mammalian cells. For example, the misincorporation of methionine in response to oxidative stress reduces reactive oxygen species (Netzer *et al.* 2009; Jones *et al.* 2011; Wiltrout *et al.* 2012; Lee *et al.* 2014; Schwartz and Pan 2017). We refer to altered use of the genetic code as


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[1]Corresponding authors: Department of Biochemistry, University of Western Ontario, 1151 Richmond St., London, ON N6A 5C1, Canada. E-mail: mberg2@uwo.ca; and cbrandl@uwo.ca

mistranslation, though we recognize that it may be a programmed event (Moghal *et al.* 2014).

The fidelity of translation is maintained primarily at two levels. The first specificity step is aminoacylation of the 2′ or 3′ hydroxyl of the 3′-terminal adenosine of a cognate tRNA. Aminoacylation is carried out by two protein families of aminoacyl-tRNA synthetases [aaRS; reviewed in Pang *et al.* (2014)]. To discriminate chemically similar amino acids, translation fidelity and aaRS specificity is enhanced by aaRS-associated or -independent editing activities that hydrolyze misaminoacylated tRNAs (Martinis and Boniecki 2010; Perona and Gruic-Sovulj 2014). The second factor in translation fidelity involves recognition of codon–anticodon interactions of an aminoacyl tRNA in association with the elongation factor EF-Tu on the ribosome before amino acid transfer to the growing polypeptide chain (Nissen *et al.* 1995; Ogle *et al.* 2001). Lack of specificity in aminoacylation (Guo *et al.* 2014), defects in editing of misaminoacylated tRNAs (Reynolds *et al.* 2010), mutations (McClory *et al.* 2014), or drugs that alter aminoacyl-tRNA decoding on the ribosome (Hainrichson *et al.* 2008) contribute to enhanced mistranslation.

Nearly all tRNA molecules share a common L-shaped tertiary structure needed for interaction with EF-Tu and recognition by aaRSs and the ribosome. In two dimensions, tRNAs are represented as a cloverleaf structure where base pairing generates stem-loops that form the three arms of the cloverleaf. From 5′ to 3′, the major structures are an acceptor stem, a dihydrouridine (D) arm, an anticodon stem-loop, and the ribothymidine (T) arm [see Figure 1A; reviewed in Rich and RajBhandary (1976)]. Mature tRNAs terminate with a 3′-end CCA sequence. Some tRNAs, including $tRNA^{Ser}$ (tS) and $tRNA^{Leu}$ (tL), contain a fourth stem-loop structure (variable arm) located between the anticodon stem and T arm.

The tRNAs also contain unique features required for aminoacylation by a specific aaRS, called identity elements. Identity elements include single nucleotides, nucleotide pairs, and structural motifs (de Duve 1988; Hou and Schimmel 1988; Giegé *et al.* 1998). For many tRNAs, but not all, aaRS recognition requires identity elements within the anticodon (Commans *et al.* 1998). Notable exceptions in *Saccharomyces cerevisiae* are tS, $tRNA^{Ala}$ (tA), and $tRNA^{Pro}$ (tP). For these tRNAs, major identity elements required for aminoacylation are found in the acceptor stem and variable loop (Giegé *et al.* 1998). An additional complicating aspect of tRNA structure and function is the prevalence of modified nucleotides. Post-transcriptional modifications act as determinants for aminoacylation (Perret *et al.* 1990), play a role in mRNA decoding and translation fidelity (Rozov *et al.* 2016), and regulate the stability of the tRNA (Dewe *et al.* 2012).

Three models suggest mechanisms for codon reassignment and genetic code evolution. The codon capture theory (Osawa and Jukes 1989) suggests that mutational pressure on a genome combined with genome reduction may skew GC or AT content (McCutcheon *et al.* 2009). Due to this pressure, a codon may be lost from the genome. For example, high AT pressure in the genome of *Mycoplasma capricolum* is thought to be responsible for reducing the CG-rich Arg CGG codon (Oba *et al.* 1991). Once the codon is lost from the genome, loss of the cognate tRNA can follow, without phenotypic consequences. Then, as the genome content continues to drift, the codon can reemerge and be decoded either by its original cognate tRNA or "captured" by a new tRNA that reassigns the meaning of the codon.

In the ambiguous intermediate theory for genetic code evolution, mutations in a tRNA lead to decoding of a single codon by two different tRNAs (Schultz and Yarus 1994). *Candida* species provide a striking example for this mechanism of code evolution. In *C. albicans*, the CUG codon has been reassigned from leucine to serine (Massey *et al.* 2003). Related species have retained an evolutionary intermediate form, where the CUG codon is ambiguously decoded. In *C. zeylanoids*, a tS(CAG) is charged efficiently with both leucine and serine (Suzuki *et al.* 1997). Massey *et al.* (2003) proposed that a novel tRNA evolved from a serine tRNA through mutation of its anticodon such that it decoded leucine CUG codons as serine. This event likely occurred prior to loss of the tL that decoded CUG. Therefore, CUG could be decoded as leucine, by native tL, and as serine by the novel tRNA. On an evolutionary timescale, CUG codons encoding residues that could not tolerate being serine were replaced by other leucine codons and the CUG decoding tL was lost.

Upon discovering Leu CUG reassignment to alanine in the yeast *Pachysolen tannophilus*, Mühlhausen *et al.* (2016) proposed an alternative to the ambiguous intermediate theory where tRNA loss drives codon reassignment. They suggested that the original CUG decoding tL was lost in an early yeast ancestor. CUG codons were then gradually lost, until the CUG codon was recaptured by a leucine, serine, or alanine tRNA.

Whereas the codon capture model does not require a period of mistranslation that may be toxic to the organism, the ambiguous intermediate model suggests that, for a period of time, a single codon is decoded ambiguously as two amino acids, leading to proteome-wide mistranslation. These models are not mutually exclusive and Massey *et al.* (2003) revised the ambiguous intermediate theory to state that the combination of ambiguously decoding tRNAs and biased GC pressure, along with a positive advantage to the codon reassignment, leads to genetic code evolution.

Under normal conditions, mistranslation is estimated to occur at a frequency of once per $10^4$ codons (Kramer and Farabaugh 2007). This frequency increases for rare codons and upon starvation of certain amino acids or in other stress conditions. Cells must therefore have mechanisms to cope with mistranslation. At high levels, mistranslation causes errors in protein folding and potentially toxic protein aggregates (Grant *et al.* 1989; Bacher *et al.* 2005; Lee *et al.* 2006). These detrimental effects of mistranslation are managed through protein quality control pathways and molecular chaperones. In eukaryotic cells, the primary pathways induced by mistranslation are the unfolded protein response and the heat shock response (Grant *et al.* 1989; Lee *et al.* 2006). Both pathways result in removing misfolded proteins

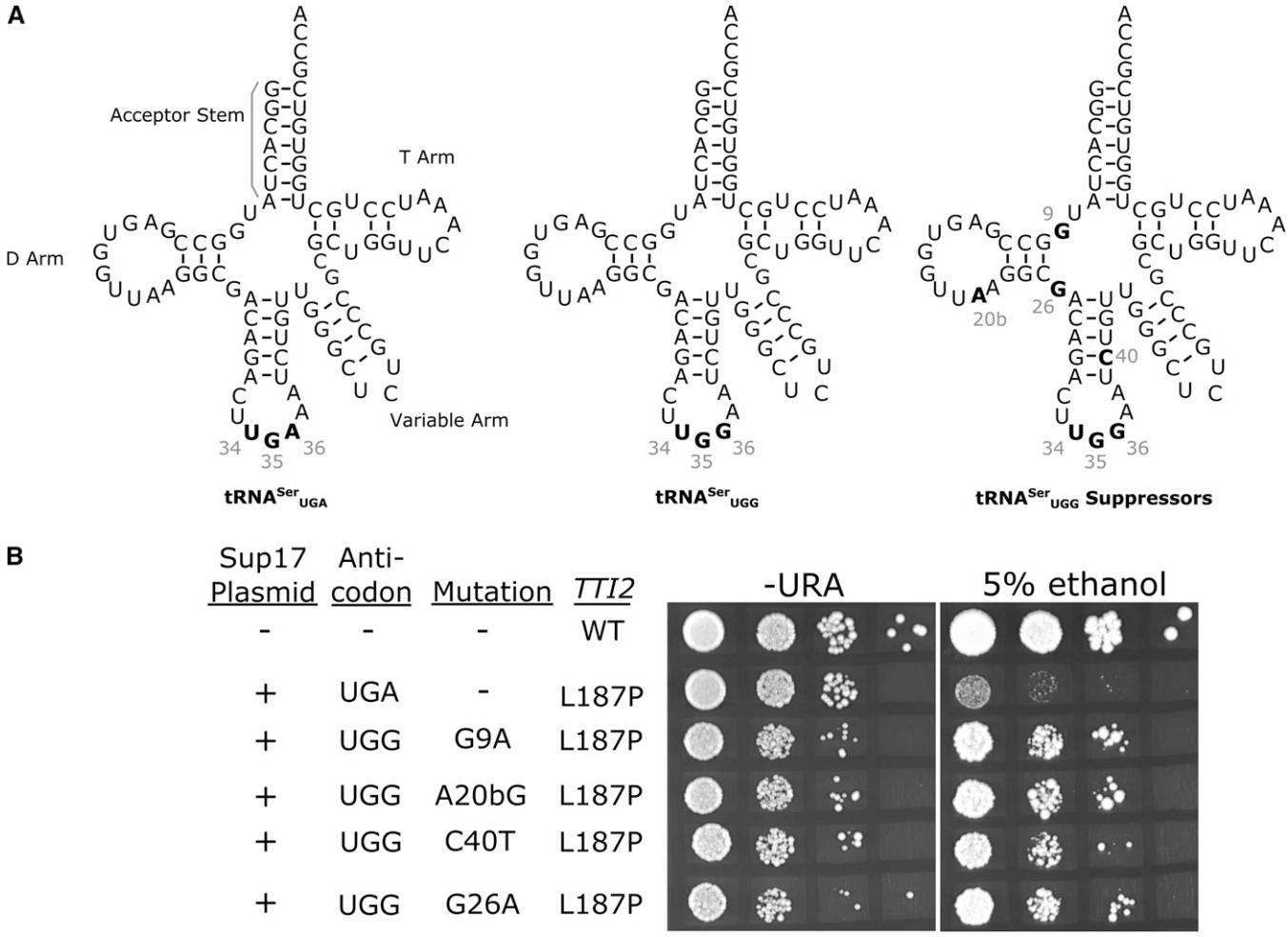


**Figure 1** tRNA$^{Ser}$ (tS) secondary structure and stress resistance of genetically selected *sup17(UGG)* alleles. (A) Secondary structures of tS(UGA), tS(UGG), and tS(UGG) with mutations that allow growth and suppress *tti2-L187P*. The anticodon and suppressor mutations are shown in bold. (B) Yeast strains CY6963 (*TTI2*), CY7673 (*tti2-L187P SUP17*), CY7619 (*tti2-L187P sup17(UGG)$_{G9A}$*), CY7620 (*tti2-L187P sup17(UGG)$_{A20bG}$*), CY7621 (*tti2-L187P sup17 (UGG)$_{C40T}$*), and CY7622 (*tti2-L187P sup17(UGG)$_{G26A}$*) were grown to stationary phase in media lacking uracil (URA). Cells were spotted in 10-fold serial dilutions onto a plate lacking URA and a YPD plate containing 5% ethanol and grown at 30°.

via proteasomal degradation (Taylor *et al.* 2014). These pathways allow a rapid response to reduce proteotoxicity, but mistranslation can be detrimental or even lethal if it occurs at levels that exceed the capacity of the quality control mechanisms.

Previously, we identified four alleles encoding a proline tRNA [tP(UGG)] as genetic suppressors of a variant of the *S. cerevisiae* cochaperone *TTI2* with a leucine to proline mutation at residue 187 (*tti2-L187P*) that results in severe slow growth under conditions of stress (Hoffman *et al.* 2016, 2017). Each tP(UGG) allele had a mutation of C70 to T, creating a G3:U70 base pair in the acceptor stem, a major identity element for alanyl-tRNA synthetase (AlaRS). The single-base change causes mischarging of tP(UGG)$_{G3:U70}$ and the decoding of UGG anticodons with alanine. In the context of a wild-type background, tP(UGG)$_{G3:U70}$ led to low levels of proteome-wide mistranslation at proline codons and induced a limited heat shock response, while reducing cell growth by 6%.

Despite the fact that interconverting proline anticodons to alanine or serine anticodons requires only a single-base change and that the anticodon is not a major determinant in the charging of these amino acids to their cognate tRNAs (Giegé *et al.* 1998), anticodon mutations were not found in our selection of suppressors of *tti2-L187P*. The present study was initiated to determine if altering the anticodon of tS to UGG would suppress *tti2-L187P*. We found that cells containing tS(UGG) encoded from a mutated *SUP17* allele were inviable. We identified four variants of tS(UGG) with single-base changes that suppress *tti2-L187P*. One of these alleles with a secondary mutation of G26A partially reduced cell growth, was unstable unless selected for, and induced a heat shock response. G26 is subject to N(2),N(2)-dimethyl modification. Loss of this modification increases turnover of the tRNA via the rapid tRNA decay (RTD) pathway (Dewe *et al.* 2012), thereby alleviating tS(UGG) toxicity. A second *sup17(UGG)* allele with a secondary mutation of G9A showed little effect on cell growth, was maintained in the

absence of selection, and induced less of a heat shock response. This mutation partially reduced aminoacylation with serine and destabilized the tRNA *in vitro*. In the context of wild-type *SUP17*, the individual mutations G26A and G9A had no effect on cell growth, were maintained in cells, and did not induce a heat shock response. We conclude that tS can evolve into a mistranslating species if the anticodon mutation is preceded by a mutation that reduces the function of the tRNA and in turn reduces the extent of mistranslation. We call the initial mutation that decreases tRNA function an ambivalent intermediate because, in isolation, it has no apparent phenotypic effect on cells. In nature, the appearance of the ambivalent intermediate would enable the subsequent anticodon mutation allowing mistranslation.

## Materials and Methods

### Yeast strains

Yeast strains are derivatives of the wild-type haploid strains BY4741 and BY4742 [Supplemental Material, Table S1 in File S1; Winzeler and Davis (1997)]. The *tti2* disruption strains covered by either *TTI2* (CY6963) or *tti2-L187P* (CY7020) on a centromeric plasmid have been described (Hoffman *et al.* 2016). CY7681 was generated by transforming *tti2-L187S* on a centromeric plasmid into CY6963 and *TTI2* on a *URA3* centromeric plasmid was lost by plating on 5-fluoroorotic acid. Strains CY7619, CY7620, CY7621, CY7622, CY7655, CY7656, CY7670, CY7673, and CY7682 contain *SUP17* or *tL(UAA)B2*-derived alleles (as indicated in Table S1 in File S1) on centromeric *URA3* plasmids in CY7020. CY7628, CY7629, CY7631, CY7632, and CY7633 contain *SUP17*-derived alleles in centromeric *URA3* plasmids in BY4742. CY7665, CY7666, CY7667, CY7668, and CY7669 contain *SUP17*-derived alleles in centromeric *LEU2* plasmids in BY4742. The *met22Δ* strain (CY7640) was derived from a spore colony of the yeast magic marker strain in the BY4743 diploid background (Tong *et al.* 2001).

Yeast strains were grown in yeast peptone media containing 2% glucose or synthetic media supplemented with nitrogenous bases and amino acids at 30° unless otherwise indicated. For spot plate assays, cells were grown to stationary phase, then spotted in 10-fold serial dilutions. For growth curves, cells were grown to stationary phase, diluted 1:400 in media lacking uracil, and grown at 30°. Every hour, the $OD_{600}$ was measured and growth rate averaged over two biological replicates per strain.

### Plasmid constructs

*SUP17* including 300 bp upstream and downstream was amplified by PCR using primers UG5953 and UG5954 (Table S2 in File S1) from wild-type yeast genomic DNA and ligated into YCplac33 and YCplac111 as a *Hin*dIII-*Eco*RI fragment to generate YCplac33-*SUP17* (CB3076) and YCplac111-*SUP17* (CB4048). The anticodon of *SUP17* was mutagenized to UGG using two-step PCR. First, *SUP17* was amplified using UG5953 with UI6807 and UG5954 with UI6806. The PCR products of these reactions were the template in the second round of PCR with primers UG5953 and UG5954. This product was cloned as a *Hin*dIII-*Eco*RI fragment into YCplac33 to generate YCPlac33-*sup17(UGG)* (CB3082). Derivatives of YCPlac33-*sup17(UGG)* with mutations at G9A (CB4020), A20bG (CB4021), C40T (CB4022), and G26A (CB4023) were obtained through genetic selection. The inserts from CB4020 and CB4023 were cloned as *Hin*dIII-*Eco*RI fragments into YCplac111 to give CB4044 and CB4046, respectively. YCplac33-$sup17(UGG)_{G9A,\ A20bG}$ (CB4070) was made by two-step PCR using YCplac33-*sup17(UGG)* as a template, outside primers UG5953/UG5954, and additional primers VB3039/VB3040. YCplac33-*tL(UAA)B2* was cloned as a *Hin*dIII-*Eco*RI fragment after being amplified by PCR using primers UL0676 and UL0677 (CB4043). The anticodon was switched to UGG to create YCplac33-*tL*(*UGG*) (CB4055) using two-step PCR and the additional primers UL1165 and UL1166.

The $sup17_{G9A}$ allele with only the secondary mutation G9A (CB4039/4047) was made by two-step PCR using $sup17(UGG)_{G9A}$ (CB4020) as a template, outside *SUP17* primers UG5953/UG5954, and additional primers UG0312/UG0313. The *Hin*dIII-*Eco*RI fragments were cloned into YCplac33/YCplac111. The $sup17_{G26A}$ allele with only the secondary mutation G26A (CB4040/4045) was made similarly using the *SUP17* (CB3076) template and additional primers UK0310/UK0311.

The yeast *TTI2* and *tti2-L187P* constructs have been described (Hoffman *et al.* 2016). The *tti2-L187S* construct was made by two-step PCR with *TTI2* as a template, outside primers 5693-1/5693-2, and additional primers VB3060/VB3061. The product was cloned into YCplac111 as a *Not*I-*Sac*I fragment (CB4071).

The yeast seryl-tRNA synthetase (SerRS) gene (*SES1*) was amplified by PCR using primers UK0550 and UK0551, digested with *Nco*I and *Sac*I, and cloned into $pP_{RO}EX$ HTa (Invitrogen, Carlsbad, CA; CB4042).

The centromeric plasmid containing heat shock element (HSE)-driven enhanced GFP (*HSE-eGFP*) was kindly provided by Martin Duennwald (Brandman *et al.* 2012).

### Selection of variants of sup17(UGG) and tL(UGG) that suppress tti2-L187P

Equal aliquots of 20 μg of YCplac33-*sup17(UGG)* or *tL(UGG)* were UV-irradiated at 302 nm from 6 to 36 sec and transformed into CY7020. Ura+ transformants were screened for growth on YPD containing 5% ethanol. Strains were mated with a wild-type strain to allow loss of the plasmid-borne *tti2-L187P*-containing plasmid. The YCplac33 plasmids were then isolated, sequenced, and transformed back into CY7020 to analyze growth.

### Plasmid stability assay

BY4742 was transformed with YCplac111-*SUP17* and one of YCplac33-*SUP17*, $sup17(UGG)_{G9A}$, $sup17(UGG)_{G26A}$, $sup17_{G9A}$,

or *sup17$_{G26A}$*. Transformants were colony purified on plates lacking uracil and leucine then grown to stationary phase in liquid media lacking uracil and leucine. Cells were diluted 1:200 into YPD medium, grown for 20 hr, and streaked for single colonies on YPD plates. Individual colonies were selected for the presence of the YCplac plasmids on plates lacking leucine or uracil. Numbers reported are the percentage of cells retaining the plasmid for four independent growths.

### Preparation and analysis of tRNAs

Wild-type *SUP17*, *sup17(UGG)*, and *sup17(UGG)$_{G26A}$* were amplified by PCR for *in vitro* transcription with primer UL0743 containing a T7 promoter upstream of the tRNA gene and VA1362 from the template plasmids CB3076, CB3082, and CB4023. Mutant *sup17(UGG)$_{G9A}$* was amplified by PCR with primer UL0972 and VA1362 from template CB4020. tRNAs were prepared as described in Hoffman *et al.* (2017). Briefly, PCR products were gel purified and used as a template for *in vitro* transcription reactions. tRNAs were gel purified, folded, 3′-end labeled with CCA adding enzyme, and [α-$^{32}$P]ATP (Perkin-Elmer [Perkin Elmer-Cetus], Norwalk, CT) and purified using BioSpin30 columns (Bio-Rad, Hercules, CA). The radiolabeled tRNAs were analyzed by gel electrophoresis. Each tRNA was incubated at 95° for 1 min, put on ice for 3 min followed by the addition of Structure Buffer (Ambion), and allowed to fold at 37° for 15 min. The RNA was precipitated and separated on a 10% sequencing gel. For the G-lane, each tRNA was incubated at 50° for 5 min before being treated with RNase T1 at room temperature for 15 min.

Stability of the tRNAs was assessed by measuring the change in absorbance during heating. After folding, tRNAs were suspended in 1 mM $MgCl_2$ and the $OD_{260}$ measured in the temperature range from 20° to 85° (Cary 100 Bio Spectrophotometer). The first derivative of the melting curve was plotted against temperature.

### Protein purification

Tti2$_{L187P}$ was purified from strains containing TAP-FLAG-*tti2-L187P* and either *SUP17* or *sup17(UGG)$_{G26A}$*, as described in Hoffman *et al.* (2017). Yeast strains were grown to stationary phase in minimal media lacking uracil, diluted 1:100 in YPD, and grown to an $OD_{600}$ = 2.0. Cells were lysed by grinding in liquid nitrogen and Tti2 purified using two-step tandem affinity purification.

Six-histidine-tagged yeast SerRS (Ses1) was purified from *E. coli* strain BL21 pRARE containing plasmid CB4042. Cells were grown to stationary phase in 2 ml LB containing 25 μg/ml chloramphenicol and 100 μg/ml ampicillin before being diluted 1:1000 into 500 ml of the same medium and grown to an $OD_{600}$ = 0.6. Expression of Ses1 was induced overnight at room temperature using 1 mM isopropyl β-D-1-thiogalactospyransoide. Cells were lysed in 25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, and 0.5 mg/ml lysozyme, and Ses1 purified using TALON resin (Clontech Laboratories) and eluted with the same buffer containing 200 mM imidazole. Purified protein was dialyzed into 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, and 40% glycerol, and stored at −20°.

### Aminoacylation assay

Aminoacylation reactions were performed, as described in O'Donoghue *et al.* (2011), with 3 μM tRNA, 150 nM $^{32}$P-labeled tRNA, 10 mM serine, 5 mM ATP (pH 7.0), and 10 μM SerRS. A 2-μl aliquot of each reaction was spotted onto polyethylenimine–cellulose thin layer chromatography (TLC) plates (Millipore, Bedford, MA) and developed in 5% acetic acid and 100 mM ammonium acetate. The TLC plates were exposed to a phosphor screen and imaged using a Storm 860 Phosphorimager (GE Healthcare Life Sciences). Densitometry analysis was competed using ImageJ 1.48v. Average values were calculated across two technical replicates.

### Fluorescence heat shock reporter

Yeast strains containing the *HSE-GFP* reporter and either *SUP17*, *sup17(UGG)*, *sup17(UGG)$_{G9A}$*, *sup17(UGG)$_{G26A}$*, *sup17$_{G9A}$*, or *sup17$_{G26A}$* were grown to stationary phase in medium lacking leucine and uracil, diluted 1:50 in the same medium, and grown for 6 hr at 30°. Cell densities were normalized to $OD_{600}$ before measuring fluorescence. Fluorescence was measured with a BioTek Synergy H1 microplate reader at an emission wavelength of 528 nm using Gen5 2.08 software. The mean relative fluorescence units were calculated across three technical and three biological replicates for each strain.

### Mass spectrometry

Samples were prepared as previously described by Hoffman *et al.* (2017) at the Functional Proteomics Facility (University of Western Ontario, http://www.uwo.ca/biohm/fpf/). Briefly, TAP-Tti2 was tandem affinity purified from BY4742 expressing either *SUP17* or *sup17(UGG)$_{G26A}$*. Tti2 was picked from a 10% polyacrylamide gel stained with Coomassie Brilliant Blue. Gel pieces were destained in 50 mM ammonium bicarbonate and 50% acetonitrile, reduced in 10 mM dithiothreitol (DTT), alkylated using 55 mM iodoacetamide (IAA), and digested with trypsin (prepared in 50 mM ammonium bicarbonate, pH 8). The Waters MassPREP Station (Perkin-Elmer) was used for in-gel digestion. Peptides were extracted in 1% formic acid and 2% acetonitrile, then lyophilized.

Liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) analysis was performed at the Yale W. M. Keck Foundation Biotechnology Resource Laboratory. LC-MS/MS analysis was performed on a Thermo Scientific Q Exactive Plus equipped with a Waters nanoAcquity ultraperformance liquid chromatography (UPLC) system utilizing a binary solvent system (Buffer A: 100% water, 0.1% formic acid; Buffer B: 100% acetonitrile, 0.1% formic acid). Trapping was performed at 5 μl/min, 97% Buffer A for 3 min using a Waters Symmetry C18 180 μm × 20 mm trap column. Peptides were separated using an ACQUITY UPLC peptide separation technology (PST) (Ethylene Bridged Hybrid (BEH)) C18

**Table 1 *sup17* alleles generated in this study**

| tRNA allele | Anticodon | Mutation |
|---|---|---|
| *SUP17* | UGA | — |
| *sup17(UGG)* | UGG | — |
| $sup17(UGG)_{G9A}$ | UGG | G9A |
| $sup17(UGG)_{A20bG}$ | UGG | A20bG |
| $sup17(UGG)_{C40T}$ | UGG | C40T |
| $sup17(UGG)_{G26A}$ | UGG | G26A |
| $sup17_{G9A}$ | UGA | G9A |
| $sup17_{G26A}$ | UGA | G26A |

nanoACQUITY Column 1.7 μm, 75 μm × 250 mm (37°) and eluted at 300 nl/min with the following gradient: 3% buffer B at initial conditions; 5% B at 1 min; 35% B at 50 min; 50% B at 60 min; 90% B at 65 min; 90% B at 70 min; and return to initial conditions at 71 min. MS was acquired in profile mode over the 300–1700 m/z range using one microscan, 70,000 resolution, automatic gain control (AGC) target of $3 \times 10^6$, and a maximum injection time of 45 msec. Data-dependent MS/MS were acquired in centroid mode on the top 20 precursors per MS scan using one microscan, 17,500 resolution, AGC target of $1 \times 10^5$, maximum injection time of 100 msec, and an isolation window of 1.7 m/z. Precursors were fragmented by higher-energy collisional dissociation (HCD) activation with a collision energy of 28%. MS/MS were collected on species with an intensity threshold of $2 \times 10^4$, charge states 2–6, and peptide match preferred. Dynamic exclusion was set to 20 sec.

Data were analyzed using Proteome Discoverer (version 1.3) software and searched in-house using the Mascot algorithm (version 2.6.0) (Matrix Science). The data were searched against a SwissProtein database with taxonomy restricted to *S. cerevisiae* as well as a custom database containing the *tti2* mutant. Search parameters used were trypsin digestion with up to two missed cleavages; peptide mass tolerance of 10 ppm; MS/MS fragment tolerance of 0.02 Da; and variable modifications of methionine oxidation, carbamidomethyl, or propionamide adduct to cysteine, and Pro → Ser substitution. Normal and decoy database searches were run, with the C.I. set to 95% ($P < 0.05$).

### *Data availability*

Yeast strains and plasmids are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

## Results

### *Variants of tS(UGG) suppress tti2-L187P*

Inserting a proline at position 187 in *TTI2* leads to a nonfunctional protein product and a stress-sensitive phenotype in *S. cerevisiae* (Hoffman *et al.* 2016). Previously, we identified four independent $tP_{G3:U70}$ alleles through genetic selection that misincorporate alanine at proline codons and restore the function of *tti2-L187P*. Interestingly, our genetic selection did not identify alleles of tA or tS. Like tP, the cognate aaRSs for tA and tS do not rely on identity elements or recognition determinants in the anticodon (Giegé *et al.* 1998). A single-base mutation in the anticodon of tA or tS could therefore potentially lead to noncognate decoding and mistranslation of proline codons. The fact that our selection for second-site suppressors of *tti2-L187P* did not identify any anticodon mutations to tA or tS suggested that the anticodon mutant tRNAs may be nonfunctional or toxic for the cell. To test this, we engineered *URA3* centromeric plasmids containing *SUP17* [tS(UGA)] plus 300 bp of upstream and downstream flanking sequence, with and without the anticodon mutated to UGG (proline). The plasmids were transformed into the haploid yeast strain CY7020 (*tti2-L187P*) to determine if the *sup17(UGG)* allele would suppress the ethanol sensitivity caused by *tti2-L187P*. Interestingly, DNA concentrations that supported robust transformation by wild-type *SUP17* resulted in very few transformants of the *sup17(UGG)* plasmid (Figure S1 in File S1). The lack of transformants suggested that *sup17(UGG)* was toxic to the cells, perhaps due to its high efficiency in mistranslating serine at proline codons.

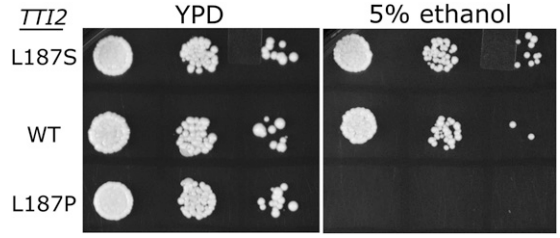


**Figure 2** *tti2-L187S* allows growth under stress conditions. Yeast strains CY7681 (*tti2-L187S*), CY6963 (*TTI2*), and CY7020 (*tti2-L187P*) were grown to stationary phase in YPD media. Cells were spotted in 10-fold serial dilutions onto a YPD plate or on a YPD plate containing 5% ethanol and grown at 30°. WT, wild-type.

We reasoned that if the efficiency of *sup17(UGG)* to mistranslate was decreased, it may no longer be toxic. Therefore, we UV-irradiated the *sup17(UGG)* plasmid and transformed the DNA pool into CY7020. Approximately 100 Ura+ transformants were obtained. These were screened for their ability to suppress *tti2-L187P*, as seen by growth on rich medium containing 5% ethanol. Four strains that suppressed *tti2-L187P* were identified. The *URA3*-containing plasmids were isolated from the strains and the *sup17*(*UGG*) alleles along with their 5′- and 3′-flanking regions were sequenced (Figure 1A and Table 1). Each of the four variants had a single-base change in the tRNA gene. To verify that the plasmids were responsible for suppression, each was retransformed into CY7020 and the transformants analyzed for growth on plates containing 5% ethanol (Figure 1B).

Consistent with our previous analysis of $tP_{G3:U70}$ (Hoffman *et al.* 2017), we predicted that the variant *sup17* alleles suppressed *tti2-L187P* through their ability to mistranslate serine for proline at residue 187. We first addressed whether serine

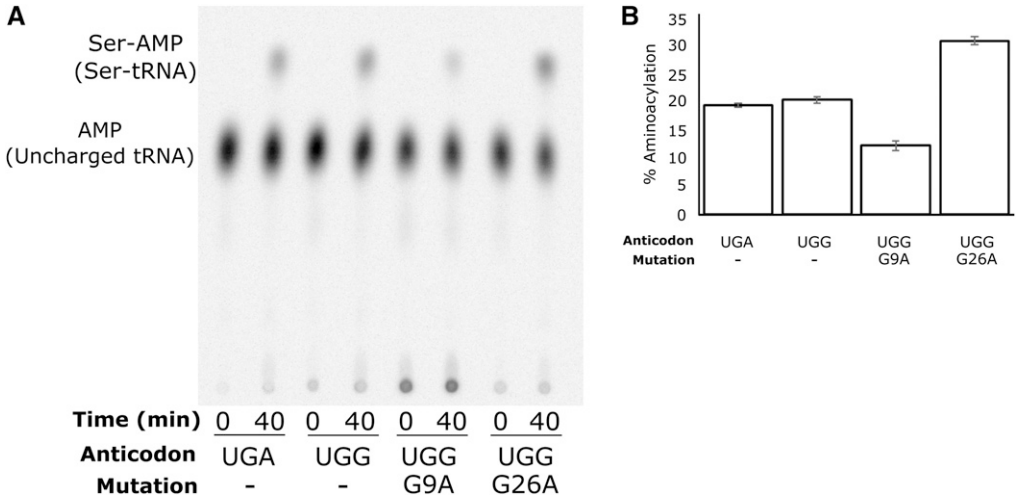


**Figure 3** *SUP17* alleles are aminoacylated with serine. (A) A total of 150 nM of radiolabeled tS, tS(UGG), tS(UGG)$_{G9A}$, or tS(UGG)$_{G26A}$ were incubated with 10 μM SerRS, 10 mM serine, and 3 μM unlabeled tS. Two microliters of each reaction was treated with nuclease P1. Reaction mixtures were then spotted on glass TLC plates and chromatographed in 0.1 M ammonium acetate and 5% acetic acid. TLC plates were imaged using a Storm 860 Phosphorimager to detect free $^{32}$P-labeled AMP and $^{32}$P-labeled AMP charged with serine. (B) Percent of tRNA that was aminoacylated after 40 min was calculated for two technical replicates. Error bars represent the maximum and minimum values. SerRS, seryl-tRNA synthetase; tS, tRNA$^{Ser}$.

at position 187 of Tti2 would support growth. As shown in Figure 2, a strain containing *tti2-L187S* as the sole copy of *TTI2* grows comparably to a wild-type *TTI2* strain.

We next addressed whether *sup17*(*UGG*)$_{G9A}$ and *sup17*(*UGG*)$_{G26A}$ could be aminoacylated by SerRS. Recombinant *S. cerevisiae* Ses1 (SerRS) was purified from *E. coli* via an N-terminal 6-histidine tag and used in aminoacylation assays with tS(UGA), tS(UGG), tS(UGG)$_{G9A}$, and tS(UGG)$_{G26A}$, which were synthesized by *in vitro* transcription and refolded. The tRNAs were 3′-end-labeled with CCA-adding enzyme and [α-$^{32}$P]ATP. Before use, the tRNAs were examined by gel electrophoresis (Figure S2 in File S1). tS(UGA), tS(UGG), and tS(UGG)$_{G26A}$ were estimated to be ∼90% full length, whereas tS(UGG)$_{G9A}$ was ∼80% full length. Aminoacylation reactions were first carried out for 0, 2, 20, and 40 min for wild-type tS(UGA) with the level of charging indicated as a ratio of charged tRNA *vs.* total tRNA. Through this time period, aminoacylation was in a linear range (Figure S3 in File S1). Reactions with the different tRNAs were performed in the presence of excess unlabeled tS(UGA). tS(UGA) and tS(UGG) aminoacylated with nearly equal efficiency, confirming that the anticodon is not an identity element required for charging by yeast SerRS (Figure 3). Aminoacylation of tS(UGG)$_{G9A}$ with serine was reduced to 60% that of the wild-type tRNA. Interestingly, tS(UGG)$_{G26A}$ was aminoacylated with serine at a level ∼50% higher than the wild-type. Though exact comparisons are difficult because the recombinant tRNAs are not modified, these results suggest that the G9A mutation, but not the G26A mutation, compromises aminoacylation.

We then examined the ability of tS(UGG)$_{G26A}$ to mistranslate serine for proline into Tti2-L187P within cells. A TAP-tagged derivative of *tti2-L187P* was introduced into strains containing either *SUP17* or *sup17(UGG)*$_{G26A}$. Tti2 was partially purified by tandem affinity purification and the copurified proteins analyzed by mass spectrometry. While we were not able to identify the peptide-containing residue 187 of Tti2 in the sample containing tS(UGG)$_{G26A}$, we did identify six other peptides from five proteins including Tti2 that contained serine at proline codons (Figure S4 and Table S3 in File S1). In all cases, both the proline- and serine-containing peptides were identified from the strain containing tS(UGG)$_{G26A}$, whereas only the proline peptide was identified from the strain containing wild-type tS. Five of the mistranslation events were at CCA codons. One mistranslation event occurred at a CCU codon, which is also decoded by the UGG anticodon (Hoffman *et al.* 2017).

### *Cellular response to mistranslating tRNAs*

Our initial experiment with tS(UGG) suggested that the anticodon mutation resulted in toxicity. We predicted that the variants may affect cell growth to varying extents. We therefore analyzed the growth of strains containing *SUP17*, *sup17*(*UGG*)$_{G9A}$, and *sup17*(*UGG*)$_{G26A}$. To determine the impact of the second-site mutations in isolation, we also determined the growth of strains containing the *SUP17* allele with a cognate anticodon and the secondary mutations G9A or G26A. Plasmids were transformed into the wild-type background strain BY4742. Starter cultures were grown in uracil-depleted media and cells inoculated at a dilution of 1:400 into selective media and grown at 30°. In comparison to the wild-type *SUP17*, *sup17*(*UGG*)$_{G26A}$ decreased the growth rate as indicated by a 1.4-fold increase in doubling time (Figure 4A and Figure S5 in File S1). A plasmid containing *sup17*$_{G26A}$ with its native serine anticodon had no effect on cell growth. In addition, expression of plasmids containing *sup17*(*UGG*)$_{G9A}$ with the noncognate proline anticodon or *sup17*$_{G9A}$ with the

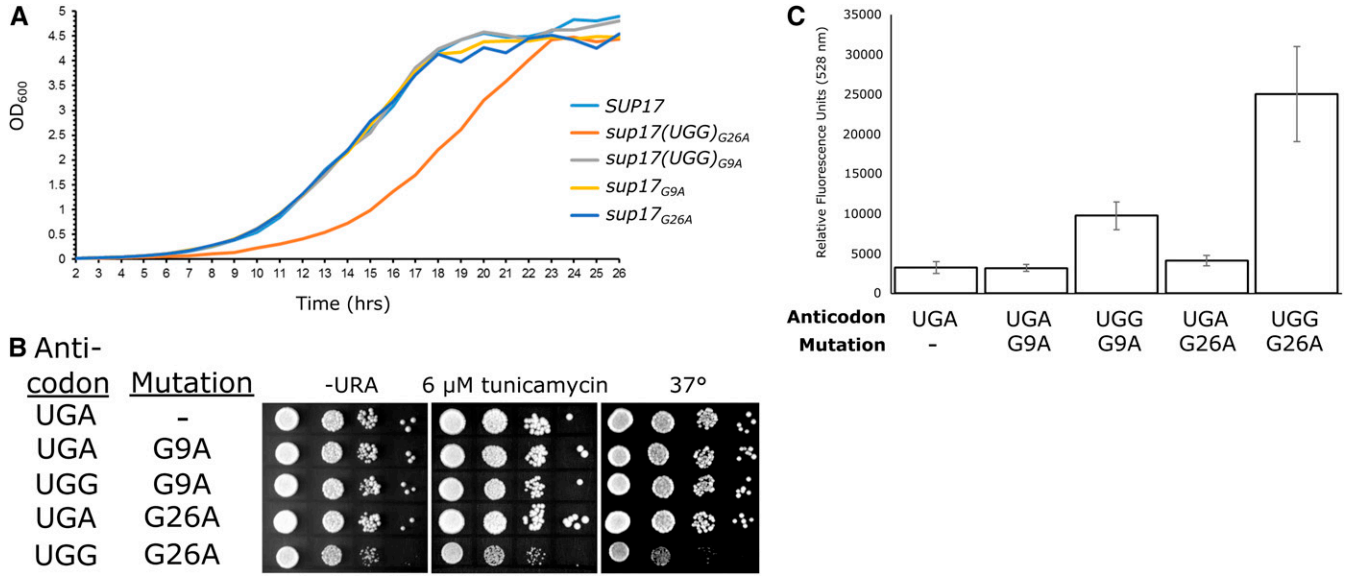


**Figure 4** Effect of *sup17(UGG)* alleles on cell growth. (A) Growth curve comparing wild-type *SUP17* to $sup17(UGG)_{G9A}$ and $sup17(UGG)_{G26A}$ suppressor mutants and $sup17_{G9A}$ and $sup17_{G26A}$ single mutants. Yeast strains CY7633 (*SUP17*), CY7632 ($sup17(UGG)_{G26A}$), CY7631 ($sup17(UGG)_{G9A}$), CY7629 ($sup17_{G26A}$), and CY7628 ($sup17_{G9A}$) were grown to stationary phase in media lacking uracil (URA), diluted 1:400 in the same media, and grown for 26 hr. Optical density measurements were taken every hour. (B) Yeast strains from (A) were grown to stationary phase in media lacking URA and spotted in 10-fold serial dilutions onto plates lacking URA and grown at 30° or 37° or on a plate lacking URA and containing 6 μM tunicamycin and grown at 30°. (C) Yeast strains CY7668 (*SUP17*), CY7666 ($sup17(UGG)_{G26A}$), CY7669 ($sup17(UGG)_{G9A}$), CY7665 ($sup17_{G26A}$), and CY7667 ($sup17_{G9A}$) containing *HSE-GFP* were grown in media lacking URA and leucine to stationary phase, diluted 1:100, and grown for 6 hr at 30°. Cell densities were normalized and fluorescence was measured at an emission wavelength of 528 nm. A BY4742 strain lacking *HSE-GFP* was used to subtract background fluorescence from each strain. Heat shock response was measured in three biological and three technical replicates for each strain. Error bars indicate 1 SD. *HSE-GFP*, heat shock element-driven enhanced GFP.

native codon did not affect cell growth. The growth of the strains was confirmed by spot assay on an uracil-depleted plate grown at 30° (Figure 4B), and again in this assay only the $sup17(UGG)_{G26A}$ strain grew at a reduced rate. On plates containing tunicamycin, which induces the unfolded protein response, and when grown at 37°, the slow growth of the $sup17(UGG)_{G26A}$ strain was exaggerated, whereas the $sup17(UGG)_{G9A}$ allele still did not reduce growth.

We then measured heat shock induction resulting from the expression of the mistranslating tRNAs and their single secondary mutations. A reporter plasmid containing GFP expressed from a Hsf1-activated promoter (*HSE-GFP*) was transformed into BY4742 also containing *SUP17*, $sup17(UGG)_{G9A}$, $sup17_{G9A}$, $sup17(UGG)_{G26A}$, or $sup17_{G26A}$ on *LEU2* centromeric plasmids (Figure 4C). Starter cultures were grown in minimal medium, diluted into fresh selective medium, and grown for 8 hr. Induction of *HSE-GFP* was measured by fluorescence at 528 nm. As expected from its ability to reduce growth, $sup17(UGG)_{G26A}$ elevated expression of *HSE-GFP* to the greatest extent, ~7.7-fold. *HSE-GFP* expression increased threefold with the $sup17(UGG)_{G9A}$ allele. Neither of the second-site mutations (G9A nor G26A) in isolation induced a heat shock response. Induction of a heat shock response by $sup17(UGG)_{G9A}$ and $sup17(UGG)_{G26A}$ is also noteworthy since it is a further indication that mistranslation of serine for proline is occurring in the cells and that the cells are responding to it.

### *tS alleles with mutations G9A and G26A are maintained in cells*

Whereas tS(UGG) is toxic to cells, mutations at G9A or G26A enable the cell to tolerate the noncognate anticodon mutated tRNA. Independent of the UGG anticodon, alleles carrying the G9A and G26A mutations did not reduce cell growth or result in a heat shock response. To obtain another quantifiable measure of the toxicity for each *sup17* variant, we determined the plasmid stability in cells under nonselective conditions. BY4742 was transformed with wild-type *SUP17* on a *LEU2* centromeric plasmid and one of wild-type *SUP17*, $sup17(UGG)_{G9A}$, $sup17(UGG)_{G26A}$, $sup17_{G9A}$, or $sup17_{G26A}$ on a *URA3* centromeric plasmid. Cultures were grown to stationary phase in medium depleted of leucine and uracil, then diluted at a ratio of 1:200 into rich medium (YPD) and grown for 20 hr. Cells were streaked for single colonies onto YPD plates. The resulting colonies were analyzed for retention of *URA3* (mutant) and *LEU2* (wild-type) plasmids by plating on medium depleted for uracil or leucine (Figure 5). In all of the strains, the wild-type *SUP17* on the *LEU2* plasmid was retained in 70% of the cells. The *URA3* plasmids containing wild-type *SUP17*, $sup17(UGG)_{G9A}$, $sup17_{G9A}$, and $sup17_{G26A}$ were retained at a similar frequency and not statistically different from one another. This indicates that the secondary mutations G9A and G26A in *SUP17* are not selected against. In contrast, $sup17(UGG)_{G26A}$ was retained at only a 15% frequency, thus confirming its toxicity.

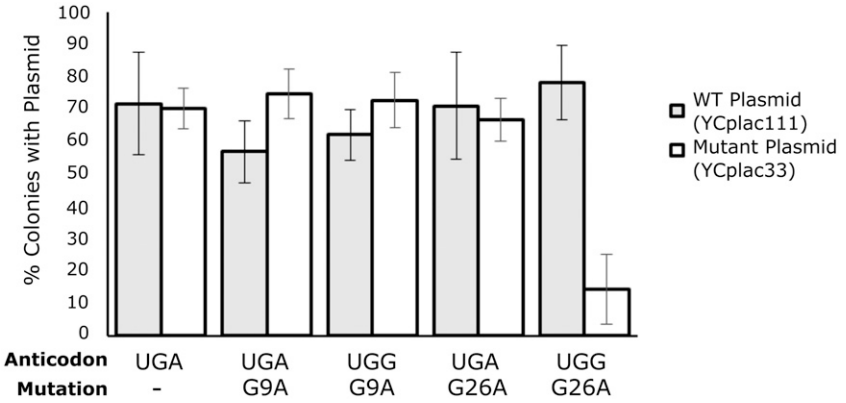


**Figure 5** *sup17*$_{G9A}$ and *sup17*$_{G26A}$ are not selected against. CY7633 (*SUP17*), CY7632 (*sup17(UGG)*$_{G26A}$), CY7631 (*sup17(UGG)*$_{G9A}$), CY7629 (*sup17*$_{G26A}$), and CY7628 (*sup17*$_{G9A}$) were transformed with YCplac111-*SUP17* and grown to stationary phase in media lacking uracil and leucine. Cells were diluted 1:200 in YPD media, grown for 20 hr, and streaked for single colonies on YPD plates. Colonies that contained either YCplac111 or YCplac33 were identified by growing on plates lacking leucine or uracil, respectively. The percentage of colonies that maintained the plasmid was calculated for four independent growths. Error bars indicate 1 SD. WT, wild-type.

### *Stability of sup17 variants*

Hypomodified tS(UGA) lacking N(2),N(2)-dimethylguanosine and 5-methylcytidine are subject to turnover by the RTD pathway (Dewe *et al.* (2012); see Figure 6A). The exonucleases Xrn1 and Rat1 are components of the RTD pathway and are responsible for degrading hypomodified tRNA (Chernyakov *et al.* 2008). Adenosine 3′,5′ bisphosphate (pAp) inhibits both Xrn1 and Rat1 (Dichtl *et al.* 1997). Met22 dephosphorylates pAp and the cellular levels of pAp accumulate when Met22 is inhibited (Murguía *et al.* 1996). The *sup17*(*UGG*)$_{G26A}$ allele cannot be modified to dimethylguanosine at position 26 in tS. The G9 position that is mutated in *sup17*(*UGG*)$_{G9A}$ is not a naturally methylated position *in vivo* (Machnicka *et al.* 2013). To examine if the secondary mutations in *sup17*(*UGG*)$_{G9A}$ and *sup17*(*UGG*)$_{G26A}$ decrease the toxicity of *sup17*(*UGG*) by targeting the mutant tRNA for turnover by the RTD pathway, we analyzed their toxicity in a strain deleted for *MET22*. As shown in Figure 6B, slow growth resulting from *sup17*(*UGG*)$_{G26A}$ expression was more evident in the *met22Δ* strain compared to a wild-type strain, suggesting that the G26A mutation targets tS(UGG)$_{G26A}$ for degradation by the RTD pathway. In contrast, *sup17*(*UGG*)$_{G9A}$ did not cause slow growth in the *met22Δ* strain, indicating that the G9A mutation does not target tS(UGG)$_{G9A}$ for turnover by the RTD pathway.

The structure of the tS D-arm visible in the *Thermus thermophilus* crystal structure of tS in complex with SerRS (Biou *et al.* 1994) shows G9 base-stacks with A21 (Figure 7A). Though there is an additional inserted base in the *T. thermophilus* tS, A21 is homologous to A20b in *S. cerevisiae* tS, another position we identified in one of the mistranslating variants of tS(UGG) (see Table 1). Therefore, we predicted that the G9A mutation may result in a poorly folded tRNA, due to the loss of base-stacking interactions in the tRNA core that help orient the D-arm. This would be consistent with its reduced aminoacylation and the subtle alterations in the degradation pattern seen for tS(UGG)$_{G9A}$ at base 18 and 22 (Figure S2 in File S1). To test this prediction, we compared the melting curves for tS(UGG) and tS(UGG)$_{G9A}$ by analyzing melting transitions through UV absorbance at 260 nm (Figure 7B). The absorbance for tS(UGG)$_{G9A}$ underwent a subtle transition over the range from 20 to 85°, with no obvious unfolding peak, while tS(UGG) underwent a sharp unfolding at ~60°. This result suggests that tS(UGG)$_{G9A}$ poorly adopts the native tRNA structure and/or is relatively unstable due to loss of a base-stacking interaction in the D-arm. We also examined the effect of combining both the G9A and A20bG mutations. As shown in Figure S5 in File S1, the two mutations did not restore function as the tRNA was not toxic. In fact, the double-mutant tRNA did not suppress *tti2-L187P*, indicating that it is not functional, likely due to further disruption of the intramolecular interactions of the tRNA core.

### *tL variants with proline anticodons can suppress tti2- L187P*

Aminoacylation by leucyl-tRNA synthetase (LeuRS), like SerRS, is largely dependent on the variable arm, but also depends partially on identity nucleotides A35 and G37 in the anticodon arm and the discriminator base A73 (Soma *et al.* 1996). To address whether tL can be engineered to mistranslate proline codons, we cloned *tL(UAA)B2* including 300 bp of upstream and downstream sequence with

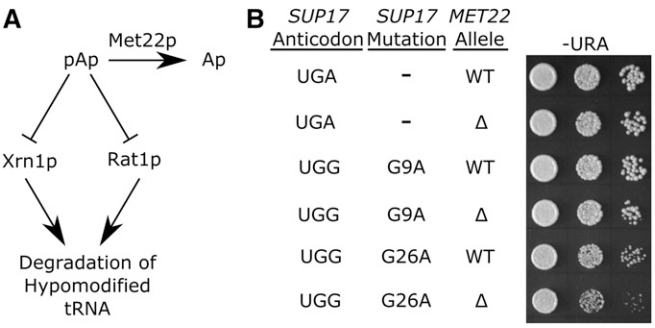


**Figure 6** *sup17(UGG)*$_{G26A}$ is degraded by the RTD pathway. (A) Deletion of *MET22* inhibits the RTD pathway. Met22p dephosphorylates pAp. When *MET22* is deleted, pAp builds up in the cell and inhibits Xrn1p and Rat1p, which are responsible for degradation of hypomodified tRNAs. (B) BY4742 (*MET22*) and CY7641 (*met22Δ*) containing either *SUP17*, *sup17(UGG)*$_{G9A}$, or *sup17(UGG)*$_{G26A}$ were grown to stationary phase in media lacking URA. Cell densities were normalized and then cells spotted in 10-fold serial dilutions onto a plate lacking URA and grown at 30°. RTD, rapid tRNA decay; URA, uracil.

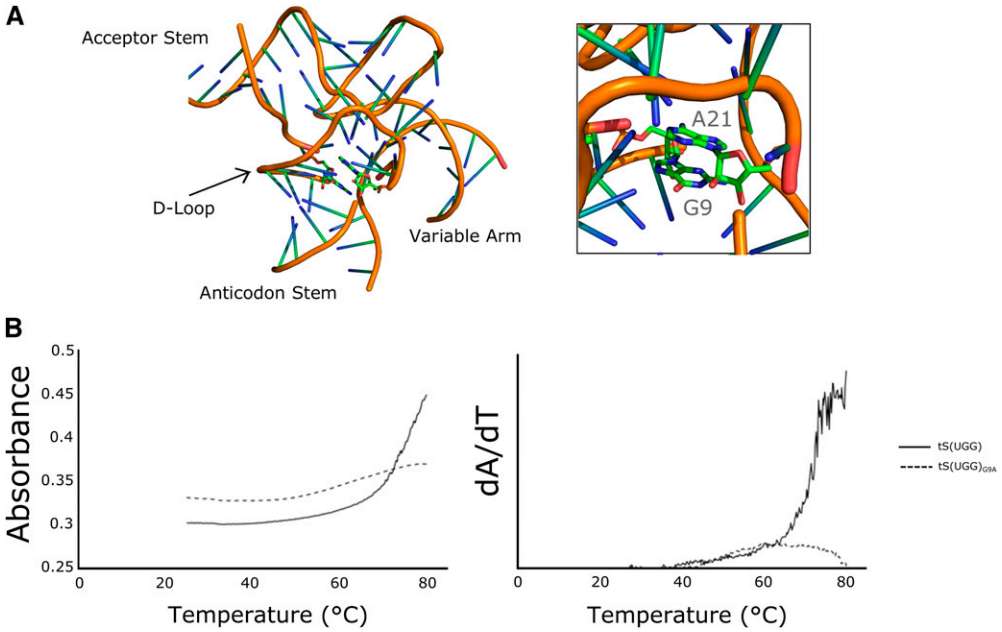


**Figure 7** Stability of the *sup17(UGG)$_{G9A}$* variant. (A) The structure of *T. thermophilus* tS [PDB 1SER; Biou *et al.* (1994)] shows G9 in proximity with A20b with the potential to base-stack. (B) The absorbance of *in vitro*-transcribed tRNA$^{Ser}$ (tS) (UGG) and tS(UGG)$_{G9A}$ in 1 mM $MgCl_2$ was measured at 260 nm from 20° to 85°. The absorbance and its first derivative (dA/dT) is plotted *vs.* temperature.

the anticodon mutated from UAA to UGG into a *URA3* centromeric plasmid. As we observed for *sup17*(*UGG*), Ura+ transformants were not obtained with this plasmid. We mutagenized the *tL*(*UGG*) plasmid and obtained Ura+ transformants of CY7020 (*tti2-L187P*) at a low frequency. These transformants were analyzed for their ability to grow in medium containing 5% ethanol. Two of ~100 transformants suppressed *tti2-L187P*. The plasmids were isolated, retransformed to verify their role in suppression (Figure 8A), and sequenced. One of the alleles contained a single-base deletion that altered the variable loop (Figure 8B). The other allele had a deletion of 32 bp, 8-bp downstream of the *tL(UAA)B2* gene (Figure S6 in File S1).

## Discussion

### *An ambivalent intermediate allows evolution of mistranslation*

Suppression of the *tti2-L187P* allele provides a sensitive method to select for tRNAs that mistranslate proline codons (Hoffman *et al.* 2017). This system allowed us to show that mistranslating tRNAs for serine and leucine can be derived through mutations to the anticodon if they are accompanied by secondary mutations that reduce the functionality of the tRNA. Interestingly, Murgola (1985) first proposed that mistranslation from a mutant tRNA must be low, to obtain missense suppression at a level that will not inhibit cell viability. He noted that glycine tRNA suppressors of *trpA* in *E. coli* were relatively easy to isolate, likely because their ability to mistranslate was low due to inefficient aminoacylation. Santos *et al.* (1996) reached a similar conclusion when characterizing a naturally evolved *C. albicans* tRNA that mistranslates leucine CUG codons with serine. Introducing *C. albicans* tS(CAG) into *S. cerevisiae* slowed growth, supporting the idea that this tRNA also mistranslates leucine CUG codons as serine in *S. cerevisiae*. U33 is a base that is conserved in nearly all tRNAs, yet the *C. albicans* tS(CAG) contains a noncanonical G33. Mutating the G33 to U in the *C. albicans* tS(CAG) resulted in a toxic tRNA. Santos *et al.* (1996) proposed that the mutation of U33G permitted the anticodon change to tS and allows nontoxic levels of mistranslation.

In both our study and the Santos study, mutations that produced noncognate anticodons in tRNAs in an otherwise native context are likely to be toxic to the cell as the result of extensive mistranslation. We were able to manipulate tS and tL through genetic selection to create mistranslating tRNAs that are tolerated by the cell. As the occurrence of two simultaneous mutations is unlikely, this suggests that an evolutionary pathway to create a mistranslating tRNA containing a noncognate anticodon can be achieved through a nontoxic or phenotypically ambivalent intermediate that reduces tRNA function (Figure 9).

A key aspect of our study was demonstrating that tRNAs with the secondary mutations alone do not have a visible phenotype under the conditions of our experiments. We refer to tRNAs with only these secondary mutations as ambivalent intermediates because, in the context of the multiple copies of isodecoders, the decreased function of the ambivalent intermediate tRNA does not affect the cell. These ambivalent intermediates could arise spontaneously and be maintained with no selective disadvantage. In turn, they provide the raw material for an anticodon change that would allow mistranslation and under certain conditions provide a selective advantage. Interestingly, if the ambivalent mutation disables the tRNA to the correct level (*e.g.*, tS(UGG)$_{G9A}$) the mutation to a noncognate anticodon may have a relatively modest effect on cell growth even in nonselective conditions.

The anticodon of many of the tRNAs plays a major role in aminoacylation such that mutation of the anticodon

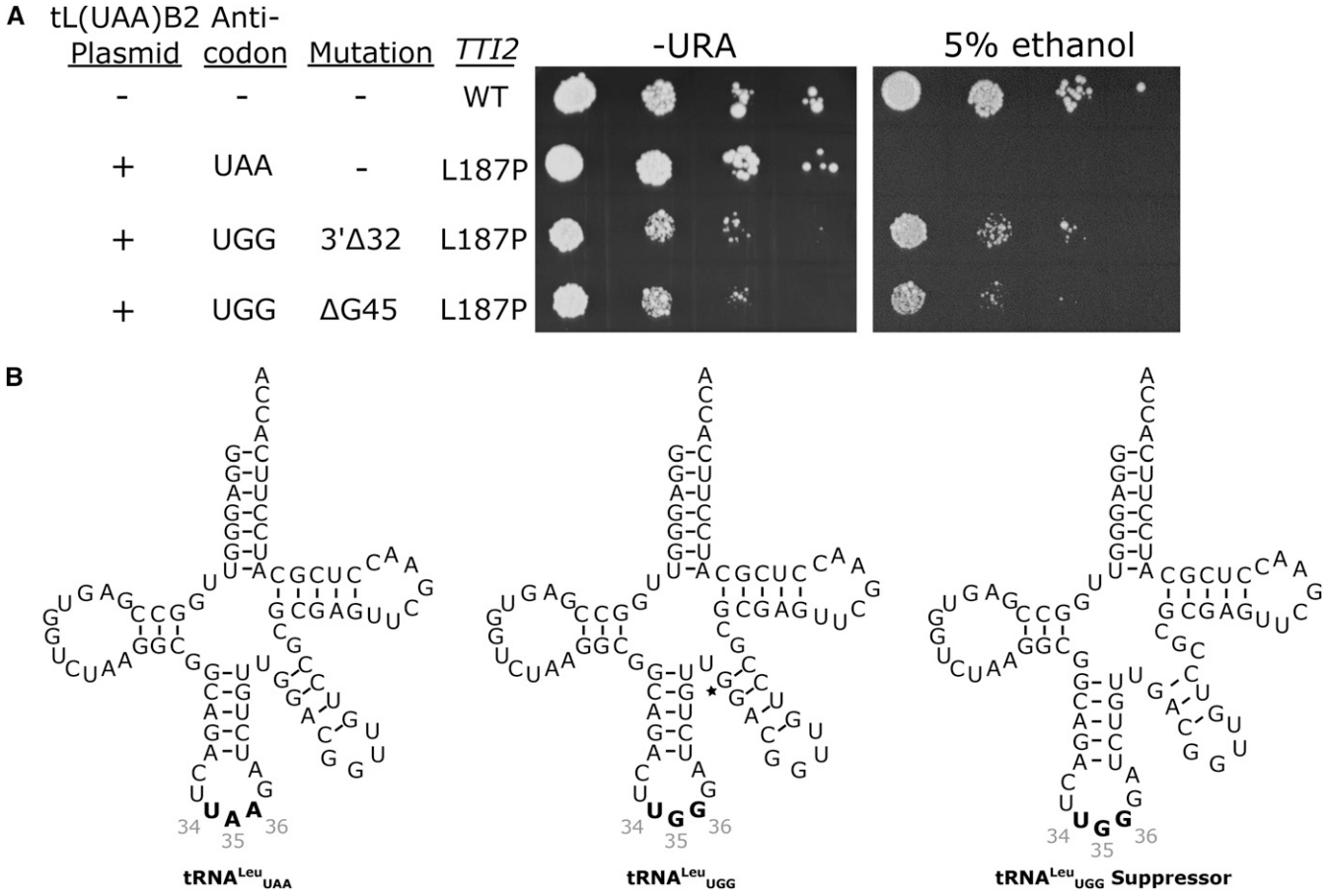


**Figure 8** tRNA$^{Leu}$ (tL) secondary structure and stress resistance of genetically selected *tL(UGG)* alleles. (A) Yeast strains CY6963 (*TTI2*), CY7655 (*tti2-L187P tL(UAA)B2*), CY7670 (*tti2-L187P tL(UGG)*$_{3'\Delta32}$), and CY7656 (*tti2-L187P tL(UGG)*$_{\Delta G45}$) were grown to stationary phase in media lacking uracil (URA). Cells were spotted in 10-fold serial dilutions onto a plate lacking URA and a YPD plate containing 5% ethanol and grown at 30°. (B) Secondary structures of tL(UAA), tL(UGG), and tL(UGG)$_{\Delta G4}$. The anticodon is shown in bold and the deletion is highlighted with a star.

significantly diminishes or abolishes aminoacylation (Giegé *et al.* 1998). If a mutation to a noncognate anticodon abolishes aminoacylation by the cognate aaRS completely, mistranslation would not occur. If the mutation only partially decreases aminoacylation, the anticodon mutation alone might sufficiently reduce function and allow the tRNA to remain below a toxic threshold in cells. Our analysis with tL, where anticodon nucleotide A35 plays a role in aminoacylation (Soma *et al.* 1996), suggests that mutation of the anticodon to UGG was not sufficient to reduce toxicity. Secondary mutations were required. However, suppressor genetics provides several examples, in addition to tRNA$^{Gly}$ mentioned above, where an anticodon change alone is sufficient for nontoxic levels of mistranslation. For example, in *E. coli*, an anticodon mutation in a phenylalanine tRNA suppresses a *trpA* mutation when the copy number of the tRNA is low [Pages *et al.* 1991; see also Murgola (1985)]. Furthermore, *SUP17* was originally identified as a suppressor of ochre (UAA) codon nonsense mutations (Ono *et al.* 1979). The toxicity of the anticodon mutation was likely alleviated through competition for the stop codon between the nonsense suppressing tRNA and its release factor. As suggested by Yona *et al.* (2013), mutation of the anticodon provides a mechanism to adapt translation to a changing environment, but it should also be noted that anticodon mutations are not the only mechanism to achieve ambiguous decoding. The major identity element for AlaRS, the G3:U70 base pair, is alone sufficient to result in misacylation of an otherwise noncognate tRNA with alanine (Imura *et al.* 1969; Hou and Schimmel 1989; Sun *et al.* 2016; Hoffman *et al.* 2017).

Our ambivalent intermediate model for the origin of mistranslating tRNAs is a possible companion to the ambiguous intermediate model (Schultz and Yarus 1994), which suggests that tRNA evolution includes a period of time in which a single codon is decoded as two different amino acids. An individual tRNA that mistranslates can arise through single-base substitutions to the anticodon. As long as the mutant tRNA is still aminoacylated, ambiguous decoding will occur with the caveat that if the tRNA is too efficient, then such an abrupt change to the genetic code will likely be toxic. The ambivalent intermediate is a tRNA with a second mutation outside of the anticodon that decreases function to avoid, or at least minimize, the toxic effects of mistranslation induced by an anticodon mutation.

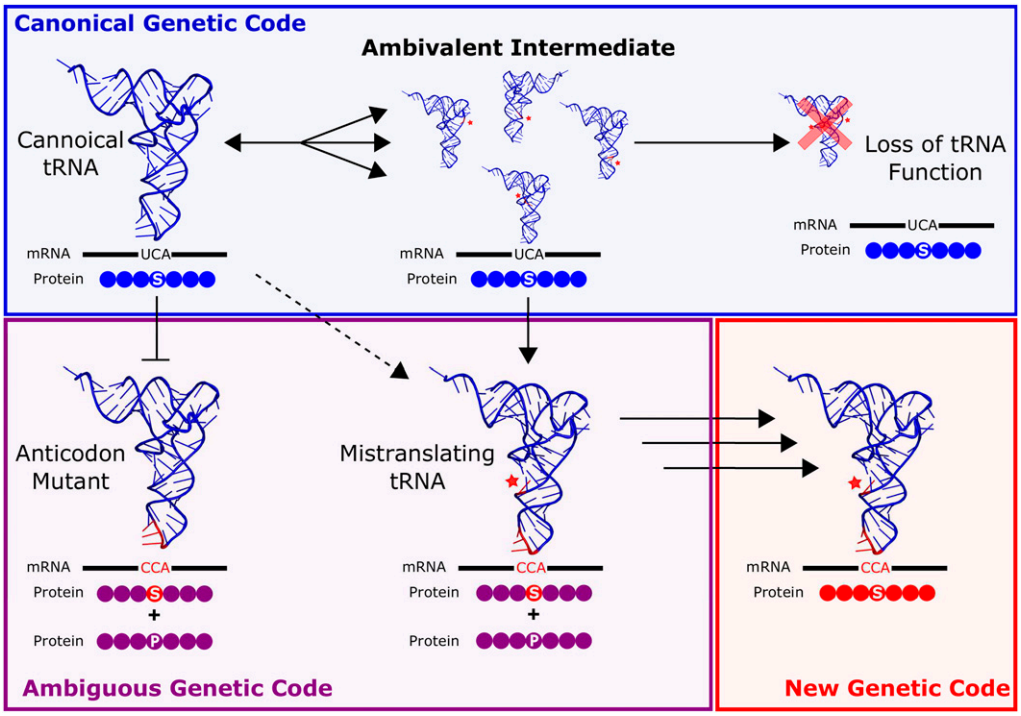


**Figure 9** Ambivalent intermediate model of genetic code evolution through tRNA anticodon mutation. Mutations to a tRNA that reduce function, which we call ambivalent intermediates, can be tolerated for tRNAs with multiple isodecoder copies. The ambivalent intermediates can revert to wild-type, or acquire additional mutations that make them nonfunctional. However, if they acquire a noncognate anticodon through mutation, their functionality may be sufficiently low that the resulting mistranslation is tolerated by the cell. In contrast, an anticodon mutation for tRNAs where the anticodon is not an identity element will be toxic. A spontaneous double mutation, to both reduce function and switch the anticodon, is possible but statistically unlikely. Over many generations, the ambiguous decoding can lead to reassignment of the codon by the mistranslating tRNA and a new genetic code.

### *Nature of the ambivalent intermediate*

In our study, we identified four ambivalent intermediate mutations that allow for nontoxic serine mistranslation of proline codons. We characterized two of the mutants in detail to identify possible mechanisms by which these mutations contribute to genetic code evolution. The efficiency of translation by a tRNA can be reduced at many stages of expression, maturation, and translation. Many of these points of control in the tRNA life cycle are well-described and include reduced transcription by RNA polymerase III, errors in processing, modification, splicing or aminoacylation, decreased affinity for EF-Tu or the ribosome, or an increase in tRNA degradation (Reynolds *et al.* 2017). *sup17*(*UGG*)$_{G26A}$ contained a mutation of a dimethylated G to A at position 26. We suggest that the toxicity of the UGG anticodon mutation is reduced by increased turnover of tS(UGG)$_{G26A}$, since loss of dimethylation at position 26 leads to turnover by the RTD pathway (Dewe *et al.* 2012) and we found that tS(UGG)$_{G26A}$ is more toxic when the RTD pathway is inhibited. Surprisingly, tS(UGG)$_{G26A}$ was aminoacylated more efficiently by serine *in vitro* than the wild-type tRNA. It is difficult to evaluate if this increase reflects the situation in cells because of differences in recombinant tS and native tS with its base modifications (Himeno *et al.* 1997), including the dimethylation of G26. It is possible that the unmethylated wild-type tS synthesized *in vitro* is less efficiently aminoacylated than the modified form and that the G26A mutation represents a more favorable substrate for aminoacylation.

The second mutation characterized in detail was *sup17*(*UGG*)$_{G9A}$. This mutation decreased aminoacylation *in vitro*. We suggest that the G9A mutation may reduce the toxicity in cells of the anticodon mutation by impacting the fold and/or stability of the tRNA. This interpretation results from the finding that *in vitro*-synthesized tS(UGG)$_{G9A}$ showed a higher level of degradation than the other tRNAs when analyzed on a sequencing gel, and had a slightly altered pattern of degradation in the D-arm/loop. The melting profile of tS(UGG)$_{G9A}$ also lacked the sharp unfolding profile that is characteristic of a wild-type tRNA, but rather showed minimal hyperchromicity with increasing temperature. The crystal structure of tS from *T. thermophilus* in complex with its aaRS indicates that G9 base-stacks with A20b, another position that when mutated in *sup17*(*UGG*) suppresses *tti2-L187P*. The G9-A20b base-stacking helps to stabilize the positioning of the D-loop. It is possible that mutating these bases increases the flexibility of the D-arm, that in turn leads to a poorly folded or less stable tRNA. Despite its potentially more flexible structure, tS(UGG)$_{G9A}$ was aminoacylated to a reasonable level *in vitro* and does suppresses *tti2-L197P in vivo*. The rationale for this is unclear, but we suggest that the aminoacylation could be due to the tRNA becoming more structured upon binding SerRS, and *in vivo*, RNA chaperones likely assist in the proper folding of tS(UGG)$_{G9A}$ (Huang *et al.* 2006). The fourth allele that allows mistranslation, *sup17*(*UGG*)$_{C40T}$, creates a mismatch in the anticodon stem. We suggest that this mutation also decreases tRNA stability and may affect base pairing with the codon or inhibit interactions with the ribosome. Related to this, Curran and Yarus (1986) found that reducing base pairing of positions 30 and 40 diminishes

stop codon readthrough by an *E. coli* nonsense suppressor, $tRNA^{Phe}$.

We also selected for mutations of *tL*(*UGG*) that allow for nontoxic levels of mistranslation. The first mutation was a deletion that shortens the variable arm of the tRNA by 1 bp. The long, variable arm is an identity element for yeast LeuRS, which recognizes both the orientation and the sequence of the variable arm (Breitschopf *et al.* 1995; Fukunaga and Yokoyama 2005). Deleting a base pair in the variable arm that both shifts the sequence and alters the length would be expected to decrease recognition of the tRNA by LeuRS. The second mutation was a deletion of 32 nt, from positions 9 to 41 downstream of the tRNA. We hypothesize that this mutation affects post-transcriptional processing of the tRNA. tRNAs are transcribed as pre-tRNAs with leader and trailer nucleotides on the 5′- and 3′-ends (O'Connor and Peebles 1991). At the 3′-end, both exo- and endonucleases process the tRNA [reviewed in Hopper (2013)]. In *S. cerevisiae*, Lhp1 binds the 3′-trailer resulting in endonuclease cleavage (Yoo and Wolin 1997). In the absence of Lhp1, the 3′-trailer is processed by exonucleases. Deleting 32 nt downstream of the tRNA gene may impair one of these mechanisms, thus reducing the level of functional mature tRNA in the cell. This deletion indicates that mutating positions adjacent to the tRNA gene may be sufficient to decrease the function of a tRNA to permit a nontoxic level of mistranslation upon an anticodon mutation.

For the mistranslating tRNAs to suppress *tti2-L187P*, there must be a balance between the misincorporation of serine (or leucine) at proline codons and the amount of toxicity produced from mistranslated proteins. Efficient misincorporation will produce abundant functional Tti2, but may result in toxicity due to the loss of proteostasis, evident in our experiments as an increased heat shock response. The extent of suppression is indicative of this balance, which can be achieved in different ways. For example, *sup17*$(UGG)_{G26A}$ produces the largest heat shock response of the *sup17* alleles tested and is slightly toxic in cells, indicating that it is mistranslating efficiently. In contrast, *sup17*$(UGG)_{G9A}$ is not toxic to cells and elicits a weaker heat shock response. Interestingly, it imparts a slightly greater degree of suppression of the deleterious tti2 allele than *sup17*$(UGG)_{G26A}$. Previously, we quantitated the level of mistranslation by $tP_{G3:U70}$ at 6% using a GFP reporter with a proline mutation that abolishes fluorescence unless the proline codon is mistranslated (Hoffman *et al.* 2017). Using this same reporter system, we were unable to show a statistical difference in GFP fluorescence in the presence of $tS(UGG)_{G9A}$ or $tS(UGG)_{G26A}$, suggesting that the level of mistranslation induced by these tRNAs is below the 6% level.

### *Conclusions*

We speculate that ambivalent intermediate mutations in tRNA genes, which are poised to alter the genetic code, exist ubiquitously in native populations of yeast as well as other organisms. Both the number of tRNA genes and the diversity of tRNA isodecoders increases with the complexity of an organism (Goodenbour and Pan 2006). In addition, Dittmar *et al.* (2006) found that, in humans, not all tissues express the different isodecoders at the same level and recent data from the 1000 genomes project indicates a high degree of variation in tRNA sequences in humans (Parisien *et al.* 2013). Interestingly, all four of the ambivalent intermediates we identified in yeast exist as variants in the human tS population (Figure S8 in File S1). It is likely that this variation includes ambivalent intermediates like those identified here, which have the potential to facilitate deviations from the standard genetic code. It will be interesting to determine if variant tRNAs that exist at a low frequency in the human population give rise to low levels of mistranslation that potentially contribute to disease.

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## Literature Cited

Bacher, J. M., V. de Crécy-Lagard, and P. R. Schimmel, 2005 Inhibited cell growth and protein functional changes from an editing-defective tRNA synthetase. Proc. Natl. Acad. Sci. U S A 102: 1697–1701.

Biou, V., A. Yaremchuk, and M. Tukalo, 1994 The 2.9 A crystal structure of T. thermophilus seryl-tRNA synthetase complexed with tRNA (Ser). Science 263: 1404–1410.

Brandman, O., J. Stewart-Ornstein, D. Wong, A. Larson, C. C. Williams *et al.*, 2012 A ribosome-bound quality control complex triggers degradation of nascent peptides and signals translation stress. Cell 151: 1042–1054.

Breitschopf, K., T. Achsel, K. Busch, and H. J. Gross, 1995 Identity elements of human tRNALeu: structural requirements for converting human tRNASer into a leucine acceptor in vitro. Nucleic Acids Res. 23: 3633–3637.

Chernyakov, I., J. M. Whipple, L. Kotelawala, E. J. Grayhack, and E. M. Phizicky, 2008 Degradation of several hypomodified mature tRNA species in Saccharomyces cerevisiae is mediated by Met22 and the 5′-3′ exonucleases Rat1 and Xrn1. Genes Dev. 22: 1369–1380.

Commans, S., M. Lazard, F. Delort, S. Blanquet, and P. Plateau, 1998 tRNA anticodon recognition and specification within subclass IIb aminoacyl-tRNA synthetases. J. Mol. Biol. 278: 801–813.

Crick, F., 1968 The origin of the genetic code. J. Mol. Biol. 38: 367–379.

Curran, J. F., and M. Yarus, 1986 Base substitutions in the tRNA anticodon arm do not degrade the accuracy of reading frame maintenance. Proc. Natl. Acad. Sci. U S A 83: 6538–6542.

de Duve, C., 1988 Transfer RNAs: the second genetic code. Nature 333: 117–118.

Dewe, J. M., J. M. Whipple, I. Chernyakov, L. N. Jaramillo, and E. M. Phizicky, 2012 The yeast rapid tRNA decay pathway competes with elongation factor 1A for substrate tRNAs and acts on tRNAs lacking one or more of several modifications. RNA 18: 1886–1896.

Dichtl, B., A. Stevens, and D. Tollervey, 1997 Lithium toxicity in yeast is due to the inhibition of RNA processing enzymes. EMBO J. 16: 7184–7195.

Dittmar, K. A., J. M. Goodenbour, and T. Pan, 2006 Tissue-specific differences in human transfer RNA expression. PLoS Genet. 2: e221.

Fukunaga, R., and S. Yokoyama, 2005 Aminoacylation complex structures of leucyl-tRNA synthetase and tRNALeu reveal two modes of discriminator-base recognition. Nat. Struct. Mol. Biol. 12: 915–922.

Giegé, R., M. Sissler, and C. Florentz, 1998 Universal rules and idiosyncratic features in tRNA identity. Nucleic Acids Res. 26: 5017–5035.

Goodenbour, J. M., and T. Pan, 2006 Diversity of tRNA genes in eukaryotes. Nucleic Acids Res. 34: 6137–6146.

Grant, C. M., M. Firoozan, and M. F. Tuite, 1989 Mistranslation induces the heat-shock response in the yeast Saccharomyces cerevisiae. Mol. Microbiol. 3: 215–220.

Guo, L. T., Y. S. Wang, A. Nakamura, D. Eiler, J. M. Kavran *et al.*, 2014 Polyspecific pyrrolysyl-tRNA synthetases from directed evolution. Proc. Natl. Acad. Sci. U S A 111: 16724–16729.

Hainrichson, M., I. Nudelman, and T. Baasov, 2008 Designer aminoglycosides: the race to develop improved antibiotics and compounds for the treatment of human genetic diseases. Org. Biomol. Chem. 6: 227–239.

Himeno, H., S. Yoshida, A. Soma, and K. Nishikawa, 1997 Only one nucleotide insertion to the long variable arm confers an efficient serine acceptor activity upon Saccharomyces cerevisiae tRNA(Leu) in vitro. J. Mol. Biol. 268: 704–711.

Hoffman, K. S., M. L. Duennwald, J. Karagiannis, J. Genereaux, S. Alexander *et al.*, 2016 Saccharomyces cerevisiae Tti2 regulates PIKK proteins and stress response. G3 (Bethesda) 6: 1649–1659.

Hoffman, K. S., M. D. Berg, B. H. Shilton, C. J. Brandl, and P. O'Donoghue, 2017 Genetic selection for mistranslation rescues a defective co-chaperone in yeast. Nucleic Acids Res. 45: 3407–3421.

Hopper, A. K., 2013 Transfer RNA post-transcriptional processing, turnover, and subcellular dynamics in the yeast Saccharomyces cerevisiae. Genetics 194: 43–67.

Hou, Y. M., and P. Schimmel, 1988 A simple structural feature is a major determinant of the identity of a transfer RNA. Nature 333: 140–145.

Hou, Y. M., and P. Schimmel, 1989 Evidence that a major determinant for the identity of a transfer RNA is conserved in evolution. Biochemistry 28: 6800–6804.

Huang, Y., M. a. Bayfield, R. V. Intine, and R. J. Maraia, 2006 Separate RNA-binding surfaces on the multifunctional La protein mediate distinguishable activities in tRNA maturation. Nat. Struct. Mol. Biol. 13: 611–618.

Imura, N., G. B. Weiss, and R. W. Chambers, 1969 Reconstitution of alanine acceptor activity from fragments of yeast tRNA-Ala II. Nature 222: 1147–1148.

Jones, T. E., R. W. Alexander, and T. Pan, 2011 Misacylation of specific nonmethionyl tRNAs by a bacterial methionyl-tRNA synthetase. Proc. Natl. Acad. Sci. USA 108: 6933–6938.

Kramer, E. B., and P. J. Farabaugh, 2007 The frequency of translational misreading errors in E. coli is largely determined by tRNA competition. RNA 13: 87–96.

Lee, J. W., K. Beebe, L. A. Nangle, J. Jang, C. M. Longo-Guess *et al.*, 2006 Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration. Nature 443: 50–55.

Lee, J. Y., D. G. Kim, B.-G. Kim, W. S. Yang, J. Hong *et al.*, 2014 Promiscuous methionyl-tRNA synthetase mediates adaptive mistranslation to protect cells against oxidative stress. J. Cell Sci. 127: 4234–4245.

Ling, J., P. O'Donoghue, and D. Söll, 2015 Genetic code flexibility in microorganisms: novel mechanisms and impact on physiology. Nat. Rev. Microbiol. 13: 707–721.

Machnicka, M. A., K. Milanowska, O. Osman Oglou, E. Purta, M. Kurkowska *et al.*, 2013 MODOMICS: a database of RNA modification pathways–2013 update. Nucleic Acids Res. 41: D262–D267.

Martinis, S. A., and M. T. Boniecki, 2010 The balance between pre- and post-transfer editing in tRNA synthetases. FEBS Lett. 584: 455–459.

Massey, S. E., G. Moura, P. Beltrão, R. Almeida, J. R. Garey *et al.*, 2003 Comparative evolutionary genomics unveils the molecular mechanism of reassignment of the CTG codon in Candida spp. Genome Res. 13: 544–557.

McClory, S. P., A. Devaraj, and K. Fredrick, 2014 Distinct functional classes of ram mutations in 16S rRNA. RNA 20: 496–504.

McCutcheon, J. P., B. R. McDonald, and N. A. Moran, 2009 Origin of an alternative genetic code in the extremely small and GC-rich genome of a bacterial symbiont. PLoS Genet. 5: e1000565.

Moghal, A., K. Mohler, and M. Ibba, 2014 Mistranslation of the genetic code. FEBS Lett. 588: 4305–4310.

Mühlhausen, S., P. Findeisen, U. Plessmann, H. Urlaub, and M. Kollmar, 2016 A novel nuclear genetic code alteration in yeasts and the evolution of codon reassignment in eukaryotes. Genome Res. 26: 945–955.

Murgola, E. J., 1985 tRNA, suppression, and the code. Annu. Rev. Genet. 19: 57–80.

Murguía, J. R., J. M. Bellés, and R. Serrano, 1996 The yeast HAL2 nucleotidase is an in vivo target of salt toxicity. J. Biol. Chem. 271: 29029–29033.

Netzer, N., J. M. Goodenbour, A. David, K. A. Dittmar, R. B. Jones *et al.*, 2009 Innate immune and chemically triggered oxidative stress modifies translational fidelity. Nature 462: 522–526.

Nissen, P., M. Kjeldgaard, S. Thirup, G. Polekhina, L. Reshetnikova *et al.*, 1995 Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu, and a GTP analog. Science 270: 1464–1472.

Oba, T., Y. Andachi, A. Muto, and S. Osawa, 1991 CGG: an unassigned or nonsense codon in Mycoplasma capricolum. Proc. Natl. Acad. Sci. USA 88: 921–925.

O'Connor, J. P., and C. L. Peebles, 1991 In vivo pre-tRNA processing in Saccharomyces cerevisiae. Mol. Cell. Biol. 11: 425–439.

O'Donoghue, P., K. Sheppard, O. Nureki, and D. Soll, 2011 Rational design of an evolutionary precursor of glutaminyl-tRNA synthetase. Proc. Natl. Acad. Sci. USA 108: 20485–20490.

Ogle, J. M., D. E. Brodersen, W. M. Clemons, M. J. Tarry, A. P. Carter *et al.*, 2001 Recognition of cognate transfer RNA by the 30S ribosomal subunit. Science 292: 897–902.

Ono, B. I., J. W. Stewart, and F. Sherman, 1979 Yeast UAA suppressors effective in psi+ strains serine-inserting suppressors. J. Mol. Biol. 128: 81–100.

Osawa, S., and T. H. Jukes, 1989 Codon reassignment (codon capture) in evolution. J. Mol. Evol. 28: 271–278.

Pages, D., K. Hijazi, E. J. Murgola, J. Finelli, and R. H. Buckingham, 1991 Suppression of a double missense mutation by a mutant tRNA(Phe) in Escherichia coli. Nucleic Acids Res. 19: 867–869.

Pang, Y. L. J., K. Poruri, and S. A. Martinis, 2014 tRNA synthetase: TRNA aminoacylation and beyond. Wiley Interdiscip. Rev. RNA 5: 461–480.

Parisien, M., X. Wang, and T. Pan, 2013 Diversity of human tRNA genes from the 1000-genomes project. RNA Biol. 10: 1853–1867.

Perona, J. J., and I. Gruic-Sovulj, 2014 Synthetic and editing mechanisms of aminoacyl-tRNA synthetases. Top. Curr. Chem. 344: 1–41.

Perret, V., A. Garcia, H. Grosjean, J. P. Ebel, C. Florentz *et al.*, 1990 Relaxation of a transfer RNA specificity by removal of modified nucleotides. Nature 344: 787–789.

Reynolds, N. M., B. A. Lazazzera, and M. Ibba, 2010 Cellular mechanisms that control mistranslation. Nat. Rev. Microbiol. 8: 849–856.

Reynolds, N. M., O. Vargas-Rodriguez, D. Söll, and A. Crnković, 2017 The central role of tRNA in genetic code expansion. Biochim. Biophys. Acta. DOI: 10.1016/j.bbagen.2017.03.012.

Rich, A., and U. L. RajBhandary, 1976 Transfer RNA: molecular structure, sequence, and properties. Annu. Rev. Biochem. 45: 805–860.

Rozov, A., N. Demeshkina, I. Khusainov, E. Westhof, M. Yusupov *et al.*, 2016 Novel base-pairing interactions at the tRNA wobble position crucial for accurate reading of the genetic code. Nat. Commun. 7: 10457.

Santos, M. A., V. M. Perreau, and M. F. Tuite, 1996 Transfer RNA structural change is a key element in the reassignment of the CUG codon in Candida albicans. EMBO J. 15: 5060–5068.

Santos, M. A. S., G. Moura, S. E. Massey, and M. F. Tuite, 2004 Driving change: the evolution of alternative genetic codes. Trends Genet. 20: 95–102.

Schultz, D. W., and M. Yarus, 1994 Transfer RNA mutation and the malleability of the genetic code. J. Mol. Biol. 235: 1377–1380.

Schwartz, M. H., and T. Pan, 2017 tRNA misacylation with methionine in the mouse gut microbiome in situ. Microb. Ecol. 74: 10–14.

Soma, A., R. Kumagai, K. Nishikawa, and H. Himeno, 1996 The anticodon loop is a major identity determinant of Saccharomyces cerevisiae tRNA(Leu). J. Mol. Biol. 263: 707–714.

Sun, L., A. C. Gomes, W. He, H. Zhou, X. Wang *et al.*, 2016 Evolutionary gain of alanine mischarging to noncognate tRNAs with a G4:U69 base pair. J. Am. Chem. Soc. 138: 12948–12955.

Suzuki, T., T. Ueda, and K. Watanabe, 1997 The 'polysemous' codon–a codon with multiple amino acid assignment caused by dual specificity of tRNA identity. EMBO J. 16: 1122–1134.

Taylor, R. C., K. M. Berendzen, and A. Dillin, 2014 Systemic stress signalling: understanding the cell non-autonomous control of proteostasis. Nat. Rev. Mol. Cell Biol. 15: 211–217.

Tong, A. H., M. Evangelista, A. B. Parsons, H. Xu, G. D. Bader *et al.*, 2001 Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science 294: 2364–2368.

Wiltrout, E., J. M. Goodenbour, M. Fréchin, and T. Pan, 2012 Misacylation of tRNA with methionine in Saccharomyces cerevisiae. Nucleic Acids Res. 40: 10494–10506.

Winzeler, E. A., and R. W. Davis, 1997 Functional analysis of the yeast genome. Curr. Opin. Genet. Dev. 7: 771–776.

Yona, A. H., Z. Bloom-Ackermann, I. Frumkin, V. Hanson-Smith, Y. Charpak-Amikam *et al.*, 2013 tRNA genes rapidly change in evolution to meet novel translational demands. Elife 2: e01339.

Yoo, C. J., and S. L. Wolin, 1997 The yeast La protein is required for the 3′ endonucleolytic cleavage that matures tRNA precursors. Cell 89: 393–402.

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