Evolving Mistranslating tRNAs Through a Phenotypically Ambivalent Intermediate in Saccharomyces cerevisiae

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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^ Variants. 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...
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 Guic-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 (McCloy 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. seiyanoids, 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 10^6 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.
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 \textit{in vitro}. In the context of wild-type \textit{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

\textbf{Yeast strains}

Yeast strains are derivatives of the wild-type haploid strains BY4741 and BY4742 [Supplemental Material, Table S1; Winzeler and Davis (1997)]. The \textit{tti}2 disruption strains covered by either \textit{TTI}2 (CY6963) or \textit{tti}2-\textit{L187P} (CY7020) on a centromeric plasmid have been described (Hoffman et al. 2016). CY7681 was generated by transforming \textit{tti}2-\textit{L187S} on a centromeric plasmid into CY6963 and \textit{TTI}2 on a \textit{URA3} centromeric plasmid was lost by plating on 5-fluoroorotic acid. Strains CY7619, CY7620, CY7621, CY7622, CY7655, CY7656, CY7670, CY7673, and CY7682 contain \textit{SUP17} or \textit{tl(UAA)B2}-derived alleles (as indicated in Table S1 in File S1) on centromeric \textit{URA3} plasmids in CY7020. CY7628, CY7629, CY7631, CY7632, and CY7633 contain \textit{SUP17}-derived alleles in centromeric \textit{URA3} plasmids in BY4742. CY7665, CY7666, CY7667, CY7668, and CY7669 contain \textit{SUP17}-derived alleles in centromeric \textit{LEU2} plasmids in BY4742. The \textit{met224} 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\textsubscript{600} was measured and growth rate averaged over two biological replicates per strain.

\textbf{Plasmid constructs}

\textit{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 \textit{HindIII}-\textit{EcoRI} fragment to generate YCplac33-\textit{SUP17} (CB3076) and YCplac111-\textit{SUP17} (CB4048). The anticodon of \textit{SUP17} was mutagenized to UGG using two-step PCR. First, \textit{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 \textit{HindIII}-\textit{EcoRI} fragment into YCplac33 to generate YCplac33-\textit{SUP17(UGG)} (CB3082). Derivatives of YCplac33-\textit{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 \textit{HindIII}-\textit{EcoRI} fragments into YCplac111 to give CB4044 and CB4046, respectively. YCplac33-\textit{SUP17(UGG)}G9A, A20bG (CB4070) was made by two-step PCR using YCplac33-\textit{SUP17(UGG)} as a template, outside primers UG5953/UG5954, and additional primers VB3039/VB3040. YCplac33-\textit{tl(UAA)B2} was cloned as a \textit{HindIII}-\textit{EcoRI} fragment after being amplified by PCR using primers UL0676 and UL0677 (CB4043). The anticodon was switched to UGG to create YCplac33-\textit{tl(UGG)} (CB4055) using two-step PCR and the additional primers UL1165 and UL1166.

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

The yeast \textit{TTI}2 and \textit{tti}2-\textit{L187P} constructs have been described (Hoffman et al. 2016). The \textit{tti}2-\textit{L187S} construct was made by two-step PCR with \textit{TTI}2 as a template, outside primers 5693-1/5693-2, and additional primers VB3060/VB3061. The product was cloned into YCplac111 as a NotI/SacI fragment (CB4071).

The yeast seryl-tRNA synthetase (SerRS) gene (\textit{SES1}) was amplified by PCR using primers UK0550 and UK0551, digested with Ncol and SacI, and cloned into pPROEX 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).

\textbf{Selection of variants of \textit{sup17(UGG)} and \textit{tl(UGG)} that suppress \textit{tti}2-\textit{L187P}}

Equal aliquots of 20 µg of YCplac33-\textit{SUP17(UGG)} or \textit{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 \textit{tti}2-\textit{L187P}-containing plasmid. The YCplac33 plasmids were then isolated, sequenced, and transformed back into CY7020 to analyze growth.

\textbf{Plasmid stability assay}

BY4742 was transformed with YCplac111-\textit{SUP17} and one of YCplac33-\textit{SUP17}, \textit{sup17(UGG)}G9A, \textit{sup17(UGG)}G26A, \textit{sup17}G9A, and one of YCplac33-\textit{SUP17}, \textit{sup17(UGG)}G9A, \textit{sup17(UGG)}G26A, \textit{sup17}G9A, and UG5953 with UI6807 and UG5954 with UI6806. The PCR
or sup17G26A. 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-32P]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 MgCl2 and the OD260 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**

Tti2L187P was purified from strains containing TAP-FLAG-tti2L187P 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 OD600 = 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 OD600 = 0.6. Expression of Ses1 was induced overnight at room temperature using 1 mM isopropyl β-D-thiogalactosylpyranoside. 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 32P-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, sup17G9A, or sup17G26A 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 OD600 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
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 eluted at 300 nl/min with the following gradient: 3% buffer B to cysteine, and propionamide adduct to cysteine, and 5% 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 × 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, automatic gain control (AGC) target of 1 × 10^6, 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 targets with an intensity threshold of 2 × 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 proionanamide 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 non-functional protein product and a stress-sensitive phenotype in S. cerevisiae (Hoffman et al. 2016). Previously, we identified four independent tP3AUG70 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.

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 tP3AUG70 (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

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     |
| sup17G9A    | UGA       | G9A      |
| sup17G26A   | UGA       | G26A     |

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.
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-32P]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(UGG), and tS(UGG)G26A estimated to be 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°C. 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 sup17G26A 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 sup17G9A with the
pressor mutants and CY7629 (M. D. Berg et al.)

**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 sup17G9A and sup17G26A single mutants. Yeast strains CY7633 (SUP17), CY7632 (sup17(UGG)G26A), CY7631 (sup17(UGG)G9A), CY7629 (sup17G26A), and CY7628 (sup17G9A) 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 leucine to stationary phase, diluted 1:100, and grown for 6 hr at 30°C. (C) Yeast strains CY7668 (SUP17), CY7666 (sup17(UGG)G26A), CY7669 (sup17(UGG)G9A), CY7665 (sup17G26A), and CY7667 (sup17G9A) containing HSE-GFP were grown in media lacking URA and leucine to stationary phase, diluted 1:100, and grown for 6 hr at 30°C. 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°C (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°C, 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, sup17G9A, sup17(UGG)G26A, or sup17G26A 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, sup17G9A, or sup17G26A 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(UGG)G26A, sup17G9A, and sup17G26A 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.
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°C, with no obvious unfolding peak, while tS(UGG) underwent a sharp unfolding at ~60°C. 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 tt2-187P, 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 tt2-187P

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 5](http://example.com/fig5.png)

Figure 5 sup17G9A and sup17G26A are not selected against. CY7633 (SUP17), CY7632 (sup17(UGG)G26A), CY7631 (sup17(UGG)G9A), CY7629 (sup17G26A), and CY7628 (sup17G9A) 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.

![Figure 6](http://example.com/fig6.png)

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 (met22Δ) 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°C. RTD, rapid tRNA decay; URA, uracil.
the anticodon mutated from UAA to UGG into a URA3 centromeric plasmid. As we observed for sup17(UUG), 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(UUG)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
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 tRNAGly 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 ocher (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 8 tRNA$^{Leu}_{UAA}$ (tL) secondary structure and stress resistance of genetically selected tL(UGG) alleles. (A) Yeast strains CY6963 (TTI2), CY7655 (ttt2-L187P tL(UAA)82), CY7670 (ttt2-L187P tL(UGG)3332), and CY7656 (ttt2-L187P tL(UGG)4445) 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°C. (B) Secondary structures of tL(UAA), tL(UGG), and tL(UGG)$_{DG}$. The anticodon is shown in bold and the deletion is highlighted with a star.
**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, the tRNA life cycle are well-described and include reduced maturation, and translation. Many of these points of control in the way (Dewey et al. 2012) and we found 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 (Dewey 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 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 suppress 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)G40T, 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\textsuperscript{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 exon- 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 Ttii2, 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)\textsubscript{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)\textsubscript{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)\textsubscript{G26A}. Previously, we quantitated the level of mistranslation by p\textsubscript{TG3: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)\textsubscript{G9A} or tS(UGG)\textsubscript{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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