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Research Article

Regulating Expression of Mistranslating tRNAs by Readthrough RNA Polymerase II Transcription

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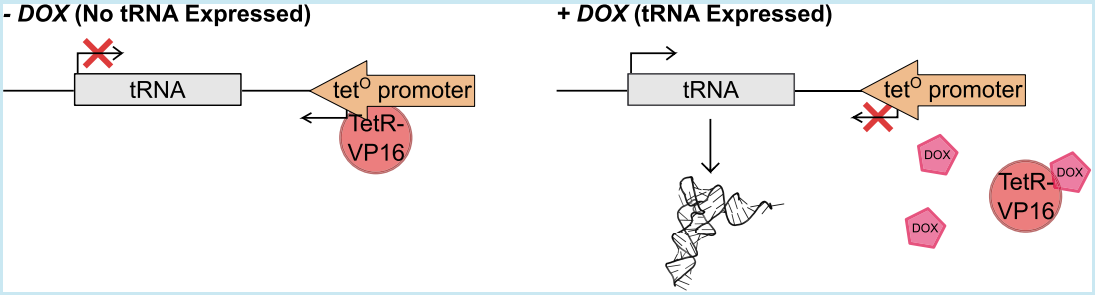

ABSTRACT: Transfer RNA (tRNA) variants that alter the genetic code increase protein diversity and have many applications in synthetic biology. Since the tRNA variants can cause a loss of proteostasis, regulating their expression is necessary to achieve high levels of novel protein. Mechanisms to positively regulate transcription with exogenous activator proteins like those often used to regulate RNA polymerase II (RNAP II)-transcribed genes are not applicable to tRNAs as their expression by RNA polymerase III requires elements internal to the tRNA. Here, we show that tRNA expression is repressed by overlapping transcription from an adjacent RNAP II promoter. Regulating the expression of the RNAP II promoter allows inverse regulation of the tRNA. Placing either Gal4- or TetR–VP16-activated promoters downstream of a mistranslating $tRNA^{Ser}$ variant that misincorporates serine at proline codons in *Saccharomyces cerevisiae* allows mistranslation at a level not otherwise possible because of the toxicity of the unregulated tRNA. Using this inducible tRNA system, we explore the proteotoxic effects of mistranslation on yeast cells. High levels of mistranslation cause cells to arrest in the G1 phase. These cells are impermeable to propidium iodide, yet growth is not restored upon repressing tRNA expression. High levels of mistranslation increase cell size and alter cell morphology. This regulatable tRNA expression system can be applied to study how native tRNAs and tRNA variants affect the proteome and other biological processes. Variations of this inducible tRNA system should be applicable to other eukaryotic cell types.

KEYWORDS: *RNA polymerase III, tRNA, inducible expression, mistranslation*

INTRODUCTION

Transfer RNAs (tRNAs) are the vehicle that bring amino acids to the growing polypeptide chain at the ribosome and read the three base codons that define protein sequences.[1−4] Both as intact molecules and as fragments, tRNAs play other essential roles in cell physiology (reviewed in ref 5). Manipulating tRNA function has numerous applications in synthetic biology and genetic code expansion, as well as in understanding genetic code evolution and the mechanisms cells use to maintain proteostasis.

One application for tRNA variants is in generating mistranslation. Mistranslation occurs when an amino acid that differs from what is specified by the "standard" genetic code is inserted into a protein during synthesis. Mistranslation occurs naturally in all cells at frequencies ranging from 1 in 3000 to 1 in 10^6 depending on the amino acid (reviewed in ref 6), with higher levels occurring in response to various environmental conditions (see, for example, refs 7−10). Excessive mistranslation leads to proteotoxic stress and slows cell growth;[11−13] however, cells use protein quality control mechanisms to tolerate mistranslation at levels approaching 8−10%.[14−16] Proteins arising from ambiguous translation caused by mistranslating tRNAs are called statistical proteins and have the potential for a broader range of function than a homogeneous protein.[17] Statistical proteins have regulatory functions in cells[18] and applications in synthetic biology and biotechnology by increasing functional diversity.[19]

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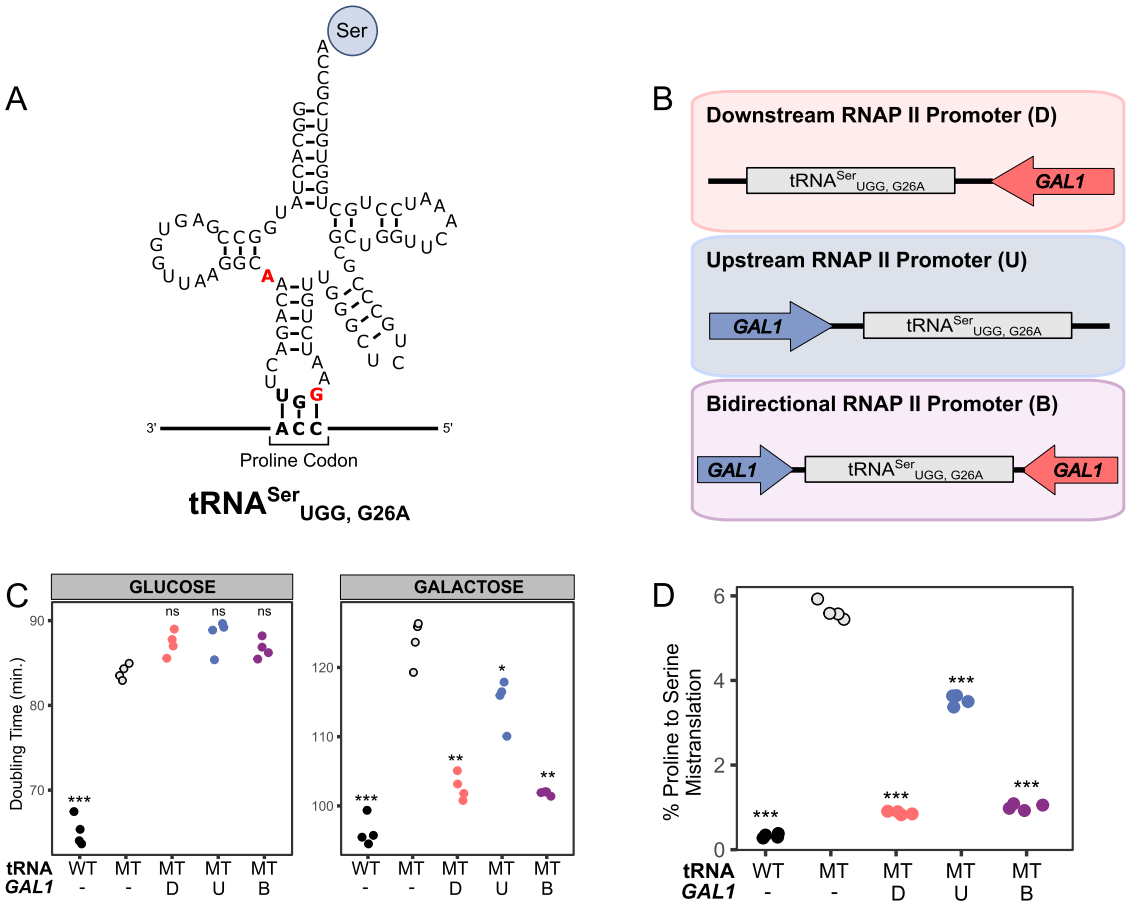

Figure 1. Transcription from a flanking RNAP II promoter decreases mistranslation derived from a mistranslating tRNA gene. (A) The structure of $tRNA^{Ser}_{UGG,G26A}$. Bases colored red were mutated to allow for nonlethal levels of serine misincorporation at proline codons. (B) The *GAL1* promoter was cloned either 300 bp downstream (D), 300 bp upstream (U), or both up and downstreams (B) of the gene-encoding $tRNA^{Ser}_{UGG,G26A}$. The sequence directly flanking the tRNA-encoding sequence is from *SUP17*. (C) Wild-type yeast strain BY4742 containing either wild-type $tRNA^{Ser}$ (WT), mistranslating $tRNA^{Ser}_{UGG,G26A}$ (MT), or the mistranslating $tRNA^{Ser}_{UGG,G26A}$ with *GAL1* promoters flanking was grown to saturation overnight in a medium lacking uracil. Strains were diluted to an OD_{600} of 0.1 in the same media containing either galactose or glucose as the carbon source and grown for 24 h at 30 °C with agitation. OD_{600} was measured every 15 min, and doubling time in minutes was calculated from the growth curves using the R package "growthcurver".[33] (D) Mass spectrometry analysis of the cellular proteome was performed on the strains described in panel (C) grown in galactose to determine the frequency of proline-to-serine substitution. In each panel, points represent biological replicates and stars indicate significant differences compared to the unregulated $tRNA^{Ser}_{UGG,G26A}$ mistranslating strain (Welch's *t*-test; $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$; ns, not statistically different).

A number of systems have been developed to regulate the expression of RNA polymerase II (RNAP II)-transcribed mRNA-encoding genes. These often involve placing transcriptional activator binding sites into 5′ promoter sequences, which then allow the gene to be regulated by a heterologous transcriptional activator protein. A classic example of this uses the Gal4 activator protein and its binding sites to regulate a specific gene. In contrast to mRNAs, in eukaryotic cells, tRNAs are transcribed by RNA polymerase III[20] (RNAP III). The key elements for RNAP III recruitment are the A box and B box.[21−23] These elements are internal to the tRNA-encoding region and thus cannot be modified without altering the tRNA structure. We have tried to block RNAP III transcription by inserting tet operator sites within tRNA intron sequences with minimal success. We do note that the Marschalek and Capone labs had some success repressing tRNA expression by placing tet or lac operator sites upstream of a tRNA gene,[24−26] while Herschbach and Johnson[27] found that the yeast α2 operator/repressor could not repress RNAP III genes. Other strategies that do not rely on transcriptional control have been used to regulate tRNA levels. One approach, developed by Zimmerman et al.,[16] is to regulate tRNA turnover through the rapid tRNA decay (RTD) pathway. The pathway is controlled through a conditional allele of *MET22*, whose substrate inhibits the endonucleases involved in tRNA decay. Disadvantages of regulation through the RTD pathway include that endogenous tRNAs may be affected, tRNA sequence needs to be altered to make them susceptible to decay, and not all tRNAs are targeted by this pathway. Similarly, we have expressed otherwise toxic mistranslating tRNAs by altering bases to destabilize tRNA structure, which, in turn, makes them more prone to decay.[15,28]

Our goal was to develop a routinely applicable system to regulate the expression of "non-native" tRNAs in yeast cells for use in synthetic biology applications and to study the impact of high levels of mistranslation. Our approach was based on the observation of Martens et al. that intergenic transcription impedes the expression of an adjacent RNAP II-transcribed gene.[29] Other work has suggested that transcription through RNAP III regulatory sequences would also inhibit their

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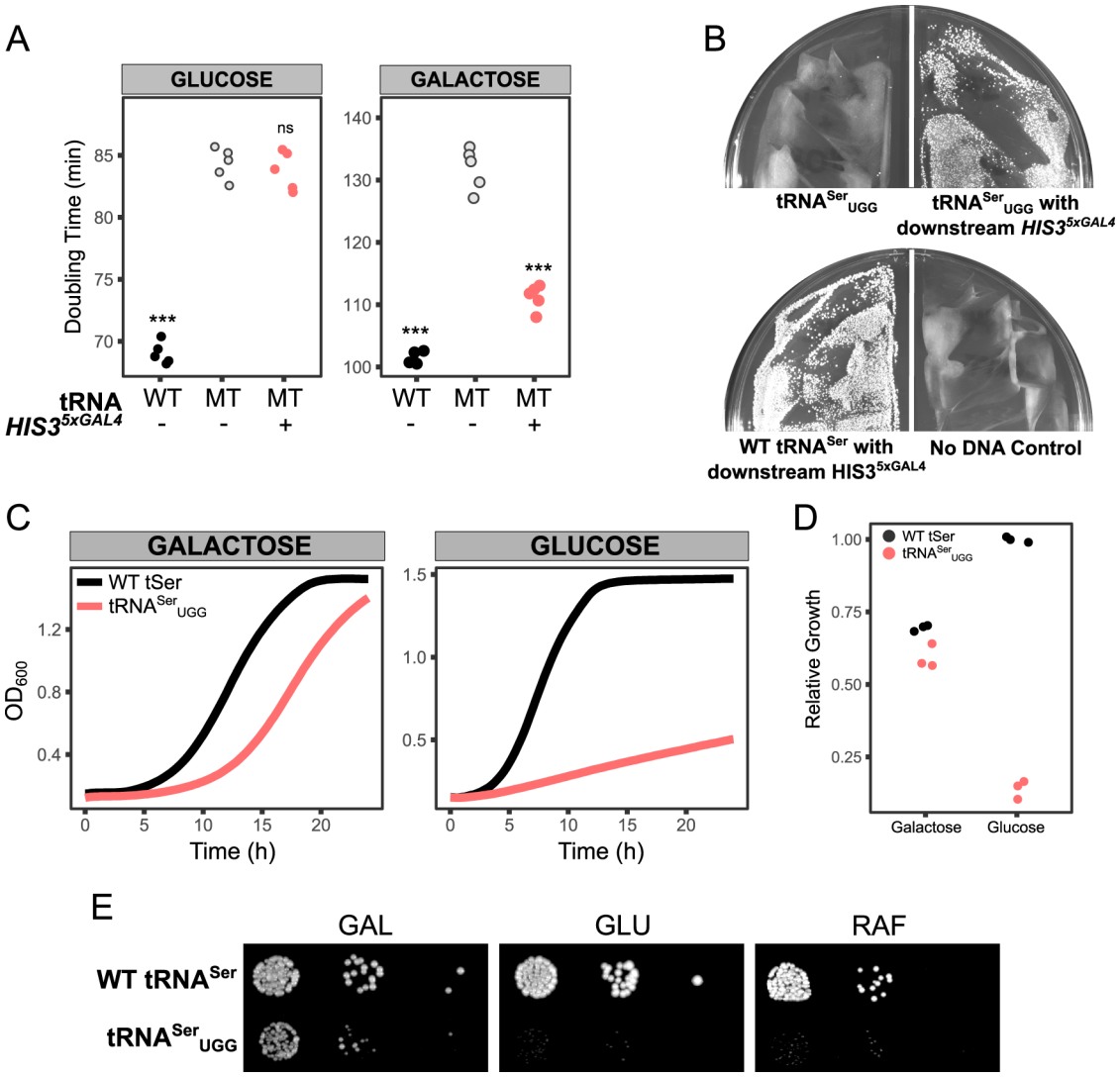

Figure 2. Regulated tRNA expression allows the transformation of a tRNA that mistranslates at otherwise lethal levels. (A) Wild-type yeast strain BY4742 containing centromeric plasmids with either wild-type $tRNA^{Ser}$ (WT), mistranslating $tRNA^{Ser}_{UGG,G26A}$ (MT), or the mistranslating $tRNA^{Ser}_{UGG,G26A}$ with a synthetic galactose-inducible promoter containing five Gal4 binding sites ($HIS3^{5xGAL4}$) were grown to saturation overnight in medium lacking uracil. Strains were diluted to an OD_{600} of 0.1 in the same media containing either glucose or galactose as the carbon source and grown for 24 h at 30 °C with agitation. OD_{600} was measured every 15 min, and doubling time in minutes was calculated from the growth curves. Points represent biological replicates, and stars indicate significant differences compared to the unregulated $tRNA^{Ser}_{UGG,G26A}$ mistranslating strain (Welch's *t*-test; ***$p < 0.0001$; ns, not statistically different). (B) Wild-type yeast strain BY4742 was transformed with either a *URA3* centromeric plasmid containing wild-type $tRNA^{Ser}$ or mistranslating $tRNA^{Ser}$ with UGG anticodon either with or without downstream $HIS3^{5xGAL4}$ promoter and plated on medium lacking uracil containing galactose. Plates were grown for 3 days at 30 °C before imaging. (C) Strains from panel (B) were grown in medium lacking uracil and containing galactose to saturation, diluted to an OD_{600} of 0.1 in medium lacking uracil and containing either glucose or galactose, and grown for 24 h at 30 °C with agitation. OD_{600} was measured every 15 min. One representative growth curve is shown for each condition. (D) Relative growth is shown for three biological replicates for each strain and condition as in panel (C). Relative growth was manually calculated from the doubling time for each strain and condition and normalized to the strain expressing wild-type $tRNA^{Ser}$ grown in glucose medium. Points represent biological replicates. (E) Strains from panel (B) were grown in medium lacking uracil and containing galactose to saturation and plated in 10-fold serial dilutions on media lacking uracil and containing either galactose (GAL), glucose (GLU), or raffinose (RAF). Plates were imaged after 2 days of growth for the glucose plate and 3 days of growth for the galactose and raffinose plates.

transcription.[30] In this work, we show that tRNA expression is efficiently repressed by placing an RNAP II promoter downstream of a tRNA-encoding gene. We use this system to regulate mistranslation in yeast cells, showing that inducing mistranslation causes growth arrest and leads to an accumulation of cells in G1. This system has applications for regulating native and synthetic tRNAs in other eukaryotic systems.

RESULTS AND DISCUSSION

RNA Polymerase II Readthrough Transcription Regulates tRNA Expression. Our goal was to engineer an inducible system to regulate tRNA expression to determine the impact of high levels of mistranslation on cells, including levels that are otherwise toxic. We hypothesized that transcription from an RNAP II promoter through the tRNA-encoding gene would interfere with RNAP III transcription and repress tRNA

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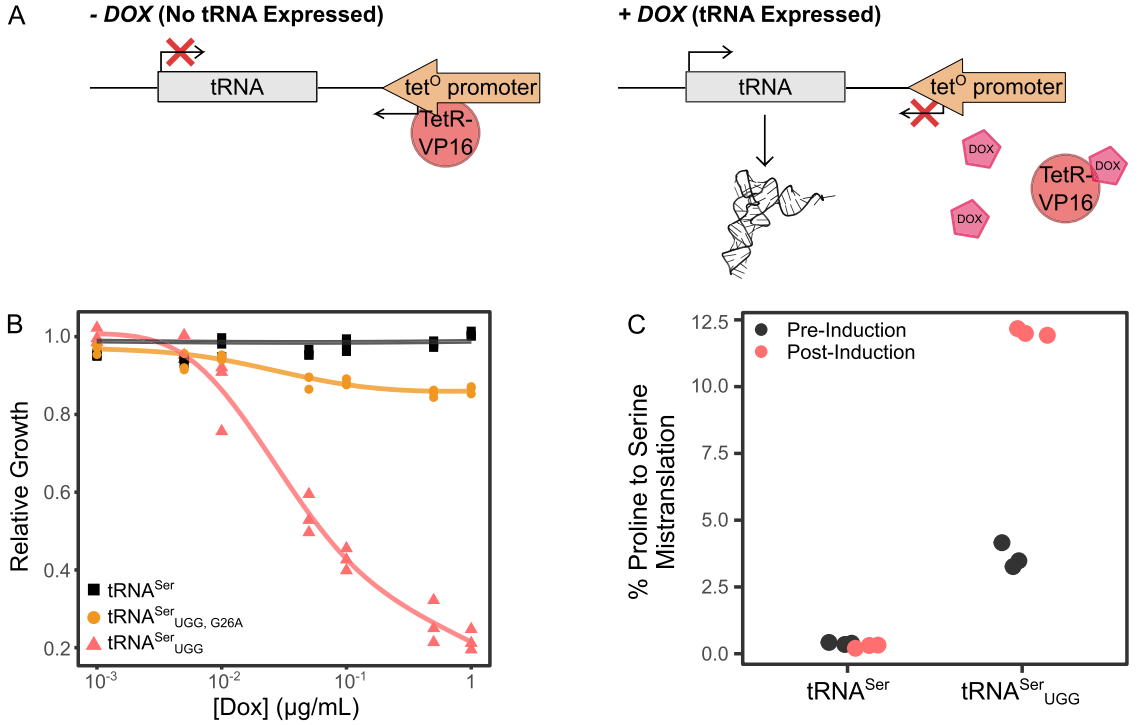

Figure 3. Doxycycline-inducible tRNA expression system. (A) Model of the tRNA expression system with or without doxycycline. In the absence of doxycycline, TetR−VP16-mediated transcription from a downstream promoter containing seven *TetO* binding sites interferes with RNAP III transcription of the tRNA. When doxycycline is added, TetR−VP16 dissociates from the promoter, which allows RNAP III to transcribe the tRNA. (B) Yeast strain CY8652 constitutively expressing the TetR−VP16 protein and containing *LEU2* plasmids expressing either wild-type $tRNA^{Ser}$ or the mistranslating variants $tRNA^{Ser}_{UGG,G26A}$ or $tRNA^{Ser}_{UGG}$ was grown to saturation in medium lacking leucine without doxycycline. Strains were diluted to OD_{600} of ∼0.1 in the same medium containing varying concentrations of doxycycline and grown for 24 h at 30 °C with agitation. OD_{600} was measured every 15 min, and doubling time in minutes was calculated from the growth curves. Doubling time was normalized to the doubling time of the wild-type strain grown without doxycycline and used to calculate the relative growth of each strain at the different doxycycline concentrations. Each point represents a biological replicate (n = 3). (C) Mass spectrometry analysis of the cellular proteome was performed on yeast strain CY8652 constitutively expressing TetR−VP16 and containing a *LEU2* plasmid expressing either wild-type $tRNA^{Ser}$−TetO or the inducible $tRNA^{Ser}_{UGG}$−TetO. Cells were grown overnight in media lacking uracil and leucine, diluted to an OD_{600} of 0.05 in the same media, and grown to an OD_{600} of ∼0.2 before they were treated with 10 μg/mL doxycycline. Aliquots were taken just before doxycycline addition (preinduction) and at 10 h after (postinduction) to determine proline-to-serine mistranslation frequency using mass spectrometry. Each point represents a biological replicate (n = 3).

expression. By making the RNAP II promoter inducible, the tRNA would come under inverse regulation.

Serylation of $tRNA^{Ser}$ requires sequences within its long variable arm (Figure 1A) and not within the anticodon.[31] Changing the $tRNA^{Ser}$ anticodon results in serine mistranslation at the codons recognized by the new anticodon. We have demonstrated this for $tRNA^{Ser}_{UGA}$, where altering the anticodon to UGG results in the incorporation of serine at proline codons.[15,28] In the context of an otherwise wild-type $tRNA^{Ser}$, constructs containing the UGG anticodon cannot be transformed into yeast, presumably because their expression causes loss of viability. However, constructs expressing $tRNA^{Ser}$ with a UGG anticodon can be transformed into yeast if they contain a secondary mutation, for example, G26A, that reduces their steady-state level.[28] Strains expressing $tRNA^{Ser}_{UGG,G26A}$ introduced on a centromeric plasmid mistranslate serine at proline codons at a frequency of approximately 5%, and cell growth rate decreases by ∼30% as compared to that seen with wild-type $tRNA^{Ser}_{UGA}$.[15] We use the effects of $tRNA^{Ser}_{UGG,G26A}$ on growth as an initial proxy for mistranslation, as decreased growth correlates with increased mistranslation.[15]

We engineered three constructs to test the regulation of a tRNA with flanking galactose-inducible promoter (Figure 1B): one positions the *GAL1* promoter, containing four binding sites for the Gal4 transcriptional activator, 300 base pairs downstream of $tRNA^{Ser}_{UGG,G26A}$, another with the *GAL1* promoter 300 bases upstream of $tRNA^{Ser}_{UGG,G26A}$, and a third construct with two copies of the *GAL1* promoter, one placed upstream and the other placed downstream of the tRNA. The *GAL1* promoter is highly expressed in medium containing galactose and repressed in medium containing glucose (reviewed in ref 32). The centromeric plasmids containing the tRNA and *GAL1* promoters were transformed into a wild-type yeast strain, and doubling time was determined in liquid growth assays. When the strains containing the mistranslating tRNA with the flanking *GAL1* promoter constructs were grown in glucose containing medium, where the *GAL1* promoter is inactive, the increase in doubling time was similar to unregulated $tRNA^{Ser}_{UGG,G26A}$ (Figure 1C). In contrast, when grown in galactose, where the *GAL1* promoter is active, strains containing the $tRNA^{Ser}_{UGG,G26A}$ construct flanked with the downstream *GAL1* promoter or flanked with *GAL1* on both sides had doubling times that approached the strain expressing wild-type $tRNA^{Ser}$. The strain expressing $tRNA^{Ser}_{UGG,G26A}$ with the upstream *GAL1* promoter had a doubling time that was intermediate between the strains expressing wild-type $tRNA^{Ser}$ and unregulated $tRNA^{Ser}_{UGG,G26A}$.

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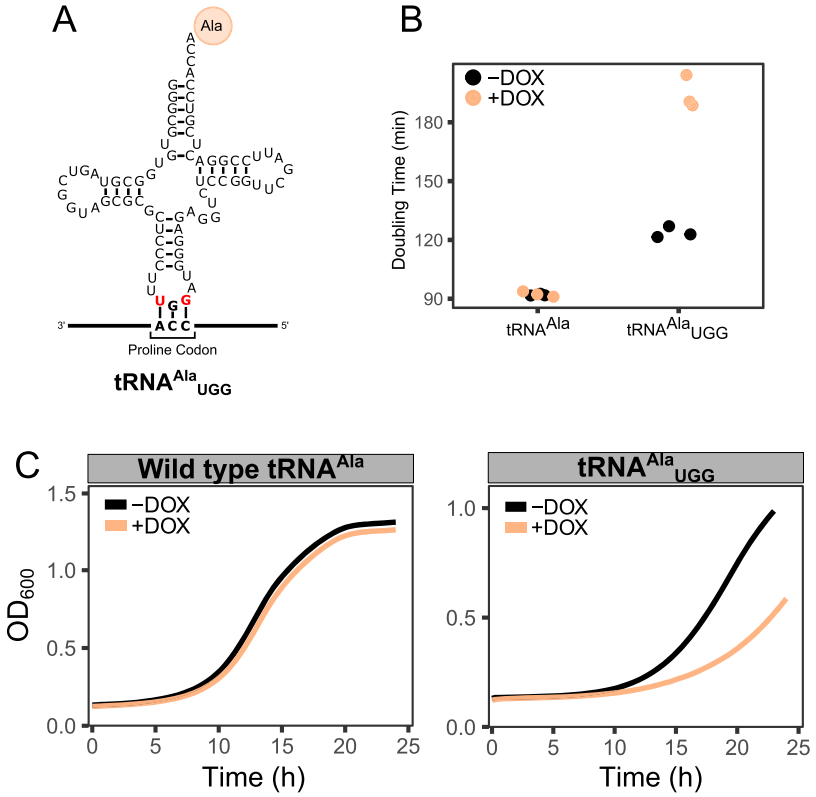

Figure 4. (A) Schematic of $tRNA^{Ala}_{UGG}$, which inserts alanine at proline codons. (B) Yeast strain CY8652 expressing either wild-type $tRNA^{Ala}$–TetO or $tRNA^{Ala}_{UGG}$–TetO was grown in medium lacking uracil and leucine, diluted to an OD_{600} of 0.1 in the same medium either with 1 μg/mL or without doxycycline, and grown for 24 h at 30 °C with agitation. OD_{600} was measured every 15 min, and doubling time in minutes was calculated from each curve using the R package "growthcurver".[33] Each point represents one biological replicate. (C) One representative growth curve from panel (B) is shown for each strain and condition.

To confirm that mistranslation was inhibited when strains were grown in galactose, we determined mistranslation frequencies in strains containing the regulated tRNA using mass spectrometry. Mistranslation frequency was calculated from the ratio of mistranslated serine peptides identified compared to peptides containing the wild-type proline residue. The observed mistranslation frequencies in each strain mirrored the results seen from strain doubling times (Figure 1D). In the strain expressing a wild-type serine tRNA, we detected 0.3% substitution of serine at proline codons, whereas in the unregulated mistranslating strain expressing $tRNA^{Ser}_{UGG,G26A}$, mistranslation frequency was 5.6%. In the strains expressing the mistranslating tRNA with the downstream *GAL1* promoter or with *GAL1* promoter flanking both sides of the tRNA, mistranslation was reduced to ∼1%. The *GAL1* promoter positioned upstream also repressed mistranslation but to a lesser extent (3.5%). These results suggest that transcription from the *GAL1* promoter inhibits the expression of the otherwise toxic tRNA, with the downstream promoter being more effective than the upstream.

To determine if the reduced toxicity of the mistranslating tRNAs was due to Gal4 activity and not another element in the promoter, we replaced the downstream *GAL1* promoter in $tRNA^{Ser}_{UGG,G26A}$ with a synthetic *HIS3* promoter containing five Gal4 binding sites[34] ($HIS3^{5xGAL4}$). Again, when grown in glucose containing medium, the doubling time of a strain containing this tRNA was similar to that containing unregulated $tRNA^{Ser}_{UGG,G26A}$ (Figure 2A). In galactose containing medium, the strain expressing $tRNA^{Ser}_{UGG,G26A}$ with downstream $HIS3^{5xGAL4}$ grew similarly to the strain containing wild-type $tRNA^{Ser}$, supporting the conclusion that Gal4 activity is responsible for inhibiting mistranslation.

Next, we determined if this system allows the expression of $tRNA^{Ser}_{UGG}$ lacking a secondary mutation to dampen its function. When unregulated, this tRNA cannot be introduced into cells.[28] The $HIS3^{5xGAL4}$ promoter was cloned downstream of the $tRNA^{Ser}_{UGG}$ gene and transformed into yeast. In contrast to unregulated $tRNA^{Ser}_{UGG}$, transformants containing the Gal4-regulated $tRNA^{Ser}_{UGG}$ were obtained when cells were plated on medium containing galactose (Figure 2B). Growth curves were performed in glucose and galactose media to measure the toxicity of $tRNA^{Ser}_{UGG}$ in expressed and repressed conditions, respectively (representative growth curves are shown in Figure 2C, and the relative growth of strains in triplicate is shown in Figure 2D). In glucose medium, the virtual lack of growth for strains containing $tRNA^{Ser}_{UGG}$ demonstrates that the mistranslating tRNA is expressed and toxic to cell growth. Growth of $tRNA^{Ser}_{UGG}$ in galactose medium demonstrates that Gal4-mediated transcription limits the expression of the otherwise toxic tRNA. Compared to a wild-type $tRNA^{Ser}$, there is some toxicity from $tRNA^{Ser}_{UGG}$ grown in galactose, likely reflecting low levels of RNAP III-mediated transcription of $tRNA^{Ser}_{UGG}$ in the repressed state.

To confirm whether Gal4-driven transcription is required to repress tRNA expression, we assayed the growth of the strain containing $tRNA^{Ser}_{UGG}$ with the downstream $HIS3^{5xGAL4}$ in raffinose medium. With raffinose as the carbon source, Gal4 is bound to its binding site but its activity is repressed by Gal80.[35] When plated on medium containing raffinose, the strain expressing $tRNA^{Ser}_{UGG}$ with downstream $HIS3^{5xGAL4}$

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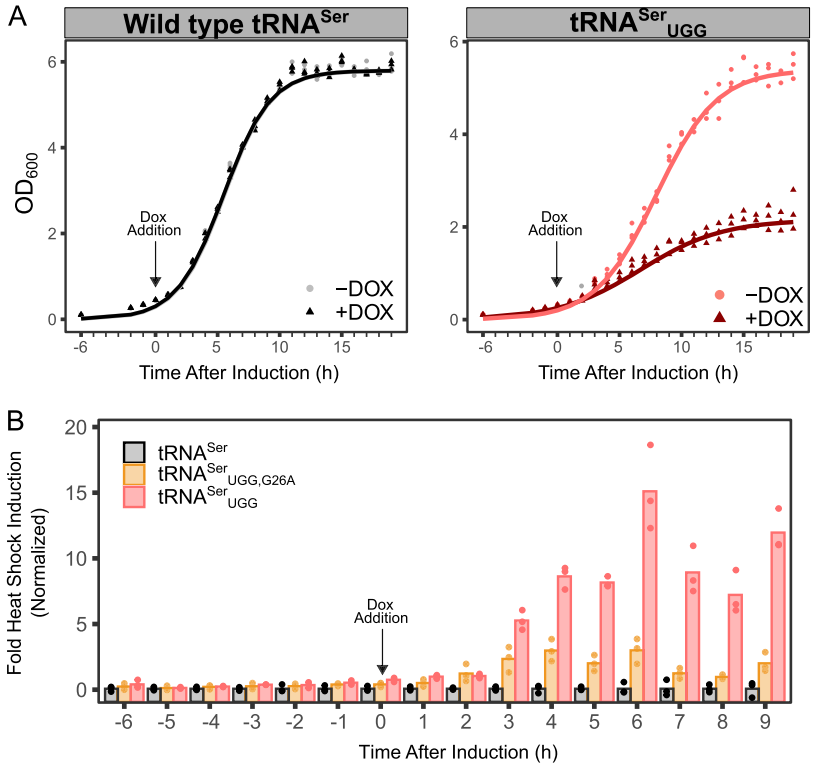

Figure 5. Effect of inducing high levels of tRNA-derived mistranslation on cell growth and heat shock response. (A) Yeast strain CY8652 constitutively expressing the TetR−VP16 protein and containing *LEU2* plasmids expressing either wild-type tRNA^{Ser}−TetO or the mistranslating variant tRNA$^{Ser}_{UGG}$−TetO was grown to saturation in medium lacking leucine without doxycycline. Strains were diluted to an OD$_{600}$ of ∼0.1 in the same medium and grown at 30 °C with agitation. At an OD$_{600}$ of ∼0.3, cultures were either left to grow (−DOX) or treated with doxycycline at a concentration of 10 μg/mL (+DOX). OD$_{600}$ was measured every hour. The arrow indicates the time of doxycycline addition. Each point represents a biological replicate (n = 3). (B) Heat shock response upon induction of the mistranslating tRNA variant. Yeast strain CY8652 constitutively expressing the TetR−VP16 protein and containing an *HSE−GFP* reporter plasmid with *LEU2* plasmids expressing either wild-type tRNA^{Ser}−TetO or the mistranslating variants tRNA$^{Ser}_{UGG,G26A}$−TetO or tRNA$^{Ser}_{UGG}$−TetO was grown to saturation in medium lacking leucine and histidine without doxycycline. Strains were diluted in the same medium to an OD$_{600}$ of 0.1 and grown to an OD$_{600}$ of ∼0.3 at 30 °C before doxycycline was added to 10 μg/mL. Every hour, an aliquot of cells was removed from the culture, cell densities were normalized to an OD$_{600}$ of 0.1, and fluorescence was measured. Fold induction was calculated as the fluorescence signal relative to the strain expressing a wild-type tRNA^{Ser} at each time point. The arrow indicates the time of doxycycline addition. Each point represents one biological replicate (n = 3).

does not grow, indicating that Gal4-driven transcription is essential to repress the transcription of tRNA$^{Ser}_{UGG}$ (Figure 2E). In agreement with this, overexpressing just the Gal4 DNA binding domain in a *gal4Δgal80Δ* strain does not suppress tRNA$^{Ser}_{UGG,G26A}$ toxicity (Figure S1).

We tested if the distance between the 3′ end of the tRNA and the regulated promoter affected tRNA expression. When cells were grown in galactose medium, the doubling time of strains with the *HIS3*^{5xGAL4} promoter 100 or 200 bp downstream from the tRNA was not statistically different from when the regulated promoter was placed 300 bp downstream (Figure S2).

To provide a titratable system that did not require switching carbon sources, we placed a minimal *CYC1* promoter with seven TetO binding sites 300 bp downstream of tRNA$^{Ser}_{UGG}$ or tRNA$^{Ser}_{UGG,G26A}$ (Figure 3A). These constructs and a similar construct containing wild-type tRNA^{Ser} were introduced into a strain containing the Tet-Off regulation system.[36] In these strains, the strong TetR−VP16 activator protein (a chimera of the Tet repressor protein and VP16) is expressed and constitutively bound to the TetO binding sites but dissociates upon binding to tetracycline or its analogue doxycycline. Therefore, the tRNA will be repressed by TetO/TetR−VP16-induced RNAP II transcription in medium lacking doxycycline and expressed when doxycycline is added. As shown in Figure S3 with β-galactosidase assays, transcription from the *CYC1−TetO* promoter decreases with increasing concentrations of doxycycline in the range of 1−100 ng/mL. The relative growth calculated from doubling times of strains containing wild-type tRNA^{Ser}−TetO, tRNA$^{Ser}_{UGG}$−TetO, and tRNA$^{Ser}_{UGG,G26A}$−TetO when grown in media containing different concentrations of doxycycline is shown in Figure 3B. In medium lacking doxycycline, the wild-type and tRNA$^{Ser}_{UGG,G26A}$ strains grow at a similar rate, while the tRNA$^{Ser}_{UGG}$-containing strain grows slightly slower. Increasing the doxycycline does not decrease the growth of the wild-type tRNA^{Ser}−TetO strain. Growth of the strain expressing tRNA$^{Ser}_{UGG,G26A}$−TetO decreased to ∼90% of the wild type at 1 μg/mL doxycycline. In contrast, the growth of the tRNA$_{UGG}^{Ser}$−TetO strain was reduced to ∼20% of the wild-type strain with 1 μg/mL doxycycline.

We performed mass spectrometry to determine the maximum amount of mistranslation from tRNA$^{Ser}_{UGG}$−TetO before and after the addition of doxycycline. Prior to the addition of doxycycline, the mistranslation of serine at proline codons by tRNA$^{Ser}_{UGG}$ was 3.6% (Figure 3C). Ten hours after the addition of doxycycline, we observed 12% substitution of serine at proline codons.

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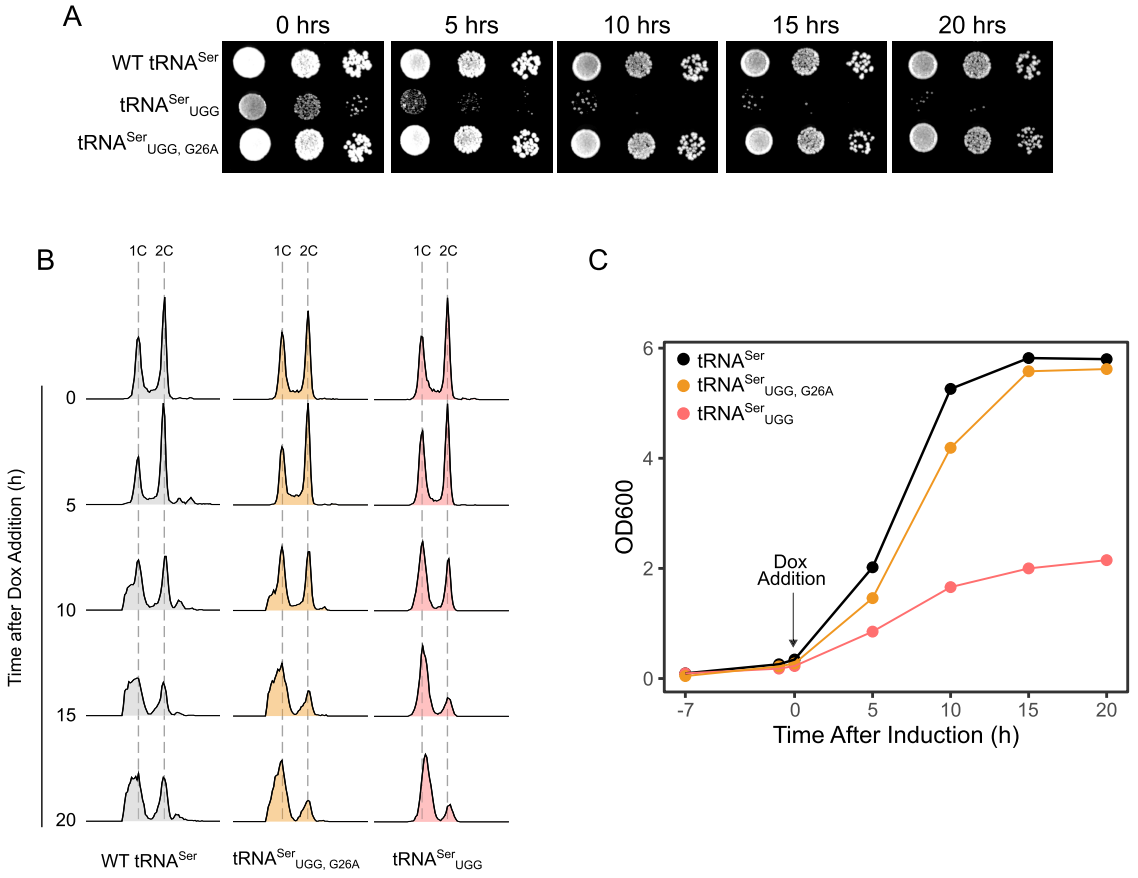

Figure 6. Growth is inhibited after high levels of tRNA-based mistranslation. (A) Yeast strain CY8652 constitutively expressing the TetR−VP16 protein and containing *LEU2* plasmids expressing either wild-type $tRNA^{Ser}$−TetO or the mistranslating variant $tRNA^{Ser}_{UGG}$−TetO or $tRNA^{Ser}_{UGG,G26A}$−TetO was grown to saturation in medium lacking leucine without doxycycline. Strains were diluted to an OD_{600} of ∼0.1 in the same medium and grown at 30 °C with agitation. At an OD_{600} of ∼0.3, cultures were treated with doxycycline at a concentration of 10 μg/mL. At various time points after doxycycline addition, an equal number of cells of each strain were washed in medium lacking leucine without doxycycline and plated in 10-fold serial dilutions on the same medium lacking doxycycline and grown at 30 °C. Plates were imaged after 2 days of growth. (B) Flow cytometric analysis of samples taken at various time points after doxycycline addition as in panel (A). Samples were taken at the times indicated in hours following doxycycline addition, prepared for analysis and stained with propidium iodide. Histograms represent ∼10 000 cells. Positions of cells with 1C and 2C DNA contents are indicated on the *x*-axis, which reflects fluorescence intensity on a linear scale. The *y*-axis represents cell frequency and has been scaled to represent the percentage of the maximum bin contained in that graph. One representative biological replicate is shown. (C) Representative growth curve of strains used for flow cytometry analysis in panel (B). The arrow indicates the time of doxycycline addition.

To determine if the regulatory system was applicable to other tRNAs, we tested if a downstream TetR−VP16-activated promoter regulated mistranslation from an alanine tRNA variant. Since a G3:U70 base pair within the acceptor stem is the principal identity element for alanylation by AlaRS,[37−40] we replaced the cognate AGC anticodon of $tRNA^{Ala}$ with UGG for proline (Figure 4A) to generate a mistranslating $tRNA^{Ala}$. The $tRNA^{Ala}_{UGG}$-encoding gene was cloned precisely in place of $tRNA^{Ser}$ to flank the $tRNA^{Ala}_{UGG}$ gene with the $tRNA^{Ser}$ 5′ and 3′ sequences (see Figure S4). Transformants were isolated and examined for growth in the presence or absence of doxycycline. $tRNA^{Ala}$ with an alanine anticodon was cloned in the same setup and used as the control. When grown in the absence of doxycycline, the strain containing $tRNA^{Ala}_{UGG}$ grew slower than the strain expressing $tRNA^{Ala}$ (Figure 4B; doubling times of 123 ± 3 and 92 ± 1 min from three biological replicates, respectively), indicative of mistranslation by $tRNA^{Ala}_{UGG}$ and again consistent with some leakiness of tRNA expression. In the presence of 1 μg/mL doxycycline, the doubling time of $tRNA^{Ala}_{UGG}$ increased to 194 ± 8 min, whereas $tRNA^{Ala}$ was unchanged. Representative growth curves are shown in Figure 4C.

Impact of High Levels of Mistranslation on Yeast Cells. Next, we used the inducible system to investigate the effect of increasing tRNA-derived mistranslation on growing cells. Yeast strains were inoculated into medium lacking doxycycline and grown for 6 h before doxycycline was added to induce tRNA expression. As shown by the growth curves in Figure 5A, there was no difference in growth rate after doxycycline addition in the strain containing wild-type $tRNA^{Ser}$, whereas $tRNA^{Ser}_{UGG}$ began to slow growth 4 h after doxycycline addition. The strain containing $tRNA^{Ser}_{UGG}$ stopped growing 15 h after doxycycline addition, reaching an OD_{600} of approximately 2.0 compared to the uninduced $tRNA^{Ser}_{UGG}$, which reached an OD_{600} of greater than 5.0 at 15 h.

Mismade proteins arising from tRNA-mediated mistranslation cause proteotoxic stress and induce a heat shock response in yeast.[11,28,37,41] Taking advantage of the inducible tRNA expression system, we investigated the dynamics of heat shock

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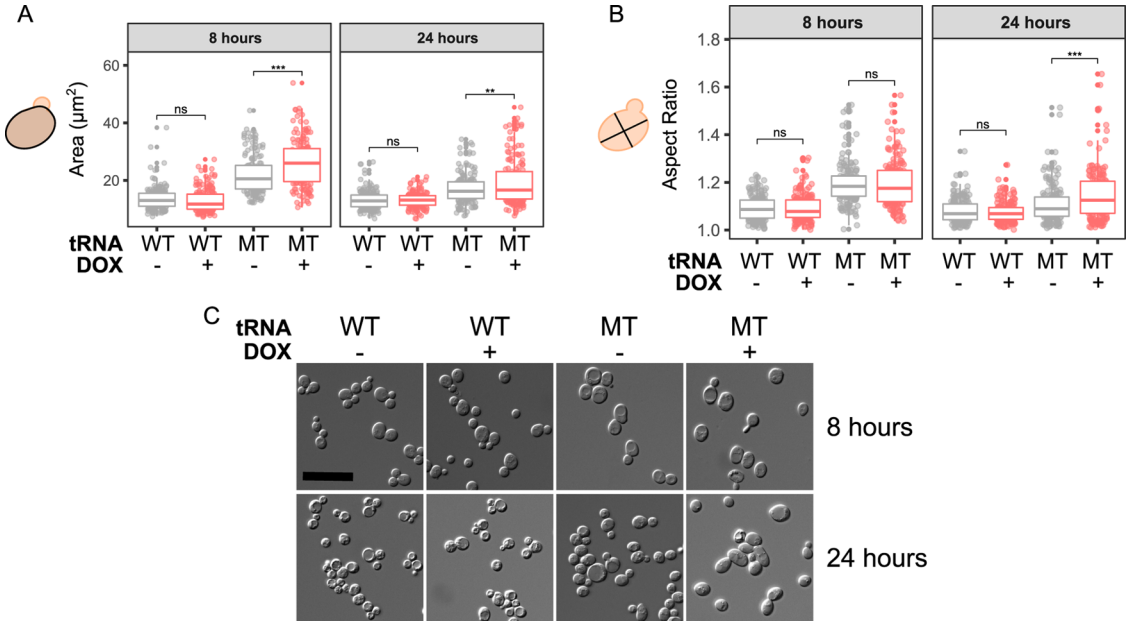

Figure 7. Cell size and shape changes upon exposure to mistranslation. Images of cells containing either an inducible wild-type $tRNA^{Ser}$ or mistranslating $tRNA^{Ser}_{UGG}$ were taken at various time points after doxycycline addition. Strains expressing either wild-type $tRNA^{Ser}$–TetO or $tRNA^{Ser}_{UGG}$–TetO were diluted to an OD_{600} of 0.1 in media lacking doxycycline. At an OD_{600} of ∼0.3, doxycycline was added to a final concentration of 10 μg/mL. Samples were taken at 8 and 24 h after doxycycline addition and imaged at 63× magnification using DIC. (A) Area and (B) aspect ratio (ratio between the longest and short diameters of each cell) were measured for 80 cells at each time point. Each point represents one cell, and stars indicate significant differences (Welch's *t*-test; $^{**}p < 0.001$, $^{***}p < 0.0001$; ns, not statistically different). (C) Representative images of cells from each strain with and without doxycycline treatment at each time point. Scale bar represents 20 μm.

induction upon the elevated expression of a mistranslating tRNA. Using a fluorescent GFP reporter driven by a promoter containing heat shock response elements, the extent of heat shock response was measured at various points after inducing either $tRNA^{Ser}_{UGG}$, which mistranslates at lethal levels, or $tRNA^{Ser}_{UGG,G26A}$, which mistranslates at levels that are tolerated by cells (Figure 5B). Three hours after doxycycline addition, GFP fluorescence increased 6.2-fold in the strain containing $tRNA^{Ser}_{UGG}$ and 3.3-fold for the less severe $tRNA^{Ser}_{UGG,G26A}$. The heat shock response reached a maximum increase of ∼16-fold and 4-fold at 6 h after tRNA induction for $tRNA^{Ser}_{UGG}$ and $tRNA^{Ser}_{UGG,G26A}$, respectively.

To determine if cells resume growth after experiencing high levels of tRNA-derived mistranslation, an aliquot of cells at various time points after doxycycline addition was washed in medium lacking doxycycline and spotted on solid medium lacking doxycycline (Figure 6A). Before adding doxycycline, strains containing $tRNA^{Ser}_{UGG}$ or $tRNA^{Ser}_{UGG,G26A}$ were viable. Viability decreased 5 h after inducing tRNA expression with doxycycline for the strain expressing $tRNA^{Ser}_{UGG}$, and few viable cells remained after 10 h. In contrast, cells expressing the less severe $tRNA^{Ser}_{UGG,G26A}$ were still viable 20 h after doxycycline addition. To determine if the high level of mistranslation causes cell death, cells were stained with propidium iodide at the same time points. Propidium iodide is a membrane-impermeable dye and only stains DNA in dead cells.[42] Interestingly, there was no increase in propidium iodide signal after increasing the expression of mistranslating $tRNA^{Ser}_{UGG}$ with the addition of doxycycline (Figure S5). This suggests that the mistranslation does not disrupt cellular membranes and the inability of the mistranslating cells to grow may not be due to typical cell death.

We hypothesized that high levels of mistranslation might cause cells to arrest at a specific cell cycle stage. We performed flow cytometry after staining with propidium iodide at different time points after doxycycline addition to assess the proportion of cells in each stage of the cell cycle (Figure 6B,C; additional biological replicates are shown in Figure S6). At 20 h after doxycycline addition, strains expressing either $tRNA^{Ser}_{UGG}$ or $tRNA^{Ser}_{UGG,G26A}$ accumulated in the G1 phase. In contrast, cells expressing wild-type $tRNA^{Ser}$ were approximately evenly split between G1 and G2 phases 20 h after doxycycline addition. Both heat shock and the misincorporation of noncanonical amino acids also cause yeast to arrest at G1.[43,44] Differing from what we see with high levels of mistranslation, cells in these conditions continue normally through the cell cycle after the stress is removed.[44] Mistranslating tRNA variants differ from heat shock and noncanonical tRNAs in that, once expressed, the tRNA variants are likely stable, resulting in mistranslation after the removal of doxycycline. The machineries to translate functional protein and turnover mismade protein continue to be crippled. We predict that with high levels of mistranslation growth is prevented because the cells are unable to sufficiently replace and/or clear the extreme levels of mismade protein.

From the cell cycle data, we noticed that in the more severe mistranslating strain expressing $tRNA^{Ser}_{UGG}$, the peaks corresponding to G1 and G2 DNA contents shifted to the right, suggesting a possible change in cell size or shape. To determine if cell size and shape change upon exposure to mistranslation, we imaged cells at various time points after doxycycline addition and measured the area of each cell, as well as the ratio between the longest and shortest diameters (aspect ratio). There was no change in cell size or aspect ratio for strains expressing wild-type $tRNA^{Ser}$ across any of the time points. In contrast, cells containing the mistranslating $tRNA^{Ser}_{UGG}$ were larger than the control wild-type $tRNA^{Ser}$ cells (Figure 7A). Cells containing $tRNA^{Ser}_{UGG}$ and treated

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with doxycycline had a greater mean area than untreated cells at both 8 and 24 h after doxycycline addition. Cells containing $tRNA^{Ser}_{UGG}$ also shifted toward a greater aspect ratio than control cells containing wild-type $tRNA^{Ser}$ (Figure 7B). This difference was exaggerated 24 h after doxycycline addition. As shown by the representative cell images in Figure 7C, and in agreement with the increased aspect ratio, mistranslation results in cells with a more oblong shape.

CONCLUSIONS

We have created regulated tRNA expression systems where tRNA expression is repressed by a downstream RNAP II promoter directed toward the tRNA-encoding gene. Since repression required a transcriptional activator and occurred only when the downstream promoter was actively transcribed, we conclude that transcription from the RNAP II promoter is required rather than just activator binding. We predict that repression is due to a readthrough transcript based on the proximity of the promoter to the tRNA-encoding gene. If the active promoter were simply leading to more open chromatin, one would expect to see increased RNAP III expression rather than repression.

When not repressed, $tRNA^{Ser}_{UGG}$ incorporated serine at proline codons at a maximum frequency of 12%. Cells are unable to grow at this frequency of mistranslation. The *GAL10*- and *TetO*-controlled tRNA systems thus allow for the regulated expression of otherwise toxic mistranslating tRNAs. In turn, they allow the efficient production of statistical proteins in yeast. The TetO system has the added benefit of being titratable. We have observed that the toxicity resulting from misincorporation from multiple mistranslating tRNA variants is additive; therefore, regulating the mistranslating tRNAs is particularly important when making statistical proteins with two or more substitutions. By placing the different mistranslating tRNAs under regulation, it is possible to mistranslate codons for several amino acids at a high level simultaneously.

Our system has applications in regulating the expression of a variety of tRNAs in addition to mistranslating tRNAs. In genetic code expansion applications, the orthogonal tRNA synthetase is normally placed under regulated control to mediate the toxic effects of misincorporation at stop codons. Using our system, the tRNA can also be regulated, allowing prior cell growth and enhanced expression of the modified protein. Ambiguous decoding has applications in biosafety because it allows the expression of dangerous products without the threat of horizontal gene transfer.[45] Allowing a high-level expression of the required tRNA will further the utility of this approach by increasing the expression of the product. Similarly, regulated ambiguous decoding will further facilitate the expression of an otherwise toxic gene product when used in concert with promoter regulation. Endogenous wild-type tRNAs can also be regulated by this system to investigate the effects of modulating cellular tRNA concentrations on translation and the proteome. This can be extended to studies of the function of essential tRNAs; e.g., $tRNA_{CGU}^{Thr}$ (*TRT2*) and $tRNA_{CUG}^{Gln}$ (*CDC65*) in yeast. Variations of this inducible tRNA system are applicable to other eukaryotes including multicellular model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*.

Using the inducible tRNA system, we investigated the effects of high levels of mistranslation on cell growth. A heat shock response was detectable ∼3 h after doxycycline addition to induce greater expression of the mistranslating tRNA. Growth rate decreased 1 h later. Cells exposed to high levels of mistranslation arrest in G1 in an irreversible manner and display an altered size and shape.

tRNA expression was not fully repressed in either the Gal4-regulated or the tetracycline-regulated system. Moving the promoter closer to the tRNA or adding an additional flanking promoter did not appreciably alter repression. It may be possible to increase repression by increasing the RNAP II promoter strength with additional activator binding sites, a stronger activator (e.g., VP64[46]), and/or increased activator expression. Flanking sequence could also be analyzed for sequences that impede the passage of RNAP II into the tRNA-encoding gene or limit RNAP II transcription. In this regard, we found that the native sequences flanking $tRNA^{Ala}$ did not permit repression. It might also be possible to obtain a wider range of tRNA expression levels using an RNAP II promoter with more gradual changes in its expression, such as the β-estradiol promoter.[47,48] For tRNAs where leaky expression still results in lethality, expression can be facilitated by incorporating a secondary mutation such as G26A that partially destabilizes the tRNA.

MATERIALS AND METHODS

Yeast Strains and Growth. Wild-type haploid yeast strains are derivatives of BY4742 (***MATα*** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*).[49] The haploid strain CY8652 (***MATα*** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tTA*-URA3*) containing the tet "off" activator (tTA*) marked with *URA3* was derived from R1158[36] after crossing with BY4741 and sporulation. The yeast two-hybrid strain PJ69-4a (***MATa*** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2::-ADE2 met2::GAL7-lacZ*) was described in James et al.[50]

Yeast strains were grown at 30 °C in yeast peptone medium or in synthetic medium supplemented with nitrogenous bases and amino acids containing 2% glucose, 2% galactose, or 2% raffinose as indicated. Transformations were performed using the lithium acetate method, as performed in Berg et al.[51] Growth curves were generated by diluting saturated cultures to an OD_{600} of ∼0.1 in synthetic medium and incubating at 30 °C. OD_{600} was measured every 15 min for 24 h in a BioTek Epoch 2 microplate spectrophotometer or by hand every hour. Doubling time was calculated using the R package "growthcurver".[33] In cases where the growth curve could not be fit with a sigmoidal curve, doubling time was calculated manually by plotting the OD_{600} on a $\log_{10}$ scale to determine the exponential phase and doubling time was determined using an exponential curve fit to the data.

DNA Constructs. *SUP17* ($tRNA^{Ser}$; pCB3076), *sup17-(UGG)* ($tRNA^{Ser}_{UGG}$; pCB3082), and *sup17(UGG)-G26A* ($tRNA^{Ser}_{UGG,G26A}$; pCB4023), including approximately 300 base pairs of 5′ and 3′ sequences, in YCplac33 have been described previously.[15,28] The *GAL1* promoter was amplified from yeast genomic DNA using primers 4588-1/XH5103 and ligated into pGEM-Teasy (Promega) to create pCB4554. *GAL1* promoter was cut with *Hin*dIII and ligated into pCB4023 to put the *GAL1* promoter upstream of *sup17-(UGG)-G26A* to create pCB4568. *GAL1* promoter was cut with *Eco*RI and ligated into pCB4023 to put the *GAL1* promoter downstream of *sup17(UGG)-G26A* to create pCB4566. To create the construct with two *GAL1* promoters, the *GAL1* promoter was cut with *Hin*dIII and ligated into pCB4566 to create pCB4851.

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The synthetic *HIS3* promoter with five Gal4 binding sites (pCB859; *his3-G4*) was previously described in Brandl et al.[34] The promoter was amplified with primers XI6247/XI6248 and cloned into pGEM-Teasy (Promega) to create pCB4594. The tRNAs *SUP17* (pCB3076),*sup17(UGG)* (pCB3082), or *sup17(UGG)-G26A* (pCB4023) were amplified with UG5953/UG5954 and cloned into pGEM-Teasy (Promega) to create pCB4652, pCB4603, and pCB4593, respectively. The synthetic *GAL* promoter was cut with *Kpn*I-*Not*I and cloned downstream of each tRNA cut *Not*I-*Hin*dIII in vector YCplac33 cut *Hin*dIII-*Kpn*I to create pCB4657, pCB4612, and pCB4598, respectively.

Constructs with different spacing between the tRNA 3′ end and promoter were created by amplifying *sup17(UGG)* (pCB3082) with upstream primer UG5953 and downstream primers YA9566 (100 bp) or YA9567 (200 bp) and ligated into pGEM-Teasy (Promega) to create pCB4633 and pCB4634, respectively. Each tRNA construct was cut with *Not*I-*Hin*dIII and cloned into a vector containing the synthetic *GAL* promoter to create pCB4660 and pCB4661, respectively.

The minimal *CYC1* promoter with seven tetracycline binding sites was amplified from the yeast Tet-Promoters collection[36] with YG4866/YG4867 and cloned into pGEM-Teasy (Promega) to create pCB4695. The TetO promoter was cut *Bam*HI-*Not*I and cloned with *SUP17*, *sup17(UGG)*, or *sup17(UGG)-G26A* cut *Hin*dIII-*Not*I into YCplac111 cut *Bam*HI-*Hin*dIII to create pCB4699, pCB4700, and pCB4701, respectively.

The Tet promoter was amplified from the yeast Tet-Promoters collection[36] with primers YG4866/YG4868 and cloned as a *Bam*HI-*Hin*dIII fragment into the *LEU2* centromeric plasmid YCplac87 to give a *his3-lacZ* fusion reporter pCB4705.[34]

The inducible wild-type $tRNA^{Ala}$ and mistranslating $tRNA^{Ala}_{UGG}$ were synthesized as a GeneString (Thermo Fisher; Figure S4) and cloned as *Hin*dIII-*Not*I fragments into pCB4699 adjacent to the Tet promoter to create pCB4787 and pCB4776, respectively.

The centromeric plasmid containing the *HSE−eGFP* was kindly provided by Onn Brandman (Stanford University).[52] The plasmid expressing the *GAL4* DNA binding domain was kindly provided by Ivan Sadowski.[53]

Mass Spectrometry. Liquid chromatography tandem mass spectrometry was performed on strains expressing mistranslating tRNA variants to identify mistranslation. For strains containing the *GAL1*-regulated tRNA constructs, starter cultures of each strain were grown to saturation in medium lacking uracil and containing 2% galactose, diluted 1:20 in the same media, and grown for 18 h at 30 °C. For the strains containing the *TetO*-regulated tRNA constructs, starter cultures of each strain were grown to saturation in media lacking uracil and leucine, diluted to an OD_{600} of 0.05 in the same media, and grown to an OD_{600} of ∼2 before 10 μg/mL doxycycline was added. Preparation of cell lysates, protein reduction, and alkylation were performed as described in Berg et al.[15] Robotic purification and digestion of proteins into peptides were performed on the KingFisher Flex using LysC and the R2-P1 method, as described in Leutert et al.[54]

Peptides were analyzed on a hybrid quadrupole orbitrap mass spectrometer (Orbitrap Exploris 480; Thermo Fisher Scientific) equipped with an Easy1200 nanoLC system (Thermo Fisher Scientific). Peptide samples were resuspended in 4% acetonitrile and 3% formic acid and loaded onto a 100 μm ID × 3 cm precolumn packed with Reprosil C18 3 μm beads (Dr. Maisch GmbH) and separated by reverse-phase chromatography on a 100 μm ID × 30 cm analytical column packed with Reprosil C18 1.9 μm beads (Dr. Maisch GmbH) housed into a column heater set at 50 °C.

Peptides were separated using a gradient of 5−30% acetonitrile in 0.125% formic acid at 400 nL/min over 95 min and online analyzed by tandem mass spectrometry with a total of 120 min acquisition time. The mass spectrometer was operated in data-dependent acquisition mode with a defined cycle time of 3 s. For each cycle, one full mass spectrometry (MS) scan was acquired from 350 to 1200 m/z at 120 000 resolution with a fill target of 3E6 ions and automated calculation of injection time. The most abundant ions from the full MS scan were selected for fragmentation using a 2 m/z precursor isolation window and beam-type collisional-activation dissociation (HCD) with 30% normalized collision energy. MS/MS spectra were acquired at 15 000 resolution by setting the AGC target to standard and injection time to automated mode. Fragmented precursors were dynamically excluded from selection for 60 s.

MS/MS spectra were searched against the *Saccharomyces cerevisiae* protein sequence database (downloaded from the Saccharomyces Genome Database resource in 2014) using Comet (release 2015.01).[55] The precursor mass tolerance was set to 50 ppm. Constant modification of cysteine carbamidomethylation (57.0215 Da) and variable modification of methionine oxidation (15.9949 Da) and proline to serine (−10.0207 Da) were used for all searches. A maximum of two of each variable modification was allowed per peptide. Search results were filtered to a 1% false discovery rate at the peptide spectrum match level using Percolator.[56] The mistranslation frequency was calculated using the unique mistranslated peptides for which the nonmistranslated sibling peptide was also observed. The frequency is defined as the counts of mistranslated peptides, where serine was inserted for proline divided by the counts of all peptides containing proline or the mistranslated serine and expressed as a percentage. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[57] partner repository with the data set identifier PXD028496.

β-Galactosidase Assay. Yeast strain CY8652 containing pCB4705 was grown to stationary phase, diluted 10-fold into media containing various concentrations of doxycycline, and grown for 8 h at 30 °C. β-Galactosidase units were determined using *o*-nitrophenyl-β-galactoside as substrate with values normalized to cell densities, as described by Ausubel et al.[58]

Cell Viability Assay. Yeast strain CY8652 containing an inducible tRNA was grown to saturation in medium lacking leucine and uracil, diluted to an OD_{600} of 0.1 in the same media, and grown for 6 h before doxycycline was added to a final concentration of 10 μg/mL. Cell viability was assessed using propidium iodide as described in Chadwick et al.[42] Briefly, cells were washed in phosphate-buffered saline (PBS) and then resuspended in PBS containing 5 μg/mL propidium iodide (Invitrogen). A sample was boiled before resuspension in propidium iodide as a positive control, and an unstained sample was used as the negative control. Samples were incubated at room temperature for 10 min before imaging on a Gel Doc system (Bio-Rad). The OD_{600} of each sample was determined using a BioTek Epoch 2 microplate reader.

Flow Cytometry. Yeast strain CY8652 containing an inducible tRNA was grown to saturation in medium lacking

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leucine and uracil, diluted to an OD_{600} of 0.1 in the same media, and grown for 6 h before doxycycline was added to a final concentration of 10 μg/mL. An aliquot of ~10^7 cells was harvested every 5 h for 20 h after doxycycline addition. Cells were prepared for flow cytometry as described in Bellay et al.[59] Briefly, cells were fixed in 70% ethanol for at least 15 min at room temperature, washed with water, and incubated sequentially in 0.2 mg/mL RNase A (Sigma-Aldrich) for 2 h at 37 °C and then 2 mg/mL proteinase K (BioBasic) for 40 min at 50 °C. Cells were resuspended in FACS buffer (200 mM Tris, pH 7.5, 200 mM NaCl, and 78 mM $MgCl_2$) and stained with 5 μg/mL propidium iodide (Invitrogen) in FACS buffer. The samples were briefly sonicated and analyzed using a BD FACSCelesta flow cytometer (Becton Dickinson Biosciences). Data were analyzed using FlowJo Flow Cytometry Analysis software and plotted on a linear scale.

Fluorescence Heat Shock Assay. Yeast strain CY8652 expressing TetR−VP16 and containing the *HSE−GFP* reporter and inducible tRNA was grown to saturation in medium lacking leucine, uracil, and histidine and diluted to an OD_{600} of 0.1 in the same medium. Every hour, cell densities were normalized to an OD_{600} of 0.1 and fluorescence was measured with a BioTek Synergy H1 microplate reader at an emission wavelength of 528 nm. Doxycycline was added 6 h after initial dilution to a final concentration of 10 μg/mL. At each time point, relative fluorescence units were calculated by subtracting background signal from a yeast strain lacking the HSE−GFP reporter. Fold induction was calculated by normalizing to the strain expressing wild-type $tRNA^{Ser}$.

Microscopy. Yeast strain CY8652 containing either wild-type $tRNA^{Ser}$−TetO or the mistranslating $tRNA^{Ser}_{UGG}$−TetO was diluted to an OD_{600} of 0.1 in medium lacking uracil and leucine and grown for 6 h at 30 °C. After 6 h, cells were either treated with 10 μg/mL doxycycline or left to grow. Cells were imaged at 8 and 24 h after the addition of doxycycline at 63× magnification using DIC on a Zeiss Axio Imager Z1 Fluorescent microscope using ZEN Blue Pro software (Zeiss Inc.). Cell size and aspect ratio were quantified by outlining living, nonbudding cells in ImageJ.[60]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00461.

List of oligonucleotides used in this study (Table S1); doubling time of strains expressing galactose-regulated tRNAs and *GAL4* DNA binding domain (Figure S1); doubling times of strains expressing regulated mistranslating tRNAs with Pol II promoter placed at different distances (Figure S2); expression of *TetO*-LacZ reporter upon doxycycline addition (Figure S3); sequence of inducible $tRNA^{Ala}_{UGG}$ construct (Figure S4); propidium iodide staining of cells expressing regulated tRNA variants after induction (Figure S5); and two additional biological replicates of the flow cytometry data (Figure S6) (PDF)

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Author Contributions

M.D.B., J.R.I., E.C., J.G., P.L., and C.J.B. contributed the data for one or more figures. J.V. and C.J.B. supervised mass spectrometry and molecular genetic experiments, respectively. M.D.B. and C.J.B. conceived the project and its methodology and wrote the manuscript. M.D.B. designed the figures. All authors participated in manuscript editing.

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Notes

The authors declare no competing financial interest.

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