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Perseverance of protein homeostasis despite mistranslation

Farah Hasan, *The University of Western Ontario*

Supervisor: O'Donoghue, Patrick, *The University of Western Ontario*

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Abstract

Transfer RNAs (tRNAs) are essential for protein synthesis and translation fidelity. Some human tRNA variants may cause amino acid misincorporation: $tRNA^{Gly}$ variants ($tRNA^{Gly}_{CCC}$, $tRNA^{Gly}_{GCC}$) have mutations that generate an alanine tRNA identity element (G3:U70), likely causing mis-aminoacylation of glycine tRNAs with alanine, while the $tRNA^{Ala}_{AGC}$ G35C ($tRNA^{Ala}_{ACC}$) variant may function similarly to mis-incorporate Ala at Gly codons by generating a Gly anticodon. I propose that these mistranslating tRNAs will disrupt protein homeostasis in mammalian cells. Although the $tRNA^{Gly}$ and $tRNA^{Ala}$ variants did not affect protein synthesis in normal growth conditions, $tRNA^{Gly}_{GCC}$ A3G depressed protein synthesis following proteasome inhibition. Mass spectrometry confirmed Ala mistranslation at multiple Gly codons caused by the $tRNA^{Gly}_{GCC}$ A3G and $tRNA^{Ala}_{AGC}$ G35C variants. Multiple mistranslation events were also observed within the same peptide. These data reveal mistranslation of Ala at Gly codons that is caused by natural human tRNA variants and tolerated under normal conditions.

Keywords

anticodon, genetic code ambiguity, identity element, mistranslation, protein aggregation, protein quality control, natural variants, neurodegenerative disease, transfer RNA (tRNA)

Summary for Lay Audience

Transfer RNAs (tRNAs) are responsible for bringing the correct amino acid to the site of protein production in cells. Each amino acid has its own set of tRNAs and an enzyme, called an aminoacyl-tRNA synthetase (aaRS) that specifically recognizes both the tRNA and amino acid to link them together. aaRS recognize tRNAs through sequences unique to tRNAs for that amino acid. Most tRNAs are recognized by their anticodon, a sequence in the tRNA that determines which amino acid goes where in the protein and is complementary to a codon or set of codons found in the messenger RNA (mRNA). However, some aaRS recognize features of the tRNA outside the anticodon. For example, to attach the amino acid alanine to its tRNAs, the alanine-aaRS recognizes the tRNA solely by the presence of a unique G3:U70 base pair close to the site of amino acid attachment in the tRNA and does not recognize the anticodon at all. For tRNAs that carry glycine, the anticodon is essential for attaching the amino acid to glycine tRNAs. Specific recognition requirements in tRNAs may allow for mutations to occur outside of unique recognition sequences that would cause them to mis-incorporate amino acids into proteins. Amino acid misplacement can cause proteins to misfold, be degraded, or build-up, leading to further problems within the cell. I studied three different tRNAs that could incorrectly place the amino acid alanine instead of glycine into proteins, a process called mistranslation. Through expression of a fluorescent protein alongside mistranslating tRNAs, I determined that glycine to alanine mistranslation does not have a significant negative impact on protein levels in mammalian cells under normal conditions. When cells were unable to degrade proteins, expression of one glycine to alanine mistranslating tRNA decreased protein levels compared to wild-type tRNA expression. I determined sites of amino acid mutations in protein isolated from mistranslating cells to conclude that mistranslating tRNA expression caused amino acid misincorporation. However, the mistranslation was conservative enough to not disrupt normal protein production in cells. My findings show that glycine to alanine mistranslation in mammalian cells is not toxic under normal conditions.

Co-Authorship Statement

Jeremy T. Lant created the plasmid that all tRNA variants were cloned into for expression. Jeremy also cloned the plasmids containing the $tRNA^{Ser}_{AGA}$ wild-type and G35A mutant that were used as controls (Fig. 2–6). Farah Hasan cloned the plasmids containing glycine and alanine tRNA variants.

The semi-denaturing detergent agarose gel electrophoresis method section (2.7) and Fig. 7 were taken from a manuscript titled "*Amyotrophic later sclerosis-associated variant cases a protein aggregation catastrophe in mistranslating cells*". The manuscript is currently under review and was co-authored by Jeremy T. Lant, Farah Hasan, Donovan McDonald, Julia Briggs, Martin Duennwald, and Patrick O'Donoghue. All authors contributed to data collection and writing, Farah Hasan wrote the methods, results, and discussion sections and performed the experiments and data analysis from which the figure was made that is shown in this manuscript.

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List of Abbreviations

Ala	Alanine
aa	Amino acid
aaRS	Aminoacyl-tRNA synthetase
AlaRS	Alanyl-tRNA synthetase
ALS	Amyotrophic lateral sclerosis
bp	Base pair(s)
DNA	Deoxyribonucleic acid
FUS	Fused in sarcoma
Gly	Glycine
GlyRS	Glycyl-tRNA synthetase
HD	Huntington's Disease
HEK 293T	Human embryonic kidney 293T cells
HTT	Huntingtin protein
Leu	Leucine
LeuRS	Leucyl-tRNA synthetase
mRNA	Messenger ribonucleic acid
MG132	Carbobenzoxy-Leu-Leu-leucinal (proteasome inhibitor)
N2a	Neuro2a (mouse neuroblastoma) cells

Phe	Phenylalanine
Pro	Proline
RNA	Ribonucleic acid
Ser	Serine
SerRS	Seryl-tRNA synthetase
Thr	Threonine
ThrRS	Threonyl-tRNA synthetase
tRNA	Transfer ribonucleic acid

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Chapter 1

1 Introduction

1.1 The Central Dogma

The central dogma of biology describes the transfer of genetic instructions stored in deoxyribonucleic acid (DNA) to allow for synthesis of proteins required for cellular function and maintenance. The central dogma was first proposed by Francis Crick in 1958 following the discovery of DNA structure in 1952 by Rosalind Franklin [1] and later by Watson and Crick [2]. Along with the deciphering of the genetic code in 1964 [3], the central dogma has become the foundational framework of understanding biological processes. We now appreciate that in order for a protein to be made in the cell, DNA from the corresponding protein coding gene must undergo transcription, generating a ribonucleic acid (RNA) intermediate called messenger RNA (mRNA), that will be translated into a protein by the ribosome. The central dogma states that transfer of information can go from DNA to RNA and back, but cannot be extracted from a protein's amino acid sequence to determine the original DNA sequence from which it originated [4].

DNA stores the genetic information for all living organisms. The genetic alphabet consists of four letters (A, C, G, T) that make up the countless genes needing to be deciphered into proteins to enable a functional cell. mRNA was identified as that intermediate between DNA and proteins, carrying the code to be read by translation machinery and produce proteins in the cell [5].

Proteins are encoded in DNA as genes, which are transcribed into mRNA by RNA polymerase II. Following splicing and post-transcriptional modification [6], mRNA transcripts are then ready to be translated into a chain of amino acids by the ribosome, and eventually folded into functioning molecules in the cell [7]. For this to happen, there must exist a carrier molecule bringing the correct amino acid to the translation machinery for a protein to be made. Transfer RNAs (tRNAs) are integral to normal cell function as they are essential components of the protein synthesis machinery. In all cells, the 20 proteinogenic amino acids are incorporated into proteins following their specific ligation to a family of

tRNAs [8]. The existence of tRNAs was first proposed in 1955 as a set of adaptor molecules that link nucleic acid sequences to the protein sequences they encode [9]. Shortly thereafter, tRNAs were discovered that functioned as the hypothesized adaptors to carry amino acids to the ribosome for protein production [10]. The discovery of tRNAs was essential to deciphering the genetic code [3, 11].

1.2 tRNA transcription and structure

tRNA genes are transcribed by RNA polymerase III. The resulting tRNA transcript then undergoes a series of cleavages and post-transcriptional modifications to become a mature tRNA (reviewed in [12]). Mature tRNAs are composed of 73 to 90 nucleotides that are arranged into 4 or 5 base-pairing stems and connecting loops [13]. In three dimensions, tRNAs fold into L-shaped structures with the anticodon and amino acid acceptor site on opposite ends. The acceptor stems contain a conserved 3'-CCA sequence that is necessary for ligation, catalyzed by a specific aminoacyl tRNA synthetase (aaRS), of the amino acid to the 3'-terminal A76 onto each tRNA [14] (Fig. 1). The anticodon loop of a tRNA contains three central nucleotides (bases 34, 35, and 36) that read a codon or set of codons found in mRNA sequences. The anticodon recognizes its appropriate codon through normal base pair interactions or wobble pairing with codons in an mRNA bound to a translating ribosome. Thus, properly aminoacylated tRNAs are essential for accurate translation of the proteome.

1.3 tRNA role in protein translation

tRNAs act as intermediate molecules between mRNA and protein: they are required to bring the appropriate amino acid to the site of protein production at the ribosome. For a protein to be accurately translated, the ribosome must recognize the mRNA, after which it can begin the three-step process of translation: initiation, elongation, and termination.

The ribosome is composed of two subunits, small and large [15, 16]. The large subunit is responsible for tRNA incorporation to allow for protein production, consisting of three sites for tRNA incorporation: A (acceptor), P (peptidyl), and E (exit) [15-18]. After recognition of an mRNA, initiation of translation begins with the first codon to be read,

usually the start codon AUG, which codes for methionine (Met). An aminoacylated Met-$tRNA^{Met}$ enters the P-site of the ribosome, where the tRNA anticodon and mRNA codon are paired, and an aminoacylated tRNA for the next amino acid to be decoded enters the A site. At the peptidyl-transfer center in the ribosome, a peptide bond is formed between the amino acids in the A- and P-sites of the ribosome, then the tRNA in the P-site releases the amino acid, Met, and the entire complex moves along the mRNA to the next codon. The $tRNA^{Met}$ in the P-site is now in the E-site, awaiting release from the ribosome upon acceptance of a new tRNA into the A-site, and the entire process of tRNA movement in the ribosome from A- to P- to E-site is then repeated during the elongation phase. As elongation is happening, the peptide chain begins to fold into its native conformation. When one of three stop codons is reached, a release factor binds to the stop codon, signaling translation termination and release of the nascent peptide chain, which completes its folding into a functional protein (reviewed in [7]).

In cells, high translational fidelity is essential to maintain error-free protein production. However, amino acid misincorporation in eukaryotes occurs at a rate of approximately 1 in every 10,000 amino acids. Calculations estimate that such an error rate would lead to roughly 15% of all proteins translated in a cell having at least one mistranslated amino acid, in standard growing conditions [19, 20]. Recent studies suggest that the translational error rate is actually much lower, citing that it potentially occurs as low as 10^{-6}, depending on mRNA codon and tRNA anticodon pairing [21, 22]. The level or rate of mistranslation has the potential to be increased depending on the presence of mistranslating tRNA variants in the genome [23, 24].

1.4 tRNA-dependent mistranslation

Mistranslation of proteins is a phenomenon in cells that causes incorporation of incorrect amino acids into proteins. The wrong amino acid can be incorporated in proteins that have mutations to the protein's coding gene which cause singular amino acid substitutions within the specific protein that is coded incorrectly. However, if mutations arise in aaRS genes, it may allow for misincorporation of amino acids in multiple proteins within the cell [25]. In order for tRNAs to become substrates for protein synthesis, aaRS enzymes must recognize the cognate tRNA and amino acid to facilitate a two-step, ATP-dependent

reaction that ligates the amino acid to its cognate tRNA (reviewed in [26]). Although some aaRS enzymes are equipped with editing features that allow for clearance of mis-charged tRNAs due to incorrect recognition of the cognate amino acid [20, 27], if an aaRS enzyme gene is mutated, that ability may be hindered and can cause disease in the individual. Charcot-Marie-Tooth disease (CMT) is a hereditary disease that causes motor and sensory neuropathy, and its cause is linked to mutations in multiple aaRS enzymes (reviewed in [28]). Mutations to aaRS enzymes associated with CMT lead to loss- or gain-of-function by the enzyme that may hinder the enzyme's ability to aminoacylate cognate tRNAs or inhibit protein translation [28-30]. Despite the amount of research on aaRS-mediated mistranslation, emerging research shows that variant tRNA genes present in the population also have the potential to facilitate mistranslation and potentially modify disease [23, 24, 31].

Here, we studied mistranslation caused directly by tRNAs, which place the amino acid in the peptide chain. Identity elements are nucleotides present in a tRNA sequence that are required for recognition by the cognate aaRS. Although many tRNAs are distinguished through anticodon recognition by a cognate aaRS enzyme, most tRNAs require additional or separate identity elements for recognition [32]. For example, the G3:U70 base pair found in the acceptor stem of alanine tRNAs ($tRNA^{Ala}$) is the major determinant for alanyl-tRNA synthetase (AlaRS), where the AlaRS enzyme recognizes $tRNA^{Ala}$ independent of the tRNA anticodon [33]. Seryl-tRNA synthetase (SerRS) [34] and pyrrolysyl-tRNA synthetase [35] also do not recognize the tRNA anticodon, while in some species leucyl-tRNA synthetase recognizes only the middle base (G35) of the anticodon as an important recognition element [36].

Among the plethora of natural human tRNA variants [24] are mutant tRNAs with altered anticodon sequences, not required for recognition by the cognate aaRS, which will not affect aminoacylation, but may alter the codon(s) decoded by a particular tRNA, resulting in mistranslations across the proteome. Further, tRNAs recognized by their anticodon could sustain a mutation elsewhere in their sequences that may result in the creation of an identity element for a non-cognate aaRS [37]. Depicted in Fig. 1 is the proposed mechanism of mistranslation caused by the alanine and glycine tRNAs described and studied here.

tRNA^{Ala} sustains an anticodon mutation that allows decoding of glycine codons in mRNA. Both glycine tRNA variants acquire the alanine tRNA identity element, G3:U70, allowing recognition by AlaRS and aminoacylation with alanine. The alanine-charged glycine tRNAs (Ala-tRNA^{Gly}) then supply alanine to the ribosome when decoding glycine codons in mRNA.

tRNA-dependent mistranslation was previously shown using a mistranslating proline (Pro) tRNA variant that places Ala at Pro codons through an A3G mutation in the tRNA^{Pro} acceptor stem to create the G3:U70 tRNA^{Ala} identity element described above [37]. Similarly, a mistranslating serine (Ser) tRNA variant with an anticodon mutation, G35A, that decodes phenylalanine (Phe) codons with Ser [31] was characterized and is used as a positive control throughout the work described here. Due to the established effects of tRNA$^{Ser}_{AAA}$ on protein synthesis and cytotoxicity, I used the wild-type and variant tRNA^{Ser} as positive controls for effects of tRNA-dependent protein mistranslation, where the tRNA$^{Ser}_{AAA}$ causes decreased protein synthesis and increased cytotoxicity [31].

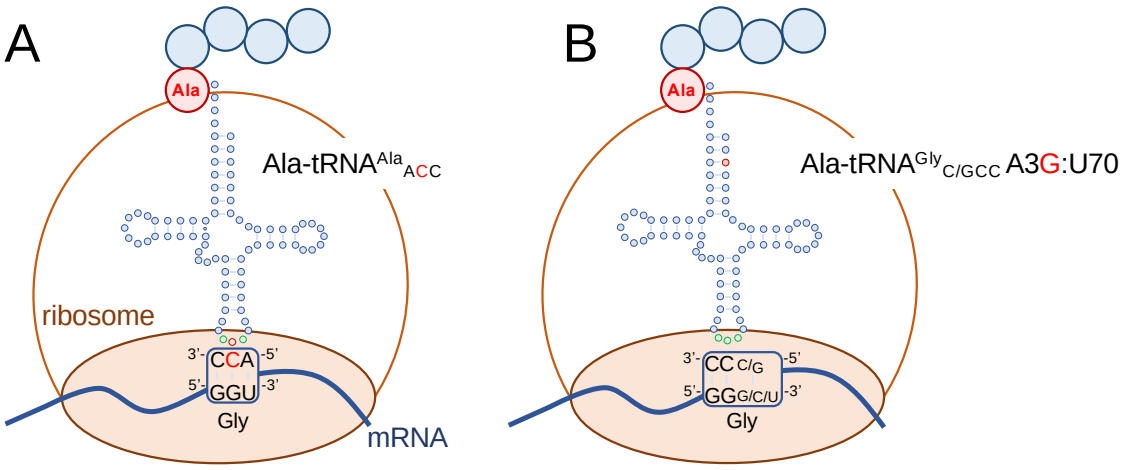

Figure 1: Schematic of tRNA-dependent mistranslation mechanisms placing Ala at Gly codons. (A) The naturally occurring tRNA^{Ala} G35C variant (red circle) acquires a Gly anticodon (tRNA$^{Ala}_{ACC}$) and decodes Gly codons, including GGU, as indicated, with Ala. (B) The natural human tRNA$^{Gly}_{C/GCC}$ A3G variants (red circle) acquire the G3:U70 major identity element for AlaRS. The Ala accepting tRNA^{Gly} also decodes the indicated Gly codons with Ala.

1.5 tRNAs can modify disease

tRNAs, like other nucleic acids, are not exempt from mutation. Some mutations in tRNAs can cause loss or gain of function [31, 38]. Initially, data from the 1000 Genomes Project suggested that individuals carry 1 or 2 tRNA gene variants compared to the reference genome [24, 39]. Targeted and deep sequencing efforts increased coverage of all human tRNA genes to show that individuals have 60–70 tRNA variants compared to reference [23], including both common and rare tRNAs with strong potential to cause mistranslation in the cell. The existence of natural tRNA variants with potential to cause mistranslation is emerging rapidly [31], yet nearly all human tRNA variants remain uncharacterized. Our lab recently characterized a $tRNA^{Ser}_{AGA}$ G35A variant that occurs in ~3% of the population and causes serine misincorporation at phenylalanine codons in mammalian cells. The data showed that a single tRNA variant inhibited protein synthesis, increased cytotoxicity, and inhibited degradation of huntingtin protein aggregates in a cellular model of neurodegenerative disease [31]. The study demonstrates that natural tRNA variants can cause amino acid misincorporation and phenotypic defects in cells. These results prompted us to continue investigation of naturally occurring human tRNA variants to determine if all mistranslating tRNAs cause similar phenotypes in mammalian cells.

1.6 ALS-associated FUS protein

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease caused by protein aggregate accumulation in the cytoplasm of motor neurons [40]. The disease manifests as muscle stiffness and twitches, gradual increase of muscle weakness and muscle wasting, before developing into loss of the ability to eat, speak, and move, eventually leading to loss of breathing ability [40]. Many proteins are associated with ALS, such as C9orf72 (chromosome 9 open reading frame 72), SOD1 (superoxide dismutase 1), TDP-43 (TAR DNA binding protein 43), and FUS (fused in sarcoma) [40]. The fused in sarcoma (FUS) protein is responsible for RNA binding, transcriptional activation, and DNA repair in the nucleus [41]. FUS has a nuclear localization signal sequence in its C-terminus that is responsible for the protein's localization to the nucleus following its translation in the cytoplasm [42]. Disturbances to the localization signal impair the ability of FUS to locate to the nucleus from the cytoplasm, causing FUS aggregation and accumulation in the

cytoplasm of motor neurons, leading to ALS in patients with localization sequence mutations [42, 43]. We studied FUS with an R521C mutation in a cellular model for ALS, alongside expression of a wild-type or mistranslating tRNA variant that is present in ~3% of individuals [23, 31]. Due to the high prevalence of the $tRNA^{Ser}$ G35A variant, we hypothesized that the coincidence of a mistranslating tRNA allele with a causative allele for ALS would exacerbate cell toxicity (Lant et al, under review). We compared the previously mentioned wild-type $tRNA^{Ser}_{AGA}$ and mistranslating $tRNA^{Ser}_{AAA}$ effects on protein synthesis, cytotoxicity, and protein aggregation in N2a cells co-expressing wild-type or ALS-causative FUS in order to determine the role of tRNA-dependent mistranslation in disease modification.

1.7 Hypothesis and goals

I investigated three tRNAs identified in publicly available sequencing databases. The $tRNA^{Ala}_{AGC}$, $tRNA^{Gly}_{CCC}$, and $tRNA^{Gly}_{GCC}$ variants (Table 1) each have the potential to mistranslate alanine at glycine codons through either anticodon or identity element mutations. The goal of my project is to determine whether these tRNAs enable amino acid misincorporation and to measure their impact on protein synthesis, protein degradation, and cell fitness.

I hypothesize that global mistranslation of alanine at glycine codons will disrupt protein homeostasis in mammalian cells. I tested this hypothesis in three aims:

1. Clone tRNAs of interest and relevant variants for expression in mammalian cells.
2. Characterize the effects of variant tRNA expression and mistranslation on cell viability and cytotoxicity under normal and stress conditions.
3. Assay the impact of mistranslation coupled with protein aggregation in a cellular model of neurodegenerative disease.

Chapter 2

2 Materials and Methods

2.1 Cloning and plasmid purification

Genomic DNA from human embryonic kidney 293T (HEK 293T) cells was used to amplify the tRNA genes of interest via polymerase chain reaction (PCR) following the Pfu Ultra II polymerase protocol (Agilent; California, USA). Primers were designed to amplify 500 base pairs (bp) up and downstream of tRNA genes of interest. PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide using a ChemiDoc MP (BioRad; California, USA). Product bands were excised, purified using the Geneaid Gel Extraction Kit (Geneaid; Taiwan, R.O.C), then used as templates for subsequent PCR reactions. For wild-type tRNAs, a second round of PCR was performed using primers designed to capture 300 bp up and downstream of the tRNA gene. *Pci*I or *Nco*I restriction sites were added to the 5'-end of each primer to allow for *Pci*I or *Nco*I (New England Biolabs; Massachusetts, USA) digestion and subsequent ligation into the *Pci*I site on pPANcherry using T4 DNA ligase (NEB). We constructed pPANcherry by removing the EGFP gene (using *Spe*I and *Nhe*I restriction sites and re-ligation) from the EGFP-mCherry construct in WT-PAN (Addgene # 99638; Massachusetts, USA). Integrating the tRNA variants into pPANcherry, thus, enables mCherry to serve as a marker for transfection and protein synthesis levels in individual cells. Mutant tRNAs were synthesized by overlap extension PCR using first-round products as template as before [31]. Mutant tRNA genes were amplified as half molecules, where the mutation was incorporated in overlapping 5' ends of the primers (Table B1). The two overlapping tRNA halves were then used as templates in a second round of PCR, amplifying the full-length mutant tRNA including 300 bp of up and downstream native sequence. These full-length mutant tRNA alleles were then cloned into the *Pci*I site of pPANCherry as above. All plasmids were verified by DNA sequencing (Azenta US; Indianapolis, USA).

2.2 Mammalian cell culture, transfection, and fluorescence microscopy

Cell culture experiments were performed in HEK 293T (ATCC # CRL-3216) or mouse neuroblastoma Neuro2a (N2a; ATCC #CCL-131) cells as indicated. Cells were grown and maintained at 37°C, with 5% CO_2 and humidity. Cells were cultured in Dulbecco's modified Eagle medium (DMEM 4.5 g/L; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco by Life Technologies) and 1% penicillin-streptomycin (P: 100 IU/mL; S: 100 µg/mL; Wisent Bioproducts, Quebec, Canada). Plasmid transfections were performed using Lipofectamine 3000 (Invitrogen, ThermoFisher Scientific, Massachusetts, USA). In brief, for a 96-well plate transfection, 0.2 µL of p3000 reagent was mixed with 100 ng of plasmid DNA and DMEM containing no FBS or antibiotics, and 0.2 µL of Lipofectamine reagent was mixed with DMEM (no FBS/antibiotics) in separate 1.5 mL microcentrifuge tubes. The solutions were incubated at room temperature for 5 minutes, mixed together, and vortexed. Following another 20-minute incubation at room temperature, 10 µL of the mixture was pipetted into a single well and the cells were incubated at 37°C with 5% CO_2 and humidity for 24 hrs before capturing fluorescent images. All fluorescent images were taken using the EVOS FL Auto Imaging System (ThermoFisher). mCherry fluorescence was visualized using the EVOS LED RFP light cube (excitation 531 ± 40 nm; emission 593 ± 40 nm). After plating and transfection, cells were incubated at 37°C with 5% CO_2 and humidity. Images were captured 24 hrs post-transfection and the level of mCherry fluorescence per cell was determined using a custom Image J script as before [31].

2.3 Cytotoxicity and proteasome inhibition assays

All cytotoxicity and proteasome inhibition assays were done with at least N = 3 biological replicates for each cell line and condition in 96-well plates. At 24-hours post-transfection with plasmids containing a tRNA variant and mCherry, cell media was replaced with media containing 0 or 10 µM proteasome inhibitor (MG132, Sigma-Aldrich), and cells were incubated at 37°C for 4 hours before images were captured using the EVOS microscope. Cytotoxicity was measured with the CytotoxGlo assay (Promega; Wisconsin, USA).

Following the manufacturer's instructions, 50 µL of assay reagent were added to each well and an initial luminescence reading was taken using the Synergy H1 Plate Reader (BioTek; Vermont, USA) before 50 µL of assay buffer including digitonin was added to each well and a final luminescence reading was taken. The initial luminescence reading quantifies the population of dead cells in each well as a result of wild-type or mutant tRNA expression, and the addition of digitonin allowed a determination of the total number of cells present in each well. A ratio of the initial luminescent reading (number of dead cells in a well) over the final luminescence reading (total number of cells in the same well) allows the quantification of dead cell percentage present in each well caused by tRNA expression.

2.4 Cell harvesting and lysis

Cells were plated and transfected in 6-well plates as described above. Media was replaced 24-hours post-transfection, and cells were incubated for an additional 24-hours before harvesting. To harvest the cells, media was aspirated, and cells were incubated for 10 minutes at room temperature in 1× phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) supplemented with 10 mM ethylenediaminetetraacetic acid (EDTA). Then, cells were resuspended and transferred to sterile 1.5 mL microcentrifuge tubes and centrifuged at 300 × g for 5 minutes. Supernatant was aspirated from cell pellets, which were stored at -80 °C. Cell lysis was performed on ice: 40 µL of mammalian cell lysis buffer (50 mM Na_2HPO_4, 1 mM $Na_4P_2O_7$, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1 mM Triton X-100, 1 mM dithiothrieitol, 0.3 mM phenylmethylsulfonyl fluoride, and 1 tablet/10 mL complete mini EDTA-free Protease Inhibitor Cocktail) [44] were added to each tube and cell pellets were resuspended before centrifugation at 21.1 × g for 10 minutes at 4°C. Supernatants were transferred to sterile 1.5 mL centrifuge tubes. The Pierce bicinchoninic acid (BCA) assay (ThermoFisher) was used to determine lysate protein concentrations to calculate appropriate loading volumes for western blotting.

2.5 Western blotting

Following harvest and lysis of cells, 15 μg of protein from each lysate was separated using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE; 15% acrylamide, 120 volts for 3 hours). Proteins were transferred from gel to methanol-activated polyvinylidene difluoride (PVDF) membranes using a Trans-Blot Turbo transfer system (25 V, 1.3 A, 14 minutes; BioRad, California, USA). All blocking and wash solutions were made using tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150mM NaCl). Following transfer, membranes were incubated in blocking solution (3% bovine serum albumin, 0.1% Tween-20, 1× TBS) for one hour at room temperature. Membranes were then incubated overnight at 4°C in primary antibody at dilutions of 1:1000 for anti-mCherry (AbCam; ab213511) or 1:5000 for anti-GAPDH (Sigma-Aldrich; MAB374). The following day, membranes were washed in 1% BSA TBS-Tween 3 × 10 minutes, before a 2-hour incubation at room temperature in IRDye 800CW donkey anti-rabbit (mCherry; Li-Cor Inc, 926-32213) or IRDye 680RD goat anti-mouse (GAPDH; Li-Cor Inc, 926–68070) fluorescent secondary antibodies at dilutions of 1:10,000. Membranes were then washed 3 × 10 minutes in TBS-Tween, and once in 1× TBS for ten minutes before imaging. Membranes were imaged using fluorescence on a ChemiDoc MP imager (Bio-rad).

2.6 Protein purification and mass spectrometry

N2a cells were plated and transfected with plasmids containing mCherry and wild-type $tRNA^{Ala}_{AGC}$, $tRNA^{Ala}_{ACC}$ variant, or $tRNA^{Gly}_{GCC}$ A3G variant in 10 cm plates as described above with N = 3 biological replicates. At 24 hrs post transfection, cells were harvested and stored in -80 °C prior to use. Cells were lysed and mCherry was purified from cell lysates through immunoprecipitation using the RFP-Trap Agarose kit (Chromotek, Munich, Germany). Agarose beads coupled with RFP nanobodies allowed for affinity purification of mCherry. The mCherry:agarose-bead complex was diluted with 2 × sodium dodecyl sulphate (SDS)-running buffer, boiled to detach the protein from the agarose beads, and purified mCherry was separated using SDS-polyacrylamide gel electrophoresis (PAGE) with 10% acrylamide. After separation of the protein, gels were stained with Coomassie blue dye. The bands corresponding to mCherry were identified at 37 kDa,

excised from the gel, placed in sterile 1.5 mL centrifuge tubes containing 5% acetic acid, and submitted for mass spectrometry analysis at the Biological Mass Spectrometry Laboratory (The University of Western Ontario, London, Canada). Concentration of protein was not measured prior to mass spectrometry analysis as the protein was not purified from the excised gel band prior to sample submission. Trypsin was used to digest mCherry and the Orbitrap Elite Velos Pro mass spectrometer (ThermoFisher) was used in FT/IT/CID configuration to identify amino acids mis-incorporated in mCherry as described before [45]. Hits corresponding to Ala misincorporation at Gly codons (Gly + 14.02 = Ala) were selected to only include peptides with an ion intensity of >1%.

2.7 Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE)

Cell lysates were prepared, and protein concentrations were measured 72 hrs post-transfection as described above. A 1.5% agarose gel and Tris-acetate EDTA (TAE) running buffer containing 40 mM Tris-acetate, 1 mM EDTA, and 0.1% w/v SDS were prepared according to established protocols [46]. Lysate samples containing 20 μg of protein were diluted in 3 × loading dye (0.5 M Tris-HCl, pH 6.8, 1.12 M sucrose; 0.025% w/v bromophenol blue; 3.8% w/v SDS) with sterile milliQ H_2O. Lysates were separated on the agarose-SDS gel for at least 3 hours at 120V. Proteins were transferred to a PVDF membrane by capillary gel transfer overnight using TAE with 0.1% SDS as a buffer. mCherry-tagged FUS aggregates were visualized by western blotting with an α-mCherry antibody.

Chapter 3

3 Results

3.1 Identifying mistranslating tRNAs

The human tRNA genes $tRNA^{Ala}_{AGC}$ (Ala-AGC-6-1), $tRNA^{Gly}_{CCC}$ (Gly-CCC-1-1), and $tRNA^{Gly}_{GCC}$ (Gly-GCC-1-5) each have naturally occurring variants in the population with the potential to mistranslate Gly codons with Ala (Table 1). Alanyl-tRNA synthetase (AlaRS) recognizes the G3:U70 base pair of $tRNA^{Ala}$ as the major identity determinant. Since AlaRS does not 'read' the $tRNA^{Ala}$ anticodon [33], mutations to the $tRNA^{Ala}$ anticodons are likely to cause mistranslation. We identified a common G35C variant in the human $tRNA^{Ala}_{AGC}$ 6-1 gene ($tRNA^{Ala}_{ACC}$) that occurs in more than 6% of individuals. This mutation is expected to retain Ala accepting activity while the ACC anticodon will decode Gly instead of Ala codons.

Conversely, glycyl-tRNA synthetase (GlyRS) recognizes the $tRNA^{Gly}$ anticodon and normally requires the C35 and C36 bases of the anticodon, the A73 discriminator base, and the C2:G71 base pair in the acceptor stem for glycylation of the tRNA [47]. Thus, while $tRNA^{Gly}$ anticodon mutations are likely to lead to loss of function, mutations at other sites can create identity elements for other tRNAs in the sequence [32]. Some $tRNA^{Gly}$ sequences naturally have a U70 base, and we identified two different $tRNA^{Gly}$ variants with an A3G mutation. The A3G mutation produces a $tRNA^{Gly}$ with a G3:U70 base pair, an AlaRS identity element, and is expected to be an efficient Ala acceptor that retains its ability to decode Gly codons. The A3G mutation occurs commonly (>5% allele frequency) in the $tRNA^{Gly}_{CCC}$ 1–1 gene and in a smaller but still substantial population (~1% of individuals) in the $tRNA^{Gly}_{GCC}$ 1–5 gene (Table 1). Each of these $tRNA^{Ala}_{ACC}$ and $tRNA^{Gly}$ A3G variants, thus, have potential to cause Ala mis-incorporation at Gly codons.

Table 1: Naturally occurring tRNA variants characterized in this study

tRNA Gene [48]	**tRNA variant**	**codons read**	**AA mis-incorporated**	**allele frequency** [23]	**allele frequency** [24, 39]
Ala-AGC-6–1	G35C	Gly $GGU_{/C/A}$	Ala	8.5%	6.5%
Gly-CCC-1–1	A3G	Gly GGG	Ala	5.2%	n.d.
Gly-GCC-1–5	A3G	Gly $GGC_{/U}$	Ala	1.2%	1.2%
Ser-AGA-2–3	G35A	Phe $UUU_{/C}$	Ser	3.0%	1.8%

n.d. – not detected

3.2 Protein synthesis in mistranslating cells

Inhibited protein synthesis is a hallmark response to mistranslation in mammalian cells [31, 49, 50]. We hypothesized that the human $tRNA^{Gly}$ and $tRNA^{Ala}$ variants will cause mistranslation of Gly codons with Ala and lead to defects in protein synthesis. To test this hypothesis, mCherry was co-expressed as a marker for protein production from a plasmid also bearing a wild-type or mutant tRNA allele. We recently showed that the human $tRNA^{Ser}_{AGA}$ (Ser-AGA-2–3 gene) G35A variant ($tRNA^{Ser}_{AAA}$) caused serine mis-incorporation at phenylalanine codons and inhibited protein synthesis in N2a cells [31]. Here, we employed $tRNA^{Ser}_{AAA}$ derived from the Ser-AGA-2–5 gene as a positive control to cause greater levels of mistranslation in cells. We co-expressed mCherry with $tRNA^{Ser}_{AGA}$ or $tRNA^{Ser}_{AAA}$ in N2a cells and measured mCherry fluorescence (Fig. 2A,B). N2a cells expressing the mutant $tRNA^{Ser}_{AAA}$ showed a substantial and significant decrease in mCherry fluorescence per cell compared to cells expressing the wild-type $tRNA^{Ser}_{AGA}$. The data are in agreement with previous results from our lab [31], confirming a significant $tRNA^{Ser}_{AAA}$-dependent reduction in protein synthesis in N2a cells.

We used the same approach to evaluate each of the wild-type and variant $tRNA^{Gly}$ or $tRNA^{Ala}$ pairs (Table 1) for their impact on mCherry production. Thus, mCherry fluorescence per cell was recorded in cells expressing the wild-type tRNA ($tRNA^{Ala}_{AGC}$ or $tRNA^{Gly}_{G/CCC}$) or their respective variants ($tRNA^{Ala}_{ACC}$ or $tRNA^{Gly}_{G/CCC}$ A3G:U70) with

the potential to decode Gly codons with Ala. The variant $tRNA^{Ala}_{ACC}$, $tRNA^{Gly}_{GCC}$ A3G, and $tRNA^{Gly}_{CCC}$ A3G each showed no significant change in mCherry fluorescence compared to cells expressing the wild-type tRNA counterpart. Western blotting confirmed these observations (Fig. 2C,D). While $tRNA^{Ser}_{AAA}$ caused a significant decrease in mCherry protein levels, none of the $tRNA^{Ala}$ or $tRNA^{Gly}$ variants led to a significant change in mCherry protein levels.

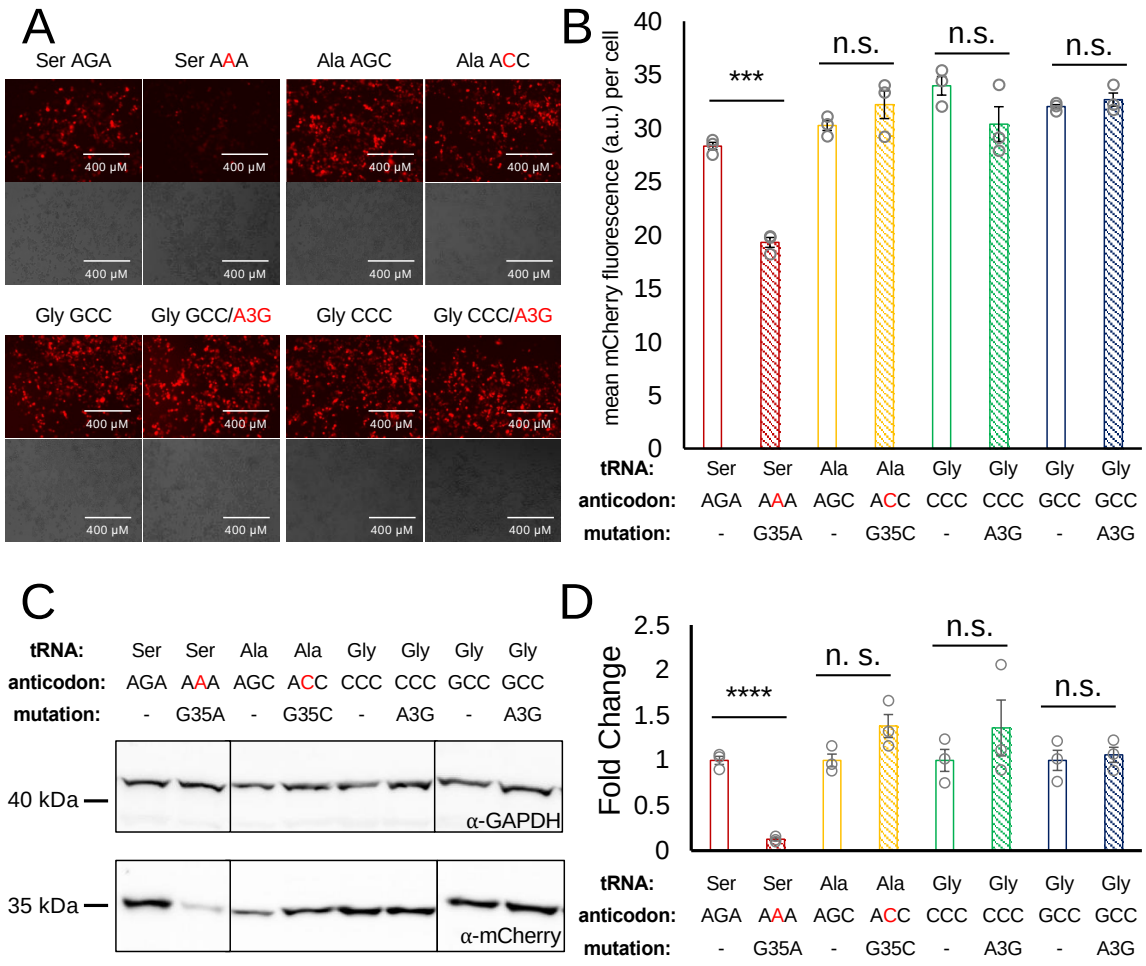

Figure 2: Protein synthesis levels in normal and mistranslating cells. (A) Brightfield and fluorescent images showing mCherry production in cells co-expressing wild-type or the indicated tRNA variants; (B) quantitation of mean mCherry fluorescence per cell. (C) Western blot of mCherry and a GAPDH loading control and (D) quantification of fold change of mCherry protein levels normalized by GAPDH. Independent sample t-tests were computed (n.s. – not significant, *** = $p \leq 0.001$, **** = $p \leq 0.0001$) based on at least N = 3 biological replicates.

3.3 Cytotoxicity in mistranslating N2a cells

Previous work established toxicity resulting from mistranslating tRNAs expressed in mammalian cells [49, 51], including from the $tRNA^{Ser}_{AAA}$ variant employed here [31] . In contrast, other mistranslating tRNAs elicit no cytotoxicity and are well tolerated in human cells [37, 49-51]. We confirmed a significant increase in cytotoxicity in cells expressing the mutant $tRNA^{Ser}_{AAA}$ compared to the wild-type tRNA allele ($tRNA^{Ser}_{AGA}$) in N2a cells (Fig. 3), while none of the $tRNA^{Ala}_{ACC}$ or $tRNA^{Gly}_{G/CCC}$ A3G:U70 variants caused a significant change in cytotoxicity compared with the wild-type tRNA allele (Fig. 3). Interestingly, cells expressing any of these wild-type tRNAs or their potential mistranslating variants showed relatively less cell death than cells expressing either of the wild-type or variant $tRNA^{Ser}$.

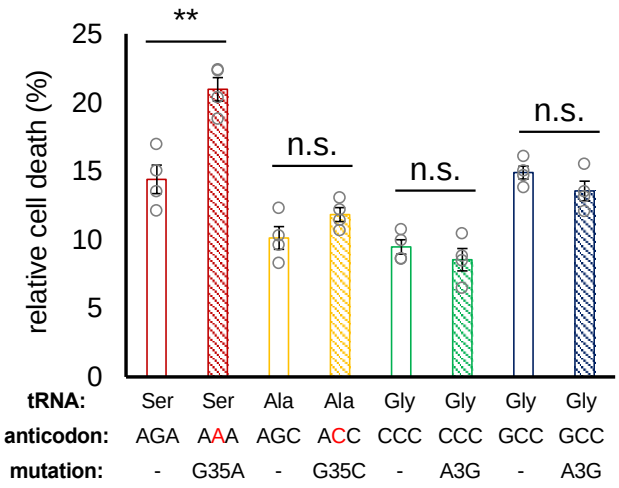

Figure 3: Cytotoxicity in N2a cells expressing wild-type or mistranslating tRNA variants. Quantification of cytotoxicity (Cytotox-Glo) observed in N2a cells expressing wild-type or the indicated tRNA variant. The $tRNA^{Ser}_{AGA}$ 2–5 gene G35A variant ($tRNA^{Ser}_{AAA}$) causes mis-incorporation of Ser at Phe codons [31] and significant toxicity in N2a cells. The $tRNA^{Ala}$ and $tRNA^{Gly}$ variants caused no significant changes in toxicity compared to cells expressing the wild-type counterpart. Independent sample t-tests were computed (n.s. – not significant, ** = $p \leq 0.01$) based on at least N = 3 biological replicates.

3.4 Mass spectrometry identification of Ala mistranslation at Gly codons

Although under normal conditions the $tRNA^{Ala}$ and $tRNA^{Gly}$ variants did not show detectible changes in protein synthesis or cytotoxicity, we used mass spectrometry to identify mis-incorporation in mCherry caused by the $tRNA^{Ala}_{ACC}$ variant and the $tRNA^{Gly}_{GCC}$ A3G mutant. As a control, mCherry was isolated from cells expressing wild-type $tRNA^{Ala}_{AGC}$. LC-MS/MS analysis of mCherry proteins purified from cells expressing potentially mis-translating tRNAs revealed abundant evidence of Ala mis-incorporation at multiple Gly codons.

In all transfected cells, we identified peptides indicating Ala mis-incorporation at G73, G107 and G108. The peptide hits at G73 may represent a fragmentation product of the cyclized red fluorophore, which is composed of residues M71, Y72, and G73. We also identified a consistent signal for mis-incorporation of Ala at positions G107 and G108 in all cells. Finally, in the wild-type tRNA-expressing cells, we found just one peptide each supporting mis-incorporation at positions G121 and G196 (Fig. 4A, S1, Table 2). Compared to peptides identified in cells expressing mutant tRNAs (Fig. 4, Table 2), peptide quality scores [52] were generally lower in the wild-type cell line (-10logP = 30 - 46), indicating greater uncertainty in the match of peptide to spectrum. Thus, these spectra may represent natural Ala mis-incorporation at Gly codons, a methylation at a nearby site, or a mis-identification of the peptide, which is also suggested by the low peptide quality scores.

Cells expressing the $tRNA^{Ala}$ or $tRNA^{Gly}$ mistranslating variants showed ample evidence of Ala mis-incorporation at multiple Gly codons in mCherry. Gly sites that showed a greater level of mis-incorporation (>1% ion intensity) were annotated with a small and red boxed ‘g’ symbol above the sequence (Fig. 4, C1-C3). Nearly all mistranslated peptide hits in the wild-type tRNA-transfected cells were less than 1% of the maximal ion intensity (Fig. C1, 4A,B), indicating a low background level of potential Ala mis-incorporation. In contrast, cells expressing the $tRNA^{Ala}$ (Fig. 4C,D, S2) or $tRNA^{Gly}$ (Fig. 4E,F, C3) variants demonstrated evidence of mis-incorporation at several different Gly residues, supported by multiple and high-quality peptide hits observed at >1% ion intensity. While we saw some evidence of mistranslation at 4 of the 25 Gly sites in wild-type cells, cells expressing the

$tRNA^{Ala}_{ACC}$ variant showed strong evidence of mistranslation at 7/25 Gly sites, and in cells expressing the $tRNA^{Gly}$ A3G variant we found Ala at 15/25 Gly residues (Fig. 4, C1-C3, Table 2). The peptide to spectra match quality scores were higher in cells expressing $tRNA^{Ala}$ and $tRNA^{Gly}$ variants compared to wild-type tRNA (Table 2). The higher ion intensity and higher quality scores of peptides from cells expressing the mutant tRNAs indicate confident identification of mistranslated peptides.

The mCherry construct contains nearly all GGC Gly codons and only one other Gly codon (GGU). In addition to ample evidence of mistranslation at GGC, we also observed Ala mis-incorporation at G56 (GGU). We detected peptides at G56, suggesting Ala mis-incorporation as a result of variant tRNA expression (Fig. C4). However, the ion intensities at these sites were less than 1% of maximal (Fig. C2, C3), suggesting relatively lower abundance of the G56A peptide. Cells expressing $tRNA^{Ala}_{ACC}$ showed two independent peptide hits that identify Ala at G56 (Fig. C2, C4A) albeit with a lower quality score (-10logP = 38.8). Cells expressing $tRNA^{Gly}_{GCC}$ A3G showed stronger evidence (-10logP = 58.5) of mis-incorporation at G56 (GGU) (Fig. S4B), and the same peptide indicated a double mis-incorporation of Ala at both G56 and G57 (Fig S4B).

Interestingly, mistranslating cells expressing the $tRNA^{Gly}_{GCC}$ A3G variant showed additional evidence of double (Fig. C5A,B) and triple mis-incorporation events in individual peptides from mCherry (Fig. C5C). In cells transfected with wild-type tRNA, we recorded only a single peptide with dual mis-incorporation of Ala at G107, G108, with ion intensity of less than 1%. Cells expressing $tRNA^{Gly}_{GCC}$ A3G produced peptides representing multiple dual mis-incorporation events at the same site (Fig. C3). We identified peptide hits displaying dual Ala mis-incorporation at G106, G164 (Fig. C5A) and at G175, G176 (Fig C5B).

Together the data demonstrate that both naturally occurring $tRNA^{Ala}_{ACC}$ and $tRNA^{Gly}_{GCC}$ A3G:U70 variants induce mistranslation of Ala at Gly codons. Thus, naturally occurring anticodon or identity element mutations are viable routes to amino acid mis-incorporation that is tolerated under normal conditions.

Table 2: Summary of Ala mis-incorporation at Gly codons identified by MS/MS

tRNA gene	tRNA variant	mCherry residue	maximal -10logP	ion counts Ala/Gly
Ala-AGC-6-1	WT	G107A	69.37	3 A / 7 G
	WT	G108A	64.26	3 A / 7 G
	WT	G121A	46.99	1 A / 14 G
	WT	G196A	35.12	1 A / 17 G
	G35C	G5A	55.78	2 A / 9 G
	G35C	G73A	67.90	5 A / 10 G
	G35C	G107A	63.70	3 A / 10 G
	G35C	G108A	69.02	3 A / 11 G
	G35C	G138A	48.41	1 A / 6 G
	G35C	G160A	42.33	1 A / 8 G
	G35C	G196A	58.42	2 A / 18 G
Gly-GCC-1-5	A3G	G5A	61.30	4 A / 11 G
	A3G	G29A	52.94	2 A / 16 G
	A3G	G95A	56.59	5 A / 19 G
	A3G	G107A	75.15	4 A / 15 G
	A3G	G108A	69.41	5 A / 15 G
	A3G	G121A	44.13	2 A / 29 G
	A3G	G138A	58.43	1 A / 17 G
	A3G	G147A	48.34	3 A / 7 G
	A3G	G160A	66.97	3 A / 18 G
	A3G	G164A	69.48	3 A / 15 G
	A3G	G175A	54.35	4 A / 18 G
	A3G	G176A	57.70	5 A / 18 G
	A3G	G196A	72.08	2 A / 28 G
	A3G	G224A	64.70	2 A / 3 G
	A3G	G229A	54.54	1 A / 8 G

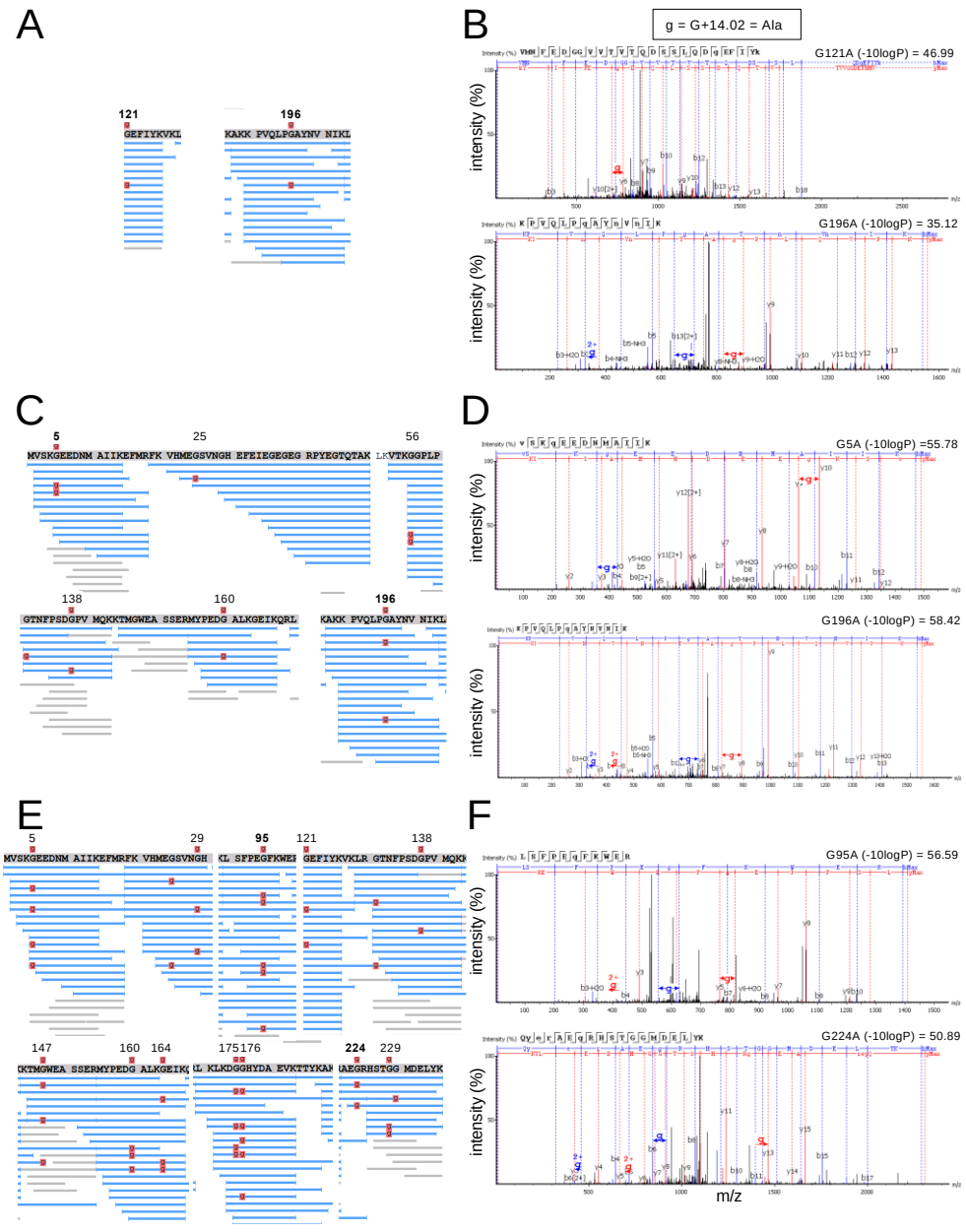

Figure 4: tRNA-dependent mis-incorporation of Ala at Gly codons detected by mass spectrometry. Tandem mass spectrometry analysis revealed alanine mis-incorporation in mCherry isolated from N2a cells expressing (A, B) the wild-type $tRNA^{Ala}_{AGC}$; (C, D) the $tRNA^{Ala}_{ACC}$ variant; and (E, F) the $tRNA^{Gly}_{GCC}$ A3G variant. Mis-incorporation events in mCherry (red boxed g = Gly + 14.02 = Ala) isolated from each cell line are depicted alongside (B, D, F) example spectra from the identified peptides. Red boxed 'g' symbols above peptide sequence annotate mistranslated residues identified with minimal ion intensities of ≥ 1%.

3.5 Protein synthesis in HEK 293T cells expressing wild-type or mistranslating tRNAs

Previous studies suggest that tRNA gene expression profiles show both cell-type and tissue-specific dependence [53]. Thus, we reasoned that differential expression of a particular mistranslating tRNA variant, or of other tRNAs that the mutant tRNA competes with for aminoacylation or decoding, may change the impact of the tRNA variant in different cell lines. To test this, we assayed protein synthesis in HEK 293T cells expressing wild-type or mistranslating tRNA variants. The mCherry fluorescence per cell in HEK 293T cells revealed an expected defect in protein synthesis in cells expressing the $tRNA^{Ser}_{AAA}$ variant (Fig. 5A,5C). In comparison to wild-type tRNA alleles, however, the $tRNA^{Ala}_{ACC}$ or $tRNA^{Gly}_{G/CCC}$ A3G:U70 variants showed no change in mCherry fluorescence per cell in HEK 293T cells (Fig. 5A,5C), similar to our observations in N2a cells described above.

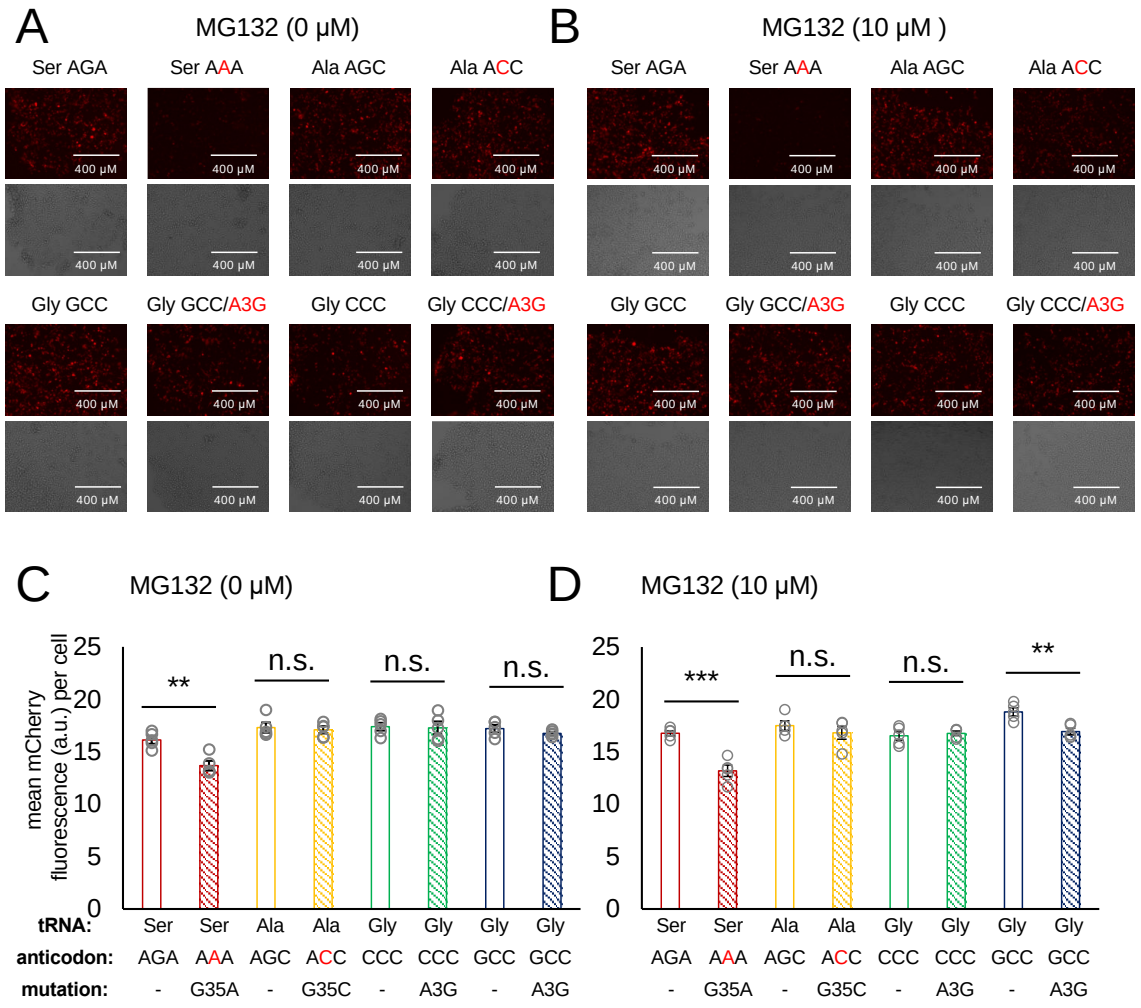

Figure 5: Proteasome inhibition reveals defective protein synthesis in cells expressing tRNA$^{Gly}_{GCC}$ A3G. Brightfield and fluorescence images showing mCherry fluorescence in cells expressing wild-type or mistranslating tRNA variants in (A) 0 μM or (B) 10 μM MG132. Quantitation of mean fluorescence per cell was plotted for cells treated with (C) 0 μM or (D) 10 μM MG132. Independent sample t-tests were computed (n.s. – not significant, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$) based on at least N = 3 biological replicates.

3.6 Cell-specific cytotoxicity of tRNA-dependent mistranslation

In N2a cells expressing $tRNA^{Ser}_{AAA}$, we observed significant cytotoxicity induced by Ser mis-incorporation at Phe codons (Fig. 3). In contrast, we found no significant change in toxicity in HEK 293T cells expressing $tRNA^{Ser}_{AGA}$ versus $tRNA^{Ser}_{AAA}$ (Fig. 6A). Repression of protein synthesis in HEK 293T cells expressing $tRNA^{Ser}_{AAA}$ (Fig 5A,C) suggests that mistranslation occurs at a level that does not increase cell death (Fig. 6A). The data indicate that the level of tRNA-dependent mistranslation or its associated phenotypic defects may be cell specific. For each of the $tRNA^{Ala}$ and $tRNA^{Gly}$ variants, we recorded no changes in cytotoxicity in HEK 293T cells (Fig. 6A), similar to our findings in N2a cells above.

3.7 Mistranslation in the context of proteasome inhibition

Since mass spectrometry confirmed Ala mis-incorporation is caused by natural human $tRNA^{Ala}$ and $tRNA^{Gly}$ variants, we hypothesized that additional stress on the cell could reveal a phenotypic defect associated with Ala mis-incorporation at Gly codons. MG132 is a potent proteasome inhibitor [54] that we used previously to measure defects in protein synthesis and protein degradation in cells expressing the mistranslating $tRNA^{Ser}_{AAA}$ variant [31]. We anticipated that inhibition of a major protein degradation pathway would cause mis-made and mis-folded proteins to accumulate and disrupt protein homeostasis in mistranslating cells.

HEK 293T cells expressing wild-type or mistranslating tRNAs were exposed to MG132 and mCherry fluorescence was measured as a marker for protein synthesis (Fig 5B,D). Cytotoxicity was monitored to identify toxic genetic interactions between proteosome inhibition and mistranslation (Fig. 6). In the presence of 10 μM MG132, both $tRNA^{Ser}_{AAA}$ and $tRNA^{Gly}_{GCC}$ A3G variants lead to significantly decreased fluorescence of mCherry per cell compared to cells expressing their wild-type tRNA counterparts. Interestingly, the $tRNA^{Gly}_{GCC}$ A3G and not the $tRNA^{Gly}_{CCC}$ A3G variant caused a reduction of protein synthesis in the context of proteasome inhibition (Fig. 5D). The $tRNA^{Ala}_{ACC}$ variant also showed no further evidence of disrupted protein homeostasis in cells treated with MG132

(Fig. 5B,D). Both the Phe-decoding $tRNA^{Ser}_{AAA}$ and the Ala mis-incorporating $tRNA^{Gly}_{GCC}$ A3G showed significant inhibition of protein synthesis under proteasome inhibition compared their wild-type tRNA counterparts.

Application of MG132 increased cell death in HEK 293T cells with each tRNA tested (Fig. 6A,B). However, we observed no apparent increase in cytotoxicity in cells expressing wild-type versus mistranslating $tRNA^{Ala}$ or $tRNA^{Gly}$ variants in the context of proteasome inhibition. Even $tRNA^{Gly}_{GCC}$ A3G, which showed defective protein synthesis in MG132 treated cells, was well tolerated in terms of cell toxicity. Interestingly, we observed a small but significant reduction in cytotoxicity in HEK 293T cells expressing $tRNA^{Ser}_{AAA}$ variant compared to the wild-type $tRNA^{Ser}_{AGA}$ under proteasome inhibition (Fig. 6B). This finding contrasts the situation in N2a cells, which showed a strong synthetic toxic interaction between $tRNA^{Ser}_{AAA}$ expression and proteasome inhibition [31]. These data again indicate cell-specific phenotypic defects associated with different mistranslating tRNAs.

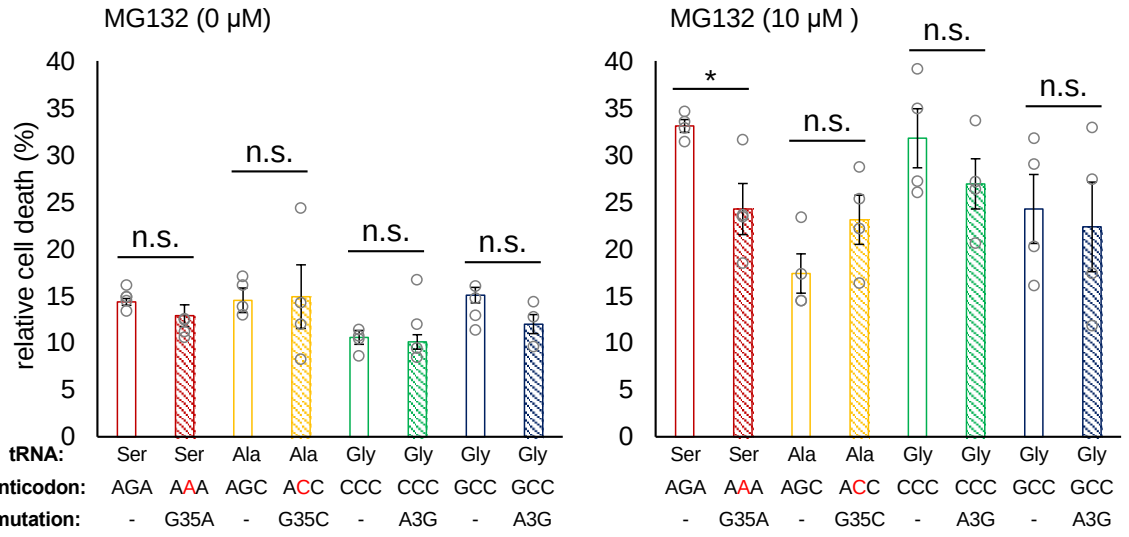

Figure 6: Proteasome inhibition reveals reduced toxicity from Ser mis-incorporation at Phe codons but not from Ala mis-incorporation at Gly codons in HEK 293T cells. Quantification of cytotoxicity (Cytotox-Glo) observed in HEK 293T cells in (A) standard conditions or (B) following treatment with 10 µM MG132 proteasome inhibitor. Independent sample t-tests were computed (n.s. – not significant, * = $p \leq 0.05$) based on at least N = 3 biological replicates.

3.8 Protein synthesis and aggregation in mistranslating cells expressing ALS-associated FUS protein

To assay the effects tRNA variants can have on protein aggregation associated with disease, N2a cells were transfected with plasmids containing genes for either $tRNA^{Ser}_{AGA}$, or the $tRNA^{Ser}_{AAA}$ variant alongside mCherry, FUS-mCherry, or R521C FUS-mCherry. Cells were grown for 72-hours to ensure aggregate development before cell harvest and lysis. Western blot analysis revealed a decrease in protein production resulting from $tRNA^{Ser}$ G35A mutant expression in both wild-type and mutant-FUS expressing cells, compared to wild-type $tRNA^{Ser}$ expression in a similar context (Fig. 7A). Semi-denaturing gel electrophoresis (SDD-AGE) was performed to determine the amount of protein aggregated in different conditions. The wild-type $tRNA^{Ser}$ did not affect the amount of wild-type or R521C mutated FUS aggregation, but $tRNA^{Ser}_{AAA}$ significantly reduced the amount of protein that was aggregated. The mutant FUS R521C was significantly more aggregated in mistranslating cells compared to the wild-type FUS allele when expressed alongside the mistranslating $tRNA^{Ser}_{AAA}$.

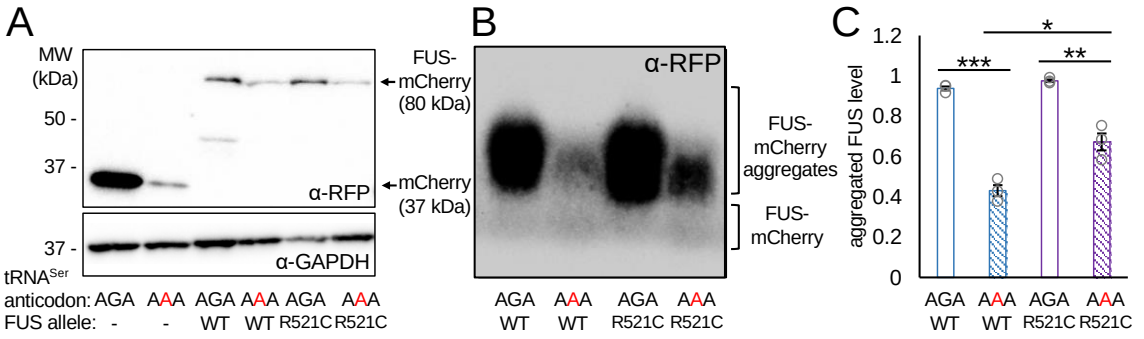

Figure 7: Analysis of total and insoluble FUS aggregates in wild-type and mistranslating cells. Lysates from N2a cells transfected with a plasmid encoding human $tRNA^{Ser}_{AGA}$ or $tRNA^{Ser}_{AAA}$ variant and mCherry, FUS-mCherry or FUS R521C-mCherry were separated on (A) SDS-PAGE or (B) SDD-AGE gels and blotted using α-mCherry antibody. (C) The percentage of aggregated protein in SDD-AGE blots was determined by densitometry. Independent sample t-tests were computed (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$) based on N = 3 biological replicates.

Chapter 4

4 Discussion

4.1 Mistranslation from natural $tRNA^{Ala}$ and $tRNA^{Gly}$ variants in human cells

The human genome contains a diverse and large family of tRNAs, encoding more than 600 tRNA genes [39]. Although some tRNA genes may be pseudogenes, estimates indicate at least 400 tRNA genes are 'high confidence' or likely functional tRNAs [55]. Evidence from human serum samples suggests expression of 411 different tRNA genes, and the human liver alone expresses at least 224 different tRNA genes [56]. A recent approach based on experimental data and sequence properties of tRNA genomic loci predicted that at least 314 tRNAs are functional and expressed [57]. Different human cells and tissues show differential regulation of tRNA genes [53, 58, 59], which include tRNAs for each proteogenic amino acid and many copies of each tRNA isoacceptor. Even tRNA isodecoders that share the same anti-codon are found in multiple copies. For example, the human $tRNA^{Gly}$ genes include 28 high confidence genes, using one of three anticodon sequences (5'-G/C/UCC-3') to decode four (5'-GGN-3') glycine codons. The human tRNAome contrasts those of yeast (275 tRNAs) and *E. coli* (88 tRNAs), which have much smaller genomic copy numbers of tRNAs. Human tRNAs are also more diverse than their counterparts in yeast [24]: the diversity of human tRNA genes is not confined to the differences between tRNA isodecoders in a single genome, but rather each individual harbours between 60 to 70 single nucleotide polymorphisms in their tRNA genes compared to the reference genome [23].

Some of these variants produce tRNAs that are likely to misread the genetic code and cause mistranslation that affects the entire proteome. While some mistranslating tRNA variants are found in exceeding rarity (1 to 2 examples per 100,000 genomes), other tRNAs that cause or may cause mistranslation are found as common variants in the population, with mean allele frequencies in excess of 5%. We previously characterized [31] and here provided additional data regarding a $tRNA^{Ser}_{AGA}$ G35A variant that mis-incorporates Ser at Phe codons. The $tRNA^{Ser}_{AAA}$ variant occurs in 2-3% of the population [23, 24], yet some

potential tRNA mistranslators are also found in up to 8.5% of the population, such as the $tRNA^{Ala}$ and $tRNA^{Gly}$ variants we characterized here (Table 1). These variants represent two different mechanisms by which a tRNA can gain function to mis-incorporate amino acids. In the case of $tRNA^{Ala}$, the anticodon mutation is ignored by AlaRS, and the resultant Ala-$tRNA^{Ala}_{ACC}$ decodes Gly codons. In contrast, the $tRNA^{Gly}$ A3G variants contain an AlaRS identity element, G3:U70 [33], which leads to Ala-$tRNA^{Gly}$ synthesis and decoding of Gly codons with Ala. Our observations here, together with our previous study of a human $tRNA^{Pro}$ G3:U70 variant [37], demonstrate that AlaRS can misaminoacylate different kinds of tRNA variants that create a G3:U70 base pair and cause Ala misincorporation in mammalian cells.

A recent study [60] focused on a similar kind of naturally occurring tRNA variant that creates a G4:U69 base pair, which is sufficient to confer Ala-accepting activity [61], in different tRNAs, including threonine (Thr) tRNAs. Although human AlaRS produces Ala-$tRNA^{Thr}$ when the G4:U69 variant is present, the threonyl-tRNA synthetase (ThrRS) found in mammalian cells and murine tissues possesses deacylation activity towards the mischarged Ala-$tRNA^{Thr}$, apparently preventing mistranslation [60]. We can confirm that no similar activity is found in mammalian (N2a) cells to proofread Ala mischarging of tRNAs that read Gly codons. Overall, our findings demonstrate that common tRNA variants can cause mistranslation of a kind or a level that cells are able to tolerate under normal conditions.

4.2 Amino acid similarity may alleviate mistranslation toxicity in cells

Here I compared a $tRNA^{Ser}$ variant that mistranslates Phe codons and a series of tRNA variants that misread Gly codons with Ala to their respective wild-type tRNAs. In terms of their chemical similarity in solution or in the context of a folded protein, Gly and Ala are much more similar amino acids than Phe and Ser. In agreement with this, I observed significant cytotoxicity only in cells expressing the Phe-decoding $tRNA^{Ser}$ variant, while the $tRNA^{Ala}$ and $tRNA^{Gly}$ variants lead to no changes in cytotoxicity, despite showing strong evidence of amino acid misincorporation.

Several examples in the literature support the idea that the similarity between an intended versus a mis-incorporated amino acid is an important factor impacting the degree and severity of cellular response to mistranslation, including resulting phenotypic defects in mistranslating cells. In yeast, $tRNA^{Ser}$ variants were assayed for the ability to misread Arg codons, along with characterization of $tRNA^{Pro}$ variants that misread Pro codons with Ala [62]. Ser mistranslation at Arg codons induced production of heat shock proteins and showed a greater decrease in growth rate compared to yeast cells that substituted Ala at Pro codons. Thus, the nature or similarity of mis-incorporated amino acids will impact the cellular response and the level of toxicity associated with mismade proteins.

Previous work in mammalian cells addressed the similarity of ambiguously encoded amino acids in the context of Ser misincorporation. A series of chimeric or synthetic $tRNA^{Ser}$ variants with several different anticodons were expressed in mammalian cells. Each variant caused Ser misincorporation at codons for several different amino acids [49]. Key evidence on the impact of mistranslation was found by surveying GFP production and fluorescence in HEK 293T cells, similar to our approach using mCherry as a marker of protein synthesis. Cells that mis-incorporated Ser at Lys codons showed only a minimal defect, while Ser mis-incorporation at Ile codons displayed the greatest defect in protein synthesis. In terms of severity of the phenotypic defect, cells mistranslating Ser for a group of amino acids showed increasing reductions in protein synthesis (Lys < Gln < Asn < Arg < Tyr < Pro < Glu < His < Asp) between 95% and 60% of wild-type levels. A $tRNA^{Ser}$ with an Ile anticodon reduced GFP levels to 20% of that in cells expressing a wild-type $tRNA^{Ser}$. We found that the $tRNA^{Ser}_{AAA}$ variant reduced mCherry levels per cell to 70% or 84% of that in N2a or HEK 293T cells expressing the wild-type tRNA, respectively. Under normal conditions, $tRNA^{Ala}$ and $tRNA^{Gly}$ variants had no effect on mCherry levels, while proteasome inhibition in HEK 293T cells reduced mCherry levels in cells expressing $tRNA^{Gly}_{GCC}$ A3G to 85% of that in cells expressing the wild-type $tRNA^{Gly}$. Thus, these data suggest that Ala misincorporation at Gly codons is less disruptive to protein homeostasis than Ser misincorporation at Phe codons.

The rate or level of mistranslation can also contribute to toxicity or growth defects associated with mistranslation [45, 63]. Two synthetic tRNA variants, one that causes more

frequent errors with Ser misincorporation at Ala codons and another that causes less frequent Ser mistranslation at Leu codons, were characterized in murine NIH3T3 cells [51]. Although both tRNA variants were well tolerated in cells, the Ser-tRNA^{Ala} mistranslator stimulated the protein kinase B (AKT) cell survival pathway and increased the growth rate of tumor cells injected into mice. The lower frequency mistranslator and less conservative Leu to Ser substitution was indistinguishable from a control lacking additional tRNA.

Another factor that can influence the level of mistranslation is related to how many codons are effectively read by a mistranslating tRNA. The tRNA$^{Ala}_{ACC}$ variant characterized here contains an A34, which is normally converted to inosine (I34) [64]. The I34 base is capable of superwobble and can read U, C, and A codons [65]. The fact that this tRNA decodes multiple GGC codons as Ala suggests the I34 modification is intact. The mCherry construct contains only one other Gly codon (GGU), and I found evidence that both tRNA^{Ala} and the tRNA^{Gly} variants direct Ala misincorporation at G56 of mCherry (GGU) (Fig. C4A). In on-going studies, we are working towards quantitative methods relying on mistranslation-sensitive fluorescent reporters [45] and absolute quantitation approaches with mass-spectrometry [66] to accurately establish the level of Ala misincorporation at Gly codons. In our future work, these approaches will also allow us to quantify the level of Ala misincorporation at each of the four Gly codons.

4.3 Cell viability and protein homeostasis despite low fidelity translation

We are only at the beginning of understanding human tRNA variants and their ability to cause mistranslation or impact health and disease [24]. Indeed, the relative abundance of some tRNA variants in the population, such as the common tRNA^{Gly} and tRNA^{Ala} variants characterized here, suggests that some mistakes in protein synthesis are less harmful than others and that our cells are robustly insensitive to significantly elevated levels of error in protein synthesis. Initial proposals, including Crick's frozen accident [67] and Orgel's error catastrophe hypothesis [68], envisioned the proteome as a fragile entity that is intolerant to errors. Crick's theory considered changes to standard genetic code or ambiguous decoding of codons as a phenotype that would most certainly be selected against during evolution. Since that time, many changes to the genetic code have been identified in natural species,

and changes to codon assignments most often require evolution of tRNA variants [69]. Indeed, the yeast *Candida albicans* decodes CUG codons ambiguously as Ser (97%) and Leu (3%) as a result of an atypical $tRNA^{Ser}_{CAG}$ that is recognized by both leucyl-tRNA synthetase (LeuRS) and SerRS [70].

Orgel's theory [68] suggests that low fidelity translation would be fatal to cells. This error catastrophe theory proposes that protein synthesis machinery produced from a low fidelity system would increasingly accumulate errors to the point where the protein components of the translation apparatus themselves would no longer function. Thus, low fidelity translation would lead to the inability to produce the proteins needed to support life. In cells expressing a natural $tRNA^{Ser}_{AAA}$ variant, mistranslation can be toxic [31] or, in the extreme limit, high levels of misincorporation can inhibit cell viability [71, 72]. However, cells from the many diverse forms of life maintain viability even in the context of dramatically increased levels of amino acid misincorporation [37, 73]. In *E. coli*, several missense suppression systems, including those that substitute Cys at Pro codons, Ser at Thr codons, Glu at Gln codons, and Asp at Asn codons, all showed negligible impacts on cell growth. Protease deficient *E. coli* strains, however, showed strong and differential sensitivity to each kind of mistranslation [74], demonstrating the importance of a protein quality control mechanism to mitigate the impact of low fidelity translation. In other contexts, including under conditions of oxidative or chemical stress, amino acid misincorporation can be beneficial or provide a selective advantage to bacterial [75], yeast [73], and mammalian cells [76]. In the case of Ala misincorporation at Gly codons in human cells, my data affirm the robust nature cells have to prevent mistakes in protein synthesis.

4.4 Protein aggregation in mistranslating cells

To assay the effects of mistranslation on protein aggregation, I expressed the $tRNA^{Ser}_{AAA}$ variant alongside ALS-causing R521C mutant FUS protein in N2a cells. $tRNA^{Ser}_{AAA}$ was previously reported to increase aggregation of a non-aggregating HTT protein and decrease the aggregation of mutant HTT protein [31]. Cells expressing the $tRNA^{Ser}_{AAA}$ variant were also defective in degradation of HTT protein aggregates following treatment with a detergent to resolve the aggregates [31]. Here we found the mistranslating $tRNA^{Ser}_{AAA}$

decreased the aggregation of both wild-type and R521C mutant FUS protein. These results are consistent with the overall decrease in protein synthesis observed in mistranslating cells caused by $tRNA^{Ser}_{AAA}$ expression shown previously [31]. The combination of misfolded protein aggregation and mistranslation is likely overwhelming protein turnover machinery in cells, therefore stalling translation altogether, in an attempt to clear the aggregates. It was further previously reported that mistranslating cells have slowed protein turnover rates and stalled ribosomal translation [31]. Stalled translation and defective protein degradation contributes to the increased toxicity and decreased protein production witnessed in mistranslating cells, regardless of protein aggregation.

Future studies will elucidate the mechanisms leading to decreased protein synthesis and increased cytotoxicity between mistranslating tRNAs and protein aggregation associated with ALS. We will next determine the rate of mistranslation, examine ribosome stalling events, and assay for other pathways of protein turnover activation (autophagy).

4.5 Impacts of mistranslation on mCherry fluorescence

Three-dimensional folded protein structure is likely predetermined by the primary amino acid sequence of a protein since each amino acid has different chemical properties that influence their placement within a protein [77]. For example, Ser contains a polar hydroxyl group, meaning it can interact with water molecules within the cell and is therefore often found on the outer surface a folded protein. In contrast, Phe is almost always found within the core of a protein due to its hydrophobic nature and aromatic ring structure. Thus, in general, polar amino acids are often situated on the surface of proteins to facilitate interactions with polar environment, while non-polar amino acids tend to be folded tightly towards the inside of proteins to ensure protein stability [78]. Due to the structural and functional differences between the amino acids, mistranslation of Phe to Ser caused by $tRNA^{Ser}_{AAA}$ resulted in the dimming of mCherry that was observed (Fig. 2B), likely caused by impaired chromophore maturation or decreased tightening of the protein core [79]. Furthermore, mistranslation causes slowed protein production [49, 50], meaning that expression of the $tRNA^{Ser}_{AAA}$ variant led to the decrease in protein levels observed (Fig. 2 C).

In contrast to Phe to Ser mistranslation events, the impact of Gly to Ala mistranslation was not severe. The chromophore of mCherry is made up of three residues – methionine, tyrosine, and glycine (Fig. D1 C). Although Gly is part of the chromophore, mass spectrometry did not reveal strong evidence to conclude mistranslation at Gly 73. Further, the population of mCherry that had a Gly73Ala mistranslation did not suffer functionally, as no decrease in fluorescence was observed when mistranslating tRNAs were expressed.

The lack of a side chain on Gly allows for high flexibility of the residue, leading to complete 360-degree range of motion, where other amino acids are more rigid in structure. The flexibility of Gly plays a role in creation of turning segments of protein beta-sheet secondary structure and is a large part of fluorescent proteins that exhibit a beta barrel 3-D conformation (Fig. D1 A,B) [80]. Gly is also found within some enzyme active sites, allowing the enzyme to change conformation more easily when unbound vs. bound to its substrate [81]. Mistranslation of Gly will always place a larger and potentially bulkier amino acid in its place as it is the smallest amino acid. These substitutions may hinder a protein's ability to fold and function correctly due to the loss of flexibility associated with other amino acids. However, we observed no impact of Gly mistranslation within mCherry, likely due to the conservative nature of the amino acid misincorporation (Gly to Ala). Ala only differs to Gly by one additional methyl group. The difference between side chains is relatively minor, does not prompt any new bond formations within the protein, and, as far as we could tell, did not hinder the ability of mCherry to fold and function as normal.

Overall, some amino acid substitutions are not as harmful as others when examining protein function (Gly to Ala vs. Phe to Ser), allowing the protein to function as if it were the wild-type protein. Future studies will look to achieve a more global understanding of how Gly to Ala mutations may affect the cell by examining glycine-rich cellular proteins such as collagen through a scratch assay. If Gly to Ala mistranslation alters collagen function, this would allow us to determine any impacts of that mistranslation by studying the cellular migration. As well, mass spectrometry of total cellular proteins as opposed to solely mCherry may reveal a more accurate level of mistranslation occurring globally in the cell as a result of variant tRNA expression.

5 Conclusion

5.1 Glycine to alanine mistranslation is not toxic in mammalian cells

I found that naturally occurring human $tRNA^{Ala}$ anticodon and $tRNA^{Gly}$ identity element variants are both viable routes to Ala misincorporation at Gly codons. These variants occur commonly in the human population, and while they cause mistranslation verified by mass spectrometry, their impact on cells under normal conditions is minimal. Thus, human cells are robust with respect to Ala misincorporation at Gly codons, suggesting that tolerance to mistranslated proteins allows for the propagation of these variants in the population. One mutant showed defective protein synthesis only in the context of inhibited protein degradation, suggesting that the accumulation of mis-made protein caused defects in protein synthesis. Our data demonstrate natural tRNAs that mistranslate Gly codons with Ala have the potential to impact cell health under conditions of stress.

5.2 Mistranslation can modify protein aggregation

I examined the effects a mistranslating $tRNA^{Ser}$ can have on protein aggregation in cells using a model of ALS caused by a mutant FUS protein. I found that expression of $tRNA^{Ser}_{AAA}$ significantly increased the amount of aggregation of the ALS-causing R521C FUS mutant compared to the wild-type protein, but reduced global aggregation compared to expression of the wild-type $tRNA^{Ser}_{AGA}$. As well, we reported that co-expression of the mistranslating tRNA and disease-causing FUS allele reduced protein synthesis and aggregation, while increasing toxicity (Lant et al, under review). We demonstrated the ability of a mistranslating tRNA to exacerbate disease phenotypes in mammalian cells. Future work will look to elucidate the mechanism of action between the mistranslating tRNA and the aggregating protein in order to better understand the synergistic effects seen in this study.

5.3 Mistranslation impacts proteostasis

Mistranslation of Phe to Ser caused by $tRNA^{Ser}_{AAA}$ altered protein levels within the cell by slowing protein synthesis, and reduced mCherry fluorescence due to improper

chromophore maturation. As well, a decrease in protein turnover caused by $tRNA^{Ser}_{AAA}$ mistranslation was reported in a previous study [31]. Although it is possible that mistranslation of all Gly residues with Ala could have resulted in similar fluorescence dimming of mCherry, our model for tRNA-dependent mistranslation was not strong enough to induce complete mistranslation within the protein. Thus, we concluded that the Gly to Ala mistranslation observed did not impact mCherry function and was not toxic to the cells.

Despite deleterious effects on protein fluorescence and cell toxicity observed in this study, mistranslation in cells is not avoidable, but can be beneficial under certain circumstances. Under conditions of oxidative stress, mammalian cells have been observed to increase mis-aminoacylation of Met onto non-cognate tRNAs by MetRS [82]. Methionine incorporation in proteins aids in the oxidative stress response as Met has antioxidant properties due to its redox ability [83]. As well, mistranslation within cells under stress conditions provides an avenue for sampling different functional protein conformations. By way of incorporating incorrect amino acids into proteins, the cell may improve protein stability, modify protein function, or strengthen stress responses in an attempt to diversify the proteome to function outside of the wild-type environment [84, 85].

5.4 Significance

We now know that humans have a greater number of tRNA gene variants in their individual genomes than previously recognized [23, 24, 39], some of which have the ability to cause protein mistranslation, yet they remain largely uncharacterized. It is important to study mistranslating tRNA variants as they lay the foundation for appropriate translation of proteins and maintenance of cellular function. tRNAs are essential to the translation of proteins, and high rates of mistranslation have the potential to be deleterious and modify disease severity. Understanding tRNA variants is important for determining their role in health and disease, as well as opening avenues for tRNAs as therapeutics for nonsense [86] and eventually missense suppression.

5.5 Future Directions

Future experiments will elucidate the effects of tRNA-dependent mistranslation on protein degradation through a cycloheximide chase assay while protein synthesis rates will be measured by a puromycin incorporation assay. As well, future work for this project will examine the impact of Ala mistranslation at Gly codons on mammalian cells in the context of an aggregating protein model of ALS and HD under normal and stress conditions. Further, this mistranslation could serve as a potential rescue for diseases caused by Ala to Gly misincorporation. Finally, I will be using CRISPR/Cas9 editing to create a stable mistranslating cell line that will allow for more effective examination of mistranslation in a native context, providing an avenue for assaying native cellular stress responses to tRNA-dependent mistranslation in mammalian cells. Otherwise, cell samples from individuals that naturally have these mistranslating tRNA variant genes in their genomes may also be acquired from the Coriell Institute Biobank. The cell samples will be assayed for the presence of the tRNA genes by genomic PCR amplification and the impacts expression of the variant tRNAs have in a true native context.

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Appendices

Appendix A: Plasmids

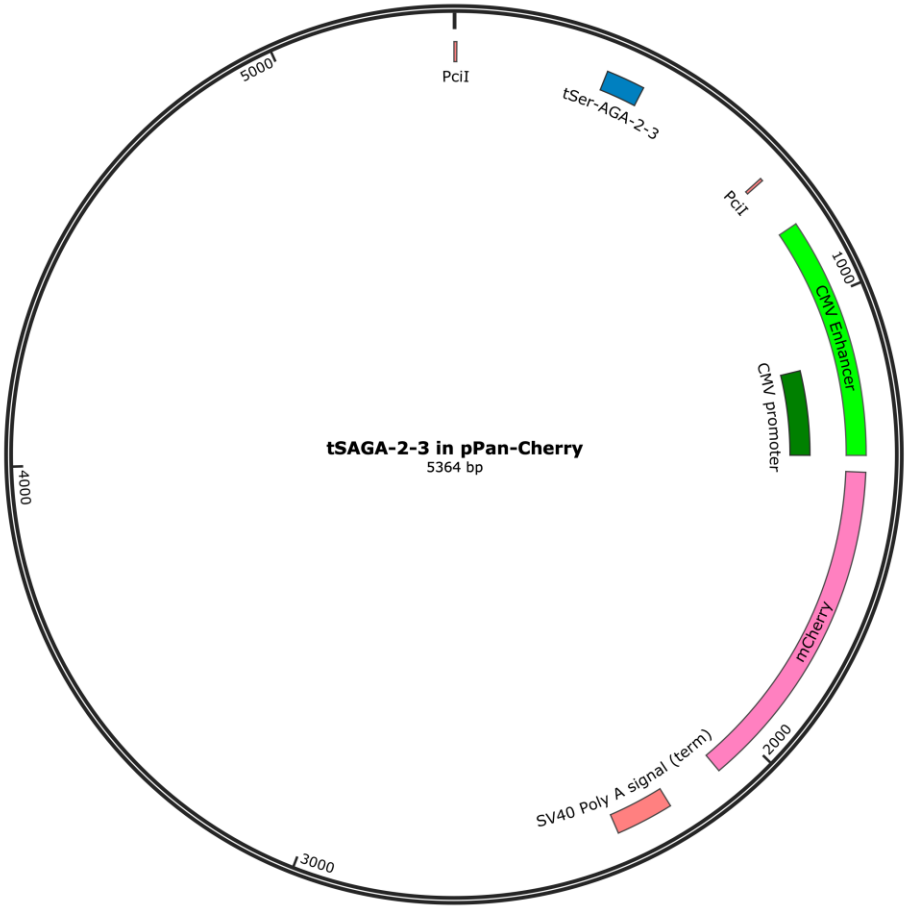

Figure A1: pPan-Cherry plasmid map. Depiction of the pPan-Cherry plasmid used in this study. $tRNA^{Ser}_{AGA}$ is shown between two *PciI* restriction sites where tRNA genes were cloned into the plasmid. tRNA genes were cloned to include ~300 bp of up- and downstream sequence to ensure transcription factor binding sequences and internal promoters were conserved for appropriate transcription by RNA polymerase III.

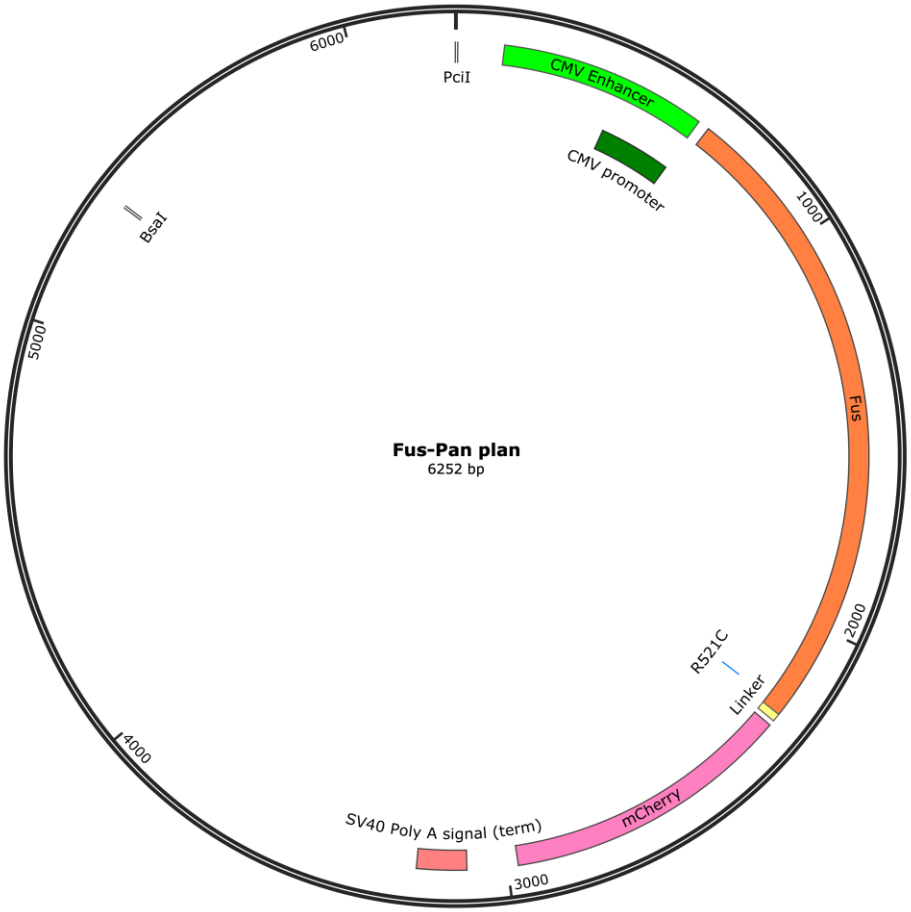

Figure A2: FUS plasmid map. Map of plasmid used in this study to express FUS fused to mCherry protein for visualization using the EVOS microscope. Wild-type or G35A mutant $tRNA^{Ser}$ were cloned into the plasmid using the *PciI* restriction site depicted. Location of the R521C mutation in FUS is highlighted.

Appendix B: Primers

Table B1: List of primers used to construct tRNA variants

tRNA gene	Primer Name	Sequence (5'-3')	Experimental Use
$tRNA^{Ala}_{AGC}$ 6–1	F-tA-AGC-6-1-Rnd1	AAATCAGTTATTTCAATGGGG	Genomic PCR of 500 bp up- and downstream of tRNA sequence
	R-tA-AGC-6-1-Rnd1	ACTATGTGGGTCTCTAATGG	
	F-tA-AGC-6-1-Rnd2-*Pci*I	CAGACT**ACATGT**AGAGAATGTTGTATGCCAGG	PCR to add the correct restriction site to ends of sequence for plasmid ligation
	R-tA-AGC-6-1-Rnd2-*Pci*I	CAGACT**ACATGT**TTAGTGATTGGTGGACTGC	
	F-tA-AGC-6-1-G35C	TGCTTACCATGCACGAGGTCCTG	SOE PCR to generate mutant tRNA variant
	R-tA-AGC-6-1-G35C	CGTGCATGGTAAGCACGCGCTCTAC	
$tRNA^{Gly}_{CCC}$ 1–1	F-tG-CCC-1-1-Rnd1	AAAAGACTAACTTAGTGGACC	Genomic PCR of 500 bp up- and downstream of tRNA sequence
	R-tG-CCC-1-1-Rnd1	TCTCCACGCAGATTCTTGC	
	F-tG-CCC-1-1-Rnd2-*Pci*I	CAGACT**ACATGT**GTTGACGATGGAAGATTTAGG	PCR to add the correct restriction site to ends of sequence for plasmid ligation
	R-tG-CCC-1-1-Rnd2-*Pci*I	CAGACT**ACATGT**GTTCTTCGGGTTTGCAGG	
	F-tG-CCC-1-1-A3G	CAGAGGCGTTGGTGGTTCAGTGGTAG	SOE PCR to generate mutant tRNA variant
	R-tG-CCC-1-1-A3G	CCACCAACGCCTCTGCGTAACAACC	
$tRNA^{Gly}_{GCC}$ 1–5	F-tG-GCC-1-5-Rnd1	TAGCAATACTTCAAGTGACC	Genomic PCR of 500 bp up- and downstream of tRNA sequence
	R-tG-GCC-1-5-Rnd1	TAAAGCAAGGTCCTCAGGC	
	F-tG-GCC-1-5-Rnd2-*Nco*I	CAGACT**CCATGG**GCAAACTCCAGATGGCTG	PCR to add the correct restriction site to ends of sequence for plasmid ligation
	R-tG-GCC-1-5-Rnd2-*Nco*I	CAGACT**CCATGG**AGTAGCTGGGACTACAGGC	
	F-tG-GCC-1-5-A3G	CGGTGCGTGGGTGGTTCAGTGG	SOE PCR to generate mutant tRNA variant
	R-tG-GCC-1-5-A3G	CACCCACGCACCGATAGAAGAGTTG	

X – mutated residue

X (bold) – restriction enzyme cut site

Appendix C: Mass Spectrometry

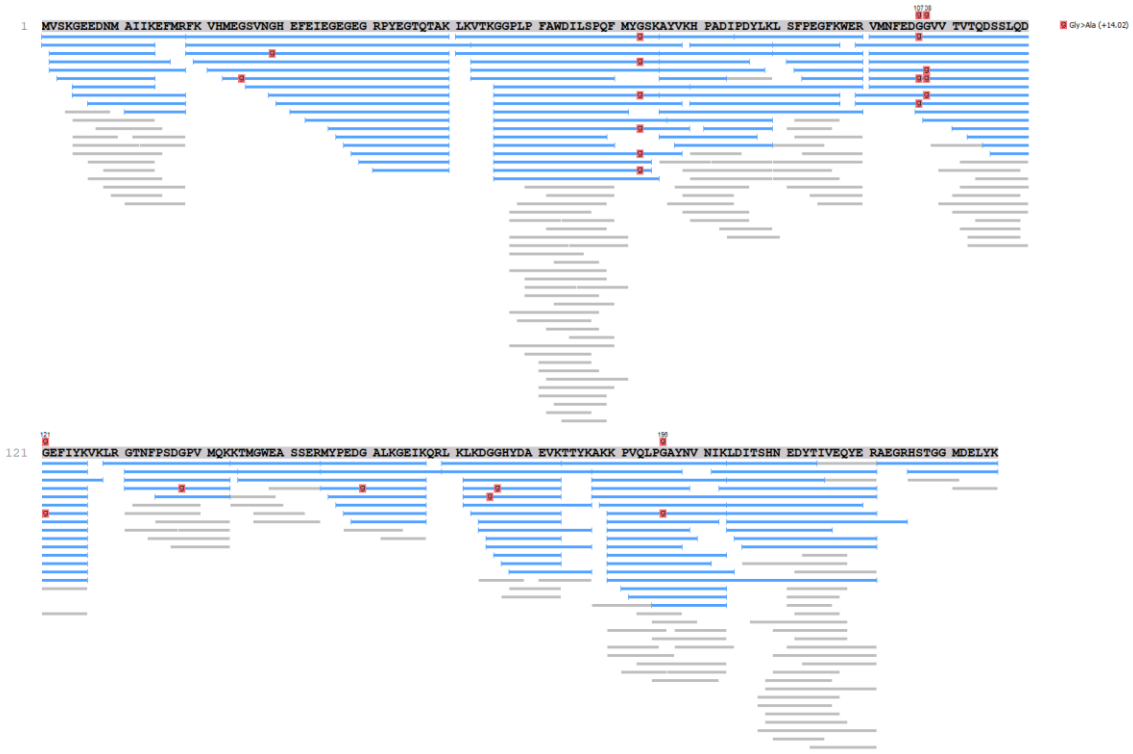

Figure C1: Peptide coverage map of mCherry produced in and isolated from cells expressing wild-type tRNA$^{Ala}_{AGC}$. MS/MS spectra supporting Ala mis-incorporation at Gly codons in mCherry were identified using PEAKS software [52] as a modified Gly (lower case g, Gly + 14.02 = Ala). Red boxes around lower case "g" symbols indicate the location of glycine to alanine mistranslation in the identified peptides. Red boxed 'g' symbols above peptide sequence annotate mistranslated residues identified with minimal ion intensities of ≥ 1%.

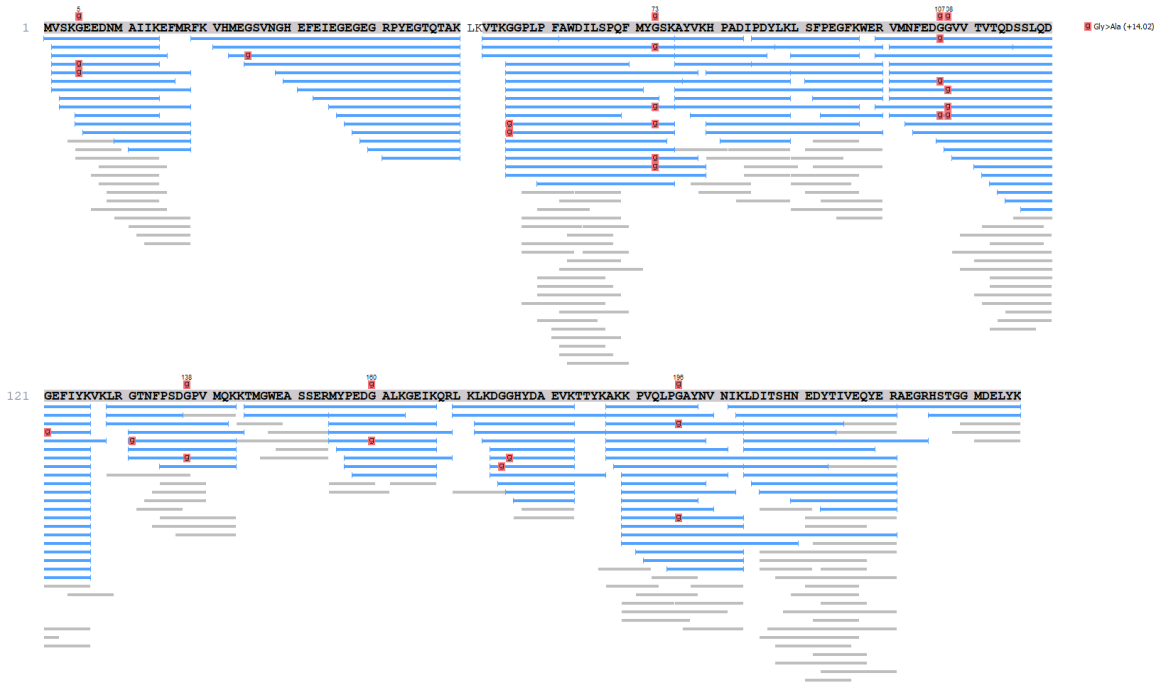

Figure C2: Peptide coverage map of mCherry produced in and isolated from cells expressing the $tRNA^{Ala}_{AGC}$ G35C variant. MS/MS spectra supporting Ala mis-incorporation at Gly codons in mCherry were identified using PEAKS software [52] as a modified Gly (lower case g, Gly + 14.02 = Ala). Red boxes around lower case "g" symbols indicate the location of glycine to alanine mistranslation in the identified peptides. Red boxed 'g' symbols above peptide sequence annotate mistranslated residues identified with minimal ion intensities of ≥ 1%.

Figure C3: Peptide coverage map of mCherry produced in and isolated from cells expressing the $tRNA^{Gly}_{GCC}$ A3G mutant. MS/MS spectra supporting Ala mis-incorporation at Gly codons in mCherry were identified using PEAKS software [52] as a modified Gly (lower case g, Gly + 14.02 = Ala). Red boxes around lower case "g" symbols indicate the location of glycine to alanine mistranslation in the identified peptides. Red boxed 'g' symbols above peptide sequence annotate mistranslated residues identified with minimal ion intensities of ≥ 1%.

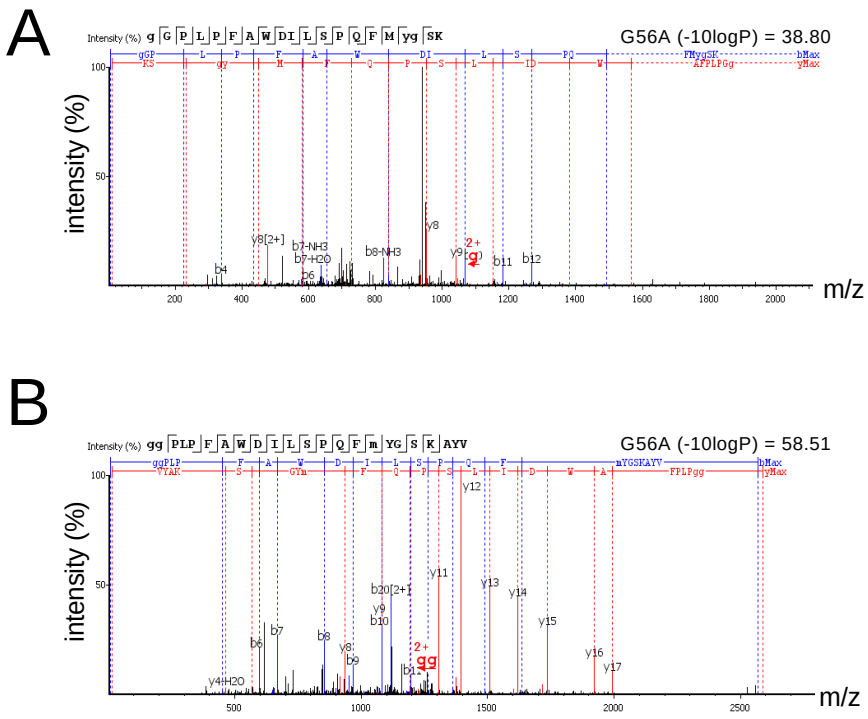

Figure C4: Tandem mass spectra documenting Ala mis-incorporation at Gly56 (GGU). MS/MS spectra for peptides demonstrating Ala misincorporation at Gly56 (GGU) in mCherry isolated from (A) N2a cells expressing mutant $tRNA^{Ala}_{AGC}$ G35C or from (B) cells expressing mutant $tRNA^{Gly}_{GCC}$ A3:G70.

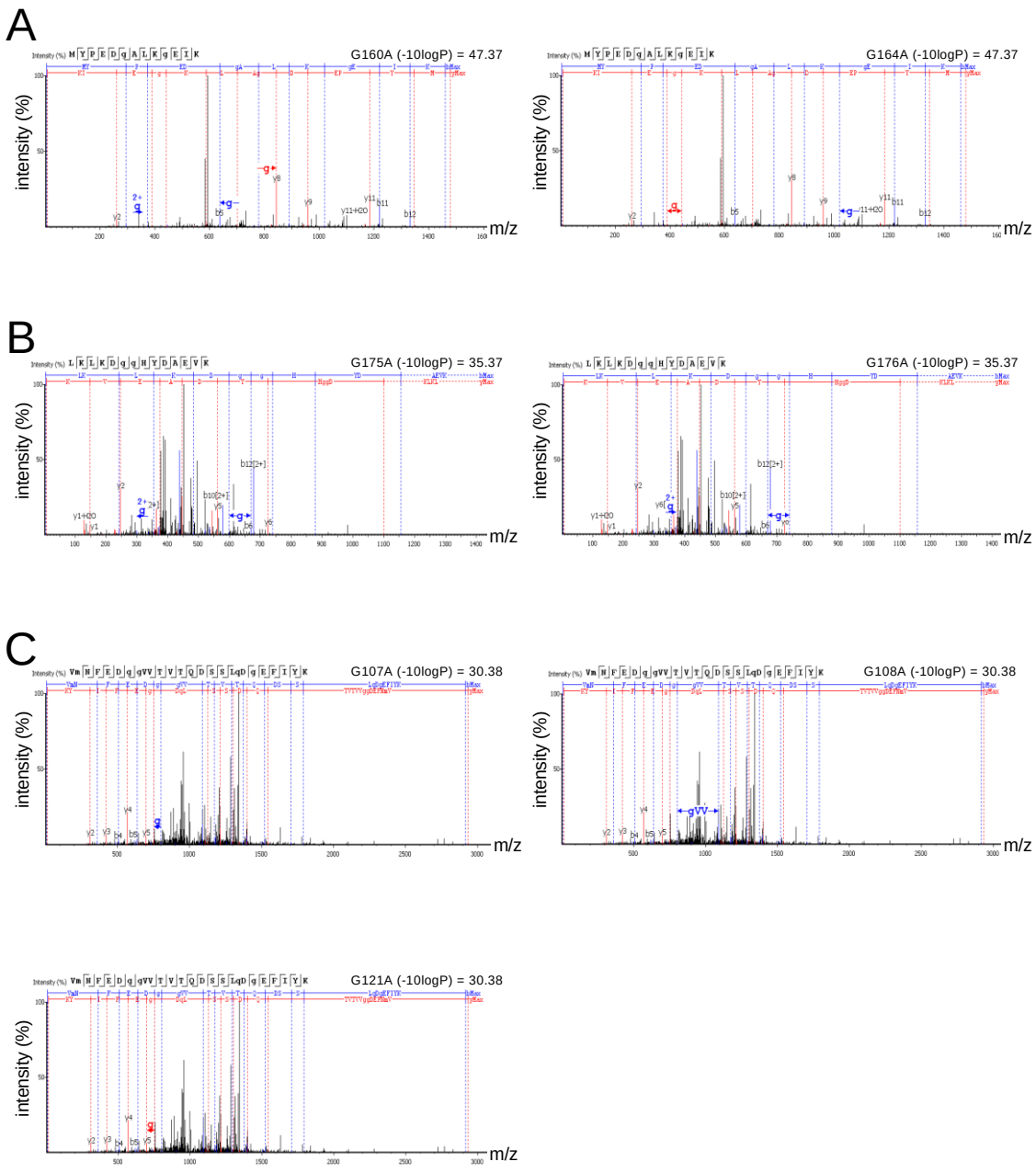

Figure C5: Tandem mass spectra of double and triple mistranslated Gly codons in individual peptides. The spectra show double or triple Ala misincorporation events at Gly residues in the same mCherry peptide isolated from N2a cells expressing the mistranslating $tRNA^{Gly}_{GCC}$ A3G:U70 mutant. The spectra support double mis-incorporation at (A) Gly residues 160 and 164 and (B) Gly residues 175 and 176. Triple mis-incorporation was identified in a peptide containing Ala at (C) Gly residues 107, 108, and 121.

Appendix D: Structure of mCherry

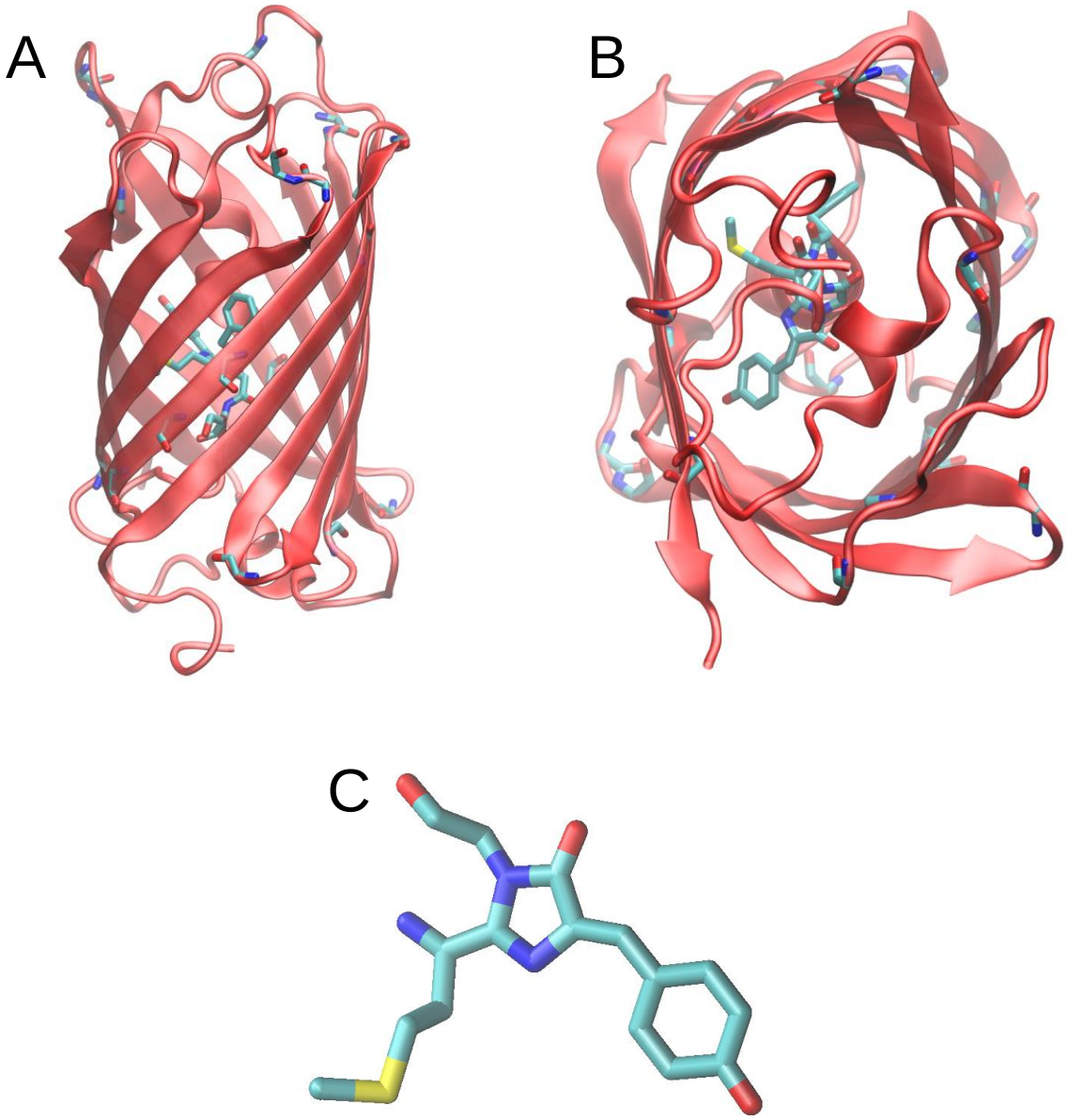

Figure D1: Protein structure of mCherry and its chromophore. 3D depiction of the protein structure of mCherry from the side (A) and from the top (B). All glycine residues are shown in both views of the structure. The mCherry chromophore (C) is depicted with the 3 residues listed. Carbon atoms are shown in light blue, oxygen atoms are shown in red, nitrogen atoms are shown in dark blue, and sulphur atoms are shown in yellow. The image was made using VMD [87]; mCherry PDB ID: 2H5Q [88].

Curriculum Vitae

Name:	Farah Hasan
Post-secondary Education and Degrees:	The University of Western Ontario, London, ON, Canada 2020–2022 MSc. Department of Biochemistry Supervisor: Patrick O'Donoghue The University of Western Ontario, London, ON, Canada 2016–2020 BSc. Honours Specialization in Genetics and Biochemistry
Honours and Awards:	Western Graduate Research Scholarship 2020–2022 Undergraduate Dean's Honour List 2018–2020 Western Scholarship of Excellence (Entrance Scholarship) 2016
Relevant Work Experience:	Teaching Assistant: Biology 1002B – Biology for Science II Molecular Biology The University of Western Ontario, London, ON, Canada Winter 2022 Teaching Assistant: Biochemistry 2280A – Biochemistry and Molecular Biology The University of Western Ontario, London, ON, Canada Fall 2021 Undergraduate Research Participant Agriculture and Agri-Food Canada, London, ON, Canada 2019–2020

Publications:

F. Hasan, J. T. Lant, P. O'Donoghue. (2022). Perseverance of protein homeostasis despite mistranslation of glycine codons with alanine. *Phil. Trans. R. Soc. B* (under review).

J. T. Lant, **F. Hasan**, D. McDonald, J. Briggs, M. Duennwald, P. O'Donoghue. (2022). Amyotrophic later sclerosis-associated variant causes a protein aggregation catastrophe in mistranslating cells. *Proc. Natl. Acad. Sci. USA* (2022–07814, under review).

In Preparation

J. T. Lant, **F. Hasan**, P. O'Donoghue. Local genetic sequence context alters the potency of identical human tRNA variants.