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# Determining the Effects of Mistranslating Transfer RNA Variants on Drosophila Melanogaster

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# Abstract

Transfer RNAs (tRNAs) play a central role in translation as adaptor molecules between mRNA and protein. Variant tRNAs can cause the misincorporation of an amino acid into a growing polypeptide. Mistranslating tRNA variants are surprisingly common in humans but the effects of mistranslating tRNA variants on eukaryotic biology are poorly understood. My thesis aimed to create a model of tRNA-induced mistranslation using the fruit fly *Drosophila melanogaster* and characterize the effects of mistranslating tRNA variants on eukaryotic biology.

I first integrated a gene encoding a serine tRNA variant that induced proline-to-serine (P→S) mistranslation into the fly genome. Proteins isolated from lines containing the mistranslating tRNA variant contained significantly more P→S substitutions than control lines. Flies containing the mistranslating tRNA variant presented with extended development, developmental lethality, more anatomical deformities, and impaired climbing performance compared to control flies. Interestingly, females presented with more deformities than males and experienced a more rapid decline in performance as they aged, indicating that females may be more susceptible to mistranslation.

To identify cellular pathways that may be affected by mistranslation, I next performed RNA-sequencing on flies containing the P→S mistranslating tRNA variant. This tRNA variant caused both sexes to downregulate metabolic genes and upregulate genes associated with gametogenesis. Males downregulated genes associated with development whereas females downregulated aerobic respiration. Females upregulated genes associated with DNA maintenance and the cell cycle, indicating that mistranslation may affect female genome integrity.

Finally, I tested whether different types of mistranslation have different effects on flies. I integrated genes encoding two new mistranslating serine tRNA variants, one that substitutes serine for valine (V→S) and another that substitutes serine for threonine (T→S). Both tRNA variants cause mistranslation at significantly higher levels than control flies. I observed extended development, developmental lethality, and increased

prevalence of anatomical deformities similar to flies containing the mistranslating P→S variants. Surprisingly, both mistranslating tRNA variants extended female lifespan and improved climbing ability in both sexes. My work shows that mistranslation has multifaceted and sex-specific effects on flies. The model I created will aid investigations into how mistranslating tRNA variants impact fitness and disease.

## Keywords

Mistranslation, tRNA, translation fidelity, RNA-sequencing, development, longevity, stress response, *Drosophila melanogaster*, aging, neurodegeneration, proteostasis

# Summary for Lay Audience

Translation describes the process by which cells produce proteins from expressed genes. Translation requires special molecules known as transfer RNAs (tRNAs) to convert the genetic code into proteins. Because of their central role in translation, if tRNA genes are mutated, then these variant tRNAs can cause proteins to be synthesized incorrectly. These translation errors are known as mistranslation.

Variant tRNAs are common in humans and associated with several diseases, but there is currently no model to study the effects of mistranslating tRNA variants in multicellular organisms. The goal of my thesis was to create a multicellular model of tRNA-induced mistranslation in the fruit fly and determine what effects mistranslating tRNA variants have on fly biology. The fruit fly is an ideal model for this research as it has a nervous system similar to humans, short generation times, and many available genetic tools.

I began by integrating a mistranslating tRNA variant into flies and characterizing its effects. This tRNA variant extended development time, increased developmental lethality, and caused anatomical deformities in adults such as missing wings or legs. Fly climbing ability, a common proxy for neurological function, was also decreased in flies containing the mistranslating tRNA variant. I was surprised to find that females were more strongly affected by mistranslation, indicating that females are particularly susceptible to translation errors. The mistranslating tRNA variant also affected gene expression of flies, causing metabolic genes to be expressed less and genes associated with reproductive processed to be expressed more, though males and females also showed different gene expression profiles.

I also tested two new mistranslating tRNA variants that induce different translation errors to see if they have different effects. These two tRNA variants replicated several of my original results, but both variants increased female lifespan and improved male and female climbing performance. My results demonstrate that I have successfully created a multicellular model of tRNA-induced mistranslation and that mistranslation has

both positive and negative effects on fly biology. Variant tRNAs are surprisingly common in humans, and the model I created represents a powerful tool to study their effect on human health.

# Co-Authorship Statement

Chapter 1 was written by Joshua Isaacson and edited by Dr. Chris Brandl and Dr. Amanda Moehring.

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> JRI created the fly lines, performed fly work, analysed data, created the figures, and wrote the manuscript. MDB performed the mass spectrometry on fly pupae and provided the data for Figure 2-1. BC and JJ helped collect data for the developmental assay in Figure 2-2. JV, CJB, and AJM edited the manuscript and provided guidance on how to conduct the experiments.

Chapter 3: JRI collected flies and extracted RNA. JRI also created figures and wrote the manuscript. MDB helped validate and analyse RNA-sequencing data in RStudio. WY extracted RNA from flies and performed qPCR. JB, CJB, and AJM edited the manuscript and provided guidance.

Chapter 4: JRI created the fly lines, performed fly work, analysed data, created the figures, and wrote the manuscript. BC designed primers used to create $tRNA^{Ser}$ variants. MDB performed mass spectrometry on fly pupae and provided the data for Figure 4-1. JJ collected data for the developmental assay represented in figures 4-2 and 4-3. JV, CJB, and AJM edited the manuscript and provided guidance.

Chapter 5 was written by JRI and edited by Dr. Chris Brandl and Dr. Amanda Moehring.

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# List of Abbreviations

| | |
|---|---|
| aaRS | Aminoacyl-tRNA synthetase |
| ADP | Adenosine diphosphate |
| AlaRS | Alanyl-tRNA synthetase |
| A-Site | Aminoacyl site |
| AspRS | Aspartyl-tRNA Synthetase |
| ATP | Adenosine triphosphate |
| BHB | Bulge-helix-bulge |
| bp | Base pair |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| CMT | Charcot-Marie-Tooth |
| CMT2D | Charcot-Marie-Tooth Disease type 2D |
| CMT2W | Charcot-Marie-Tooth Disease type 2W |
| CNS | Central nervous system |
| DNA | Deoxyribonucleic acid |
| eEF1A | Eukaryotic translation elongation factor 1A |
| eIF1 | Eukaryotic initiation factor 1 |
| eIF1A | Eukaryotic initiation factor 1A |
| eIF2 | Eukaryotic initiation factor 2 |

| | |
|---|---|
| eIF3 | Eukaryotic initiation factor 3 |
| eIF3J | Eukaryotic initiation factor 3J |
| eRF1 | Eukaryotic release factor 1 |
| eRF3 | Eukaryotic release factor 3 |
| E-Site | Exit site |
| FRT | Flippase recognition target |
| G418 | Geneticin |
| GDP | Guanosine diphosphate |
| GluRS | Glutaminyl-tRNA synthetase |
| GlyRS | Glycyl-tRNA synthetase |
| GO | Gene ontology |
| GTP | Guanosine triphosphate |
| HeLa cells | Henrietta Lacks cells |
| HisRS | Histidyl-tRNA synthetase |
| IRES | Internal ribosome entry site |
| LBSL | Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation |
| $m^5C$ | 5-methylcytosine |
| MELAS | Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes |
| MERRF | Myoclonic epilepsy with ragged red fibers |

| | |
|---|---|
| mRNA | Messenger ribonucleic acid |
| MS | Mass spectrometry |
| mt-tRNA | Mitochondrial transfer RNA |
| *mw* | *miniwhite* |
| NaCl | Sodium Chloride |
| nt | Nucleotide |
| PC | Principal component |
| PCA | Principal component analysis |
| PCR | Polymerase chain reaction |
| PheRS | Phenylalanyl-tRNA synthetase |
| PIC | Pre-initiation complex |
| ProRS | Prolyl-tRNA synthetase |
| P-site | Peptidyl site |
| PTC | Peptidyl transferase centre |
| RFP | Red fluorescent protein |
| RNA | Ribonucleic acid |
| rRNA | Ribosomal ribonucleic acid |
| SerRS | Seryl-tRNA synthetase |
| tRNA | Transfer ribonucleic acid |

# Chapter 1

# 1 General Introduction

Genetic information is encoded in DNA and must undergo transcription and translation to be converted into protein products. Translation of messenger RNA (mRNA) to protein requires the use of transfer RNA (tRNA) adaptor molecules (reviewed in Berg and Brandl 2021). These molecules base pair to mRNA at the ribosome in continuous three-nucleotide segments known as codons and provide a corresponding amino acid to the growing polypeptide. While tRNAs are best known for their role in translation, they participate in other biological processes such as stress response and RNA interference (reviewed in Mohler and Ibba 2017; Samhita *et al.* 2020; reviewed in Yu *et al.* 2021). Despite how crucial tRNAs are for a cell to function, the effects of tRNA variants are poorly understood due to challenging aspects of their molecular biology.

Existing research on tRNA variants shows that they can cause disease phenotypes in a variety of organisms. Ectopically expressed mutant tRNAs cause developmental deformities in zebrafish (Reverendo *et al.* 2014) and promote tumor growth in mouse cell lines (Santos *et al.* 2018). Two human diseases, MELAS and MERRF, are caused by mitochondrial tRNA variants (Goto *et al.* 1990; Shoffner *et al.* 1990). tRNA variants are particularly important to study given recent findings that humans have ~66 tRNA variants per individual with several of those variants predicted to cause mistranslation (Berg *et al.* 2019a).

Mistranslation is the misincorporation of an amino acid not specified by the genetic code. High levels of mistranslation induce widespread amino acid substitutions and interfere with proteostasis. However, mistranslation is also sometimes beneficial as it can suppress deleterious phenotypes or help organisms withstand stressful environmental conditions (Capecchi and Gussin 1965; Engelhardt *et al.* 1965; Chiu and Morris 1997; Murakami *et al.* 2005; Lee *et al.* 2014). Mistranslating tRNA variants are also able to lead to genetic code reassignment within an organism over time, which describes a change where a codon calls for a different amino acid than it did previously (Kawaguchi *et al.* 1989; Santos *et al.* 1993, 2011; Berg *et al.* 2017; Correia *et al.* 2023). Given the diverse

effects of mistranslation in cells and the prevalence of putative mistranslating tRNAs in humans, it is important to understand how mistranslation affects complex eukaryotes.

My work builds upon research done in the Brandl lab where they engineered serine and alanine tRNAs that recognize proline codons in yeast, thus inserting serine or alanine where there should be proline (Berg *et al.* 2017; Hoffman *et al.* 2017). These mistranslating tRNAs slowed cell growth and activated the heat-shock response, but their effects in multicellular eukaryotes were unknown. To address this, I adapted these tRNAs for use in the fruit fly *Drosophila melanogaster*. The goal of my research was to generate a multicellular model of mistranslation in *Drosophila*, understand if mistranslating tRNAs affect the development and viability of multicellular eukaryotes, and identify the pathways by which they do so. I found that proline to serine mistranslation caused developmental issues, deformities, and impaired climbing ability (Isaacson *et al.* 2022). I then analysed the proteome and transcriptome of mistranslating fly lines to identify cellular pathways that respond to mistranslation. Finally, I created new types of mistranslating serine tRNAs to determine if different kinds of mistranslation have different effects. These experiments further our understanding of how tRNA variants capable of mistranslation affect complex eukaryotes, contribute to genetic diversity and the evolution of the genetic code, and help inform how mutant tRNAs may contribute to disease.

## 1.1 Structure of tRNAs

tRNAs are relatively short RNA molecules ranging from around 70 to 100 nucleotides in length (Krahn *et al.* 2020). Mature tRNAs contain extensive internal base-pairing that produces several stem-loop motifs, giving rise to their characteristic cloverleaf secondary structure when sketched in two-dimensions (Holley *et al.* 1965). The different sections of the tRNA cloverleaf are named, from 5’ to 3’, the acceptor stem, the D-arm, the anticodon arm, the variable loop, and the TΨC-arm (Figure 1-1A). The 5’ end of the tRNA base-pairs with the 3’ end to form the acceptor stem. The acceptor stem contains the unpaired 3’ CCA trinucleotide motif which is where an amino acid is attached to the 3’ end of the tRNA by an aminoacyl tRNA synthetase (aaRS) in a process known as aminoacylation or charging (Pang *et al.* 2014). The D-arm is a stem-loop

structure containing a characteristic dihydrouridine base, which contributes to tRNA flexibility and stability (Dalluge *et al.* 1996; Dyubankova *et al.* 2015). The anticodon arm contains the anticodon, a three-base region at positions 34–36 of the tRNA that base-pairs with a mRNA codon during translation to incorporate the cognate amino acid into the growing polypeptide chain (Loveland *et al.* 2017). The anticodon is also frequently used as an identity element to ensure accurate charging of the tRNA with its cognate amino acid by an aaRS (reviewed in Giegé and Eriani 2023). The variable loop, as the name suggests, is a stem-loop structure of variable size depending on the tRNA species. In eukaryotic cells, most tRNAs have a short variable loop, but some tRNA species such as $tRNA^{Ser}$, $tRNA^{Sec}$, and $tRNA^{Leu}$ have an extended variable loop (Brennan and Sundaralingam 1976). The TΨC arm (T-arm) contains a conserved thymidine-pseudouridine-cytidine sequence (Robertus *et al.* 1974) and helps mediate ribosomal interactions (Nissen *et al.* 1995). While these features are common to most tRNAs, there exist functional tRNAs that lack some elements of the "canonical" tRNA. A mammalian mitochondrial (mt) $tRNA^{Ser}$ lacks the D-arm (Arcari and Brownlee 1980; de Bruijn *et al.* 1980), several mt-tRNAs in *Caenorhabditis elegans* lack either the D-arm or T-arm (Okimoto and Wolstenholme 1990), and a mt-$tRNA^{Ile}$ in the roundworm *Romanomermis culicivorax* lacks both the D- and T-arms and is a mere 45 nts long (Wende *et al.* 2014).

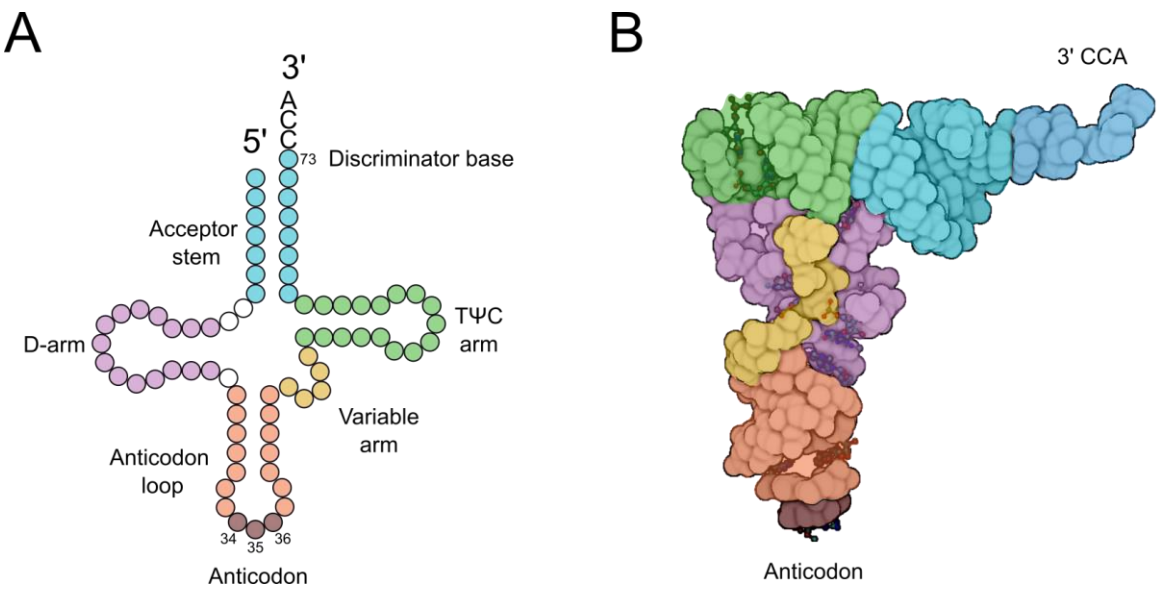


**Figure 1-1.** *Structure of a tRNA molecule in two and three dimensions.*

**A)** Representation of a tRNA in its characteristic 2D clover-leaf structure. Starting at the 5' end, there is the acceptor stem (blue), D-arm (purple), anticodon loop (orange), the anticodon at positions 34–36 (brown), variable arm (yellow), TΨC-arm (green), the discriminator base at position 73, and the universal 3' CCA motif. **B)** Representation of a tRNA in three dimensions, where it folds to form an L-shape. Colours represent the same structural feature as shown in **A)**. 3D structure of tRNA's was produced using BioRender.

Most tRNAs form an L-shape in three dimensions, with the acceptor stem and T-arm stacking into the short arm of the "L", and the D-arm and anticodon arm stacking into the long arm (Figure 1B, Rich and RajBhandary 1976; Hou 1993). Conserved nucleotides within the D-arm, T-arm, and variable loop are required to stabilize and maintain the "L" shape (Rich and Schimmel 1977). tRNAs that contain an extended variable loop, such as $tRNA^{Ser}$, maintain the overall "L" shape by having their extended variable loop pointing at a 30° angle away from the plane formed by the stacked tRNA arms (Brennan and Sundaralingam 1976). The "L" shape of tRNAs is highly conserved across organisms but it is not universally adopted (reviewed in Krahn *et al.* 2020). The 45nt mt-$tRNA^{Ile}$ in *R. culicivorax* replaces its absent D- and T-arms with extended single-stranded regions of RNA, and these regions cause the tRNA to adopt a boomerang-like shape in three dimensions (Jühling *et al.* 2018). Some proteins involved in translation adopt a tRNA-like shape, such as eukaryotic release factor 1 (eRF1), as this allows the protein to enter the ribosome to terminate translation (Song *et al.* 2000). Some viruses employ tRNA mimicry to guide translation of their genes as their genomes contain an internal ribosome entry site (IRES) whose three-dimensional shape resembles tRNAs, allowing for efficient entry into the ribosome (Kieft 2008; Ren *et al.* 2012).

How tRNAs evolved to adopt their conserved L-shape is an ongoing debate (Di Giulio 2019; Lei and Burton 2020; Burton 2020). A minimalistic *E. coli* $tRNA_{Leu}$ that lacks both the anticodon and variable loop can still be efficiently aminoacylated by LeuRS, and a CCase can add the 3' CCA to a tDNA sequence consisting of only the acceptor stem and T-arm (Shi *et al.* 1998; Larkin *et al.* 2002), suggesting that primordial

tRNAs likely lacked several structures common in contemporary mature tRNA transcripts. The existence of permuted and split tRNA genes suggests that tRNAs did not evolve as one complete gene. Rather, it seems more likely that early tRNAs were composed of two or three separate gene fragments that hybridized to form the complete tRNA. Permuted tRNAs, such as those observed in the red alga *Cyanidioschyzon merolae*, have the 3' fragment of the gene upstream of the 5' fragment (Soma *et al.* 2007). The 3', intervening sequence, and 5' sequence are then transcribed as one unit and processed by RNase P, RNase Z, and tRNA splicing machinery into a mature, functional tRNA. Split tRNAs have been observed in archaea such as *Nanoarchaeum equitans* and *Caldivirga maquilingensis* and would provide additional variability as the 5' and 3' gene fragments could be combined in different ways to produce new tRNA species (Randau *et al.* 2005; Randau and Söll 2008; Fujishima *et al.* 2009; Fujishima and Kanai 2014). The hypothesis that split-tRNAs represent the ancestral form of tRNAs is further supported by the observation that split-tRNAs and intronic pre-tRNAs share a common bulge-helix-bulge motif, are processed by the same endonuclease, and split-tRNAs are fused into a mature tRNA at the point where introns are commonly present in tRNAs (Fujishima *et al.* 2009; reviewed in Yoshihisa 2014; Kanai 2015).

Two common classifications for tRNA molecules are the terms "isoacceptor" and "isodecoder". Isoacceptors are tRNA molecules that are charged with the same amino acid, regardless of sequence or structural differences. Isoacceptors often differ at the anticodon to base pair with alternate codons corresponding to the same amino acid. There are 21 isoacceptor families, one for each amino acid and one for selenocysteine tRNAs (Chan and Lowe 2016). Isodecoders are tRNA molecules that share the same anticodon and therefore decode the same mRNA codons, but may have sequence variation outside of the anticodon. Isodecoders are often also isoacceptors, such as the two *D. melanogaster* tRNA genes $tRNA^{Ser}_{UGA\ 1\text{-}1}$ and $tRNA^{Ser}_{UGA\ 2\text{-}1}$ which are both charged with serine and share an anticodon (Chan and Lowe 2016). Some tRNA variants have altered anticodons without affecting amino acid charging, causing two different isoacceptor classes to decode the same codon. For example, native $tRNA^{Pro}_{UGG}$ and the mutant variant $tRNA^{Ser}_{UGG}$ both share a UGG anticodon, but they are charged with proline and serine, respectively. Variant tRNAs that recognize noncognate codons can cause mistranslation,

which has physiological consequences for the organism (Berg *et al.* 2017; Hoffman *et al.* 2017).

All organisms have at least one codon that does not have a cognate tRNA gene (reviewed in Ehrlich *et al.* 2021). These codons are instead decoded through noncanonical "wobble" base pairing between position 34 of the tRNA, the first position of the anticodon, and the third position of the mRNA codon (Crick 1966). U34 can base pair with G instead of its canonical A, and A34 is deaminated to inosine allowing it to base pair with U, C, and A (Crick 1966; Boccaletto *et al.* 2018; reviewed in Agris *et al.* 2018). Wobble pairing allows one tRNA to decode multiple mRNA codons. An example of this includes the *D. melanogaster* $tRNA^{Phe}_{GAA}$, which decodes both cognate UUC and noncognate UUU phenylalanine codons through wobble pairing (Chan and Lowe 2016). U34 is even capable of wobble pairing with all four bases, a phenomenon known as "superwobble", although the cost of this versatility is a decrease in overall translational efficiency (Rogalski *et al.* 2008). Because of its ability to potentially cause translation errors through wobble pairing, U34 is usually modified post-transcriptionally to limit which bases it decodes and to improve translational fidelity (Yarian *et al.* 2002; Rozov *et al.* 2016).

## 1.2 tRNA Production and Processing

tRNAs are transcribed in a manner distinct from most other classes of genes. Rather than relying on upstream promoters like protein-coding genes, eukaryotic tRNAs rely on two conserved internal promoter regions, known as the A box and B box, for transcription by RNA polymerase III (DeFranco *et al.* 1980; Hofstetter *et al.* 1981; Sharp *et al.* 1981). The A and B boxes are located within the D- and T-arm respectively, and are therefore important for tRNA function as well as transcription (Allison *et al.* 1983; reviewed in Schramm and Hernandez 2002). Transcription of tRNAs by RNA polymerase III requires several transcription factors to first bind to the tRNA (Segall *et al.* 1980). First, the transcription factor complex TFIIIC recognizes and binds to the A and B boxes of a tRNA (Lassar *et al.* 1983). TFIIIC is a very flexible protein complex which allows it to accommodate variable spacing between the A and B boxes due to extended variable arms or introns (Schultz *et al.* 1989). This DNA-protein complex is recognized and bound

by TFIIIB, which in turn recruits RNA polymerase III upstream of the tRNA gene so it can transcribe the tRNA (Han *et al.* 2018). TFIIIC is needed to recruit TFIIIB, but after recruitment TFIIIB forms a stable complex with DNA upstream of the transcription start site and directs transcription by RNA polymerase III independently of TFIIIC (reviewed in Ramsay and Vannini 2018). Transcription terminates after RNA polymerase III reaches four to six consecutive thymidine residues on the nontemplate DNA strand (Bogenhagen and Brown 1981; Allison and Hall 1985; Braglia *et al.* 2005; Arimbasseri and Maraia 2015). RNA polymerase III transcription is extremely efficient and leads to high transcript levels of its target genes. This efficiency is due in part to facilitated recycling, where transcription on preassembled TFIIIB-TFIIIC-tDNA complexes proceeds much faster than the initial transcription cycle (Dieci and Sentenac 1996).

Initial tRNA transcripts undergo substantial processing in various cellular locations to become a mature tRNA. The first step involves ribonuclease P (RNase P) cleaving the 5’ leader sequence of the precursor tRNA (pre-tRNA) transcript within the nucleus or nucleolus (Frank and Pace 2003; Walker and Engelke 2006). RNase P is an ancient ribozyme found in archaea, bacteria, and eukaryotes. RNase P is nearly unique in biology, being one of only two ribozymes common to all three domains of life—the other being the ribosome (Nissen *et al.* 2000). Bacterial RNase P recognizes various structural elements of the tRNA to direct cleavage, such as the acceptor stem, T-arm, and the 3’ CCA sequence, but also recognizes and cleaves other RNA molecules aside from tRNAs (reviewed in Walker and Engelke 2006). Several forms of RNase P exist in eukaryotic cells, including protein-only variants unique to eukaryotes (Howard *et al.* 2013; Klemm *et al.* 2016). After cleavage of the 5’ leader sequence, the pre-tRNA’s 3’ trailer sequence is cleaved by RNase Z (Frendewey *et al.* 1985; O’Connor and Peebles 1991; Dubrovsky *et al.* 2004). Other exonucleases, such as Rex1, also cleave the 3’ trailer sequence of pre-tRNAs (Copela *et al.* 2008; reviewed in Hopper and Nostramo 2019). After the 3’ trailer sequence is removed from the pre-tRNA, the 3’ CCA motif is added by tRNA nucleotidyl transferase (Aebi *et al.* 1990). This sequence is necessary for aminoacylation but is not present in the sequence of eukaryotic tRNA genes, unlike the tRNA genes of many bacterial species, such as *E. coli*. Bacteria that have genomically encoded 3’ CCA motifs

still contain tRNA nucleotidyl transferase genes, but their primary function is 3’ tRNA repair (Zhu and Deutscher 1987).

Several tRNA species contain introns that need to be spliced out. Intronic tRNAs are relatively uncommon in flies and humans, but much more common in yeast and archaea (Hopper 2013; Yoshihisa 2014; Chan and Lowe 2016; Schmidt and Matera 2020). When introns are present in tRNAs, they are nearly always present between positions 37 and 38 (Chan and Lowe 2016). This is known as the “canonical” intron position. Noncanonical introns are much more likely to be found in archaea, which tend to have greater variation in tRNA gene structure than eukarya. Introns in tRNAs usually form a bulge-helix-bulge (BHB) structure which is used to guide the splicing endonuclease (Marck and Grosjean 2003). The BHB is required for tRNA splicing in archaea, but eukaryotic tRNA splicing is not as strict. While many eukaryotic tRNA introns contain BHB motifs and eukaryotic splicing machinery can process archaeal intronic tRNAs, a BHB is not required for eukaryotic tRNA processing. Eukaryotes instead require a conserved pyrimidine-purine base pair between the anticodon and intron that is necessary (but not sufficient) for tRNA splicing. Weakening this base pair impairs or prevents splicing from occurring (Schmidt *et al.* 2019; reviewed in Schmidt and Matera 2020).

tRNAs are some of the most heavily modified RNA molecules in the cell. There exist around 150 known RNA modifications, with approximately 80% of those occurring in tRNAs (reviewed in Suzuki 2021). Between 10–20% of tRNA bases are modified, depending on the species of tRNA (Jühling *et al.* 2009; Suzuki 2021; Boccaletto *et al.* 2022). The D- and TΨC-arms are named after their characteristic dihydrouridine and pseudouridine modified bases, respectively. tRNA modifications have diverse effects on the molecule, influencing factors such as stability, ribosomal interactions, and translational fidelity (Yarian *et al.* 2002; Tavares *et al.* 2021; reviewed in Suzuki 2021). Some tRNA modifications, such as deamination of adenosine into inosine at position 34, expand the range of codons that the tRNA decodes through wobble (Senger *et al.* 1997; reviewed in Tuorto and Lyko 2016 and Agris *et al.* 2018). Other modifications have the opposite effect on decoding, instead restricting the wobble potential of the tRNA to

prevent translational infidelity (reviewed in Suzuki 2021). For example, U34 is capable of recognizing all four bases in the third position of the codon (Rogalski *et al.* 2008). When modified to $xm^5s^2U34$, its decoding potential is restricted to the purines A or G (Yokoyama *et al.* 1985; Johansson *et al.* 2008; Kurata *et al.* 2008). tRNAs also show tissue-specific modification patterns in multicellular eukaryotes, further expanding their importance and complexity (Pinkard *et al.* 2020). While tRNAs are heavily modified across the entire transcript, the universal purine at position 37, immediately 3' of the anticodon, is modified especially frequently. Modifications to position 37 can alter flexibility and conformation of the anticodon loop, helping to stabilize interactions between weak anticodons and their codons at the ribosome and increase rate of tRNA binding and dissociation from the A site of the ribosome (Konevega *et al.* 2004; Grosjean and Westhof 2016).

Cells lacking enzymes required for tRNA modifications or containing variant tRNAs that lack key modifications often present with slow growth or disease phenotypes (reviewed in Hopper 2013; Pereira *et al.* 2018; Ramos and Fu 2019; de Zoysa and Phizicky 2020). Hypomethylation of G9 caused by mutations in *TRMT10A* is linked to microcephaly and diabetes (Gillis *et al.* 2014; Cosentino *et al.* 2018). Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and myoclonus epilepsy associated with ragged red fibers (MERRF) are caused by mutations to mt-$tRNA^{Leu}_{URR}$ and mt-$tRNA^{Lys}_{UUU}$ that prevent taurine modification of U34, impairing wobble decoding and mitochondrial protein synthesis (Goto *et al.* 1990; Yasukawa *et al.* 2001; Chujo and Tomizawa 2021). An interesting case study involves a child experiencing abdominal pain and muscle weakness who was found to have a C65G $tRNA^{[Ser]Sec}$ mutation, causing a reduction of stress-related selenoproteins through hypomodification of U34 (Schoenmakers *et al.* 2016). These examples demonstrate the importance of correct tRNA modification for protein synthesis and organismal health.

Due to their importance for cellular function, cells also use tRNA modifications to identify tRNAs that were processed incorrectly. Upon detection of a defective tRNA, the molecule is marked for degradation. tRNA turnover occurs in both the cytoplasm and nucleus and through two possible pathways, the nuclear surveillance pathway and the

rapid tRNA decay pathway (RTD, Alexandrov *et al.* 2006; reviewed in Megel *et al.* 2015). The nuclear surveillance pathway identifies hypomodified or misfolded pre-tRNAs, such as pre-tRNA$^{iMet}$ lacking an m$^1$A58 modification, and polyadenylates them using the TRAMP protein complex (Anderson *et al.* 1998; Kadaba *et al.* 2004; LaCava *et al.* 2005). Polyadenylated tRNAs are then degraded by the nuclear exosome (Kadaba *et al.* 2004). The RTD monitors mature tRNAs in both the nucleus and cytoplasm and marks tRNAs for degradation independently of TRAMP (reviewed in Megel *et al.* 2015), and is mediated by Met22 (Chernyakov *et al.* 2008). Nuclear tRNAs are degraded by the exonuclease Rat1, whereas cytoplasmic tRNAs are degraded by the exonuclease Xrn1. tRNA nucleotidyl transferase can add a second CCA motif to the 3' end of an unstable tRNA to mark it for degradation by Xrn1 (Wilusz *et al.* 2011).

tRNA expression in multicellular eukaryotes has an additional layer of complexity given that codon usage and tRNA pools differ by tissue type. Cells evolved to match tRNA transcript pools with codon usage (Ling *et al.* 2015; Hanson and Coller 2018; Yang *et al.* 2021; Liu *et al.* 2021). Optimal codons improve translational efficiency and mRNA stability, whereas suboptimal codons slow translation and lead to protein misfolding. Slow codons sometimes have beneficial effects on proteins, however, as they provide time for complex protein sequences to adopt their correct conformation (Perach *et al.* 2021). Thus, to ensure accurate and efficient protein synthesis, cells must alter available tRNA pools to match their specific translational requirements. An interesting example of this phenomenon can be found in Allen *et al.* (2022), where they inserted GFP reporters into *Drosophila melanogaster* with varying proportions of rare codons and found that >50% rare codon usage caused a complete absence of visible fluorescence and a >90% reduction in GFP protein. They also found that the brain and testes were still able to produce protein from rare codon-enriched transcripts and mRNA from testes is particularly enriched for rare codons, indicating that both codon usage and translational efficiency vary by tissue. Dittmar *et al*. (2006) demonstrated that different tissues have different levels of expression of nuclear and/or mitochondrial tRNAs, with the brain showing high levels of both nuclear and mitochondrial tRNA expression compared to other tissues such as the lymph nodes. Relative abundance of tRNA species also varied dramatically by tissue but was strongly correlated to codon usage of the tissue-specific

mRNA transcripts. Certain tRNA species, such as $tRNA^{Arg}_{TCT\ 4\text{-}1}$, are expressed only in the central nervous system (CNS) and neurodegeneration results when they are mutated (Ishimura *et al.* 2014). A recent sequencing analysis found that three other tRNAs, $tRNA^{Ala}_{TGC\ 5\text{-}x}$, $tRNA^{Ala}_{TGC\ 6\text{-}1}$, and $tRNA^{Ala}_{TGC\ 7\text{-}x}$, are highly enriched in the CNS whereas $tRNA^{Gly}_{GCC\ 2\text{-}x}$ is enriched in non-CNS tissues (Pinkard *et al.* 2020). This study also found tRNA isodecoder variants with CNS-specific modification patterns, which could impact stability and translation efficiency of these tissue-specific tRNA species. Understanding how tRNA expression differs between tissues has important implications for human health, as inappropriate or reduced expression of certain tRNAs can cause conditions such as neurodegeneration or cancer (Ishimura *et al.* 2014; Goodarzi *et al.* 2016; Kirchner *et al.* 2017).

## 1.3 Aminoacylation

Each amino acid is covalently attached via an ester linkage to its corresponding tRNA isoacceptor by a specific aminoacyl-tRNA synthetase (aaRS) enzyme (reviewed in Pang *et al.* 2014). These enzymes fall into two classes based on the structure of their catalytic domain. Class I molecules contain a Rossman fold domain which contains a nucleotide-binding region with conserved “HIGH” and “KMSKS” sequences (Zelwer *et al.* 1982; Webster *et al.* 1984; Moras 1992; reviewed in Bullwinkle and Ibba 2014). These enzymes bind the minor groove of tRNAs and aminoacylate the 2’-OH of the 3’ adenosine (Sprinzl and Cramer 1975). Class II enzymes are characterized by an active domain comprised of a seven-stranded, anti-parallel beta sheet flanked by alpha-helices (Cusack *et al.* 1990; Ruff *et al.* 1991a; reviewed in Arnez and Moras 1997). These enzymes bind the tRNA major groove and aminoacylate the 3’-OH (Sprinzl and Cramer 1975; Ruff *et al.* 1991). Both aaRS classes are further divided into three subclasses based on structural and functional characteristics (reviewed in Gomez and Ibba 2020). Because of how different the functional domains are between these two aaRS classes, it is thought that they originated independently and were not derived from a common ancestor (Ribas de Pouplana and Schimmel 2004).

Accurate translation requires that aaRS enzymes are highly selective towards only their cognate tRNA isoacceptors. Aminoacyl-tRNA synthetases recognize their cognate

tRNAs through identity elements, which are specific bases, base pairs, or structural elements within tRNA isoacceptors (Hou and Schimmel 1988; Francklyn and Schimmel 1989; Normanly *et al.* 1992; Xue *et al.* 1993; Larkin *et al.* 2002). Because the anticodon directly corresponds to tRNA identity and decoding potential, most tRNA species contain identity elements within their anticodon that their cognate aaRS uses for recognition (Schulman and Pelka 1989; Ruff *et al.* 1991; Jahn *et al.* 1991; Tamura *et al.* 1992; Kholod *et al.* 1997; reviewed in Giegé *et al.* 1998; Zamudio and José 2018; reviewed in Giegé and Eriani 2023). The anticodons of some tRNA isoacceptors, including $tRNA^{Ser}$ and $tRNA^{Ala}$, do not contain identity elements and their cognate aaRS instead use other motifs such as an extended variable arm (for $tRNA^{Ser}$) or G3:U70 (for $tRNA^{Ala}$) to recognize them (McClain and Foss 1988; Francklyn and Schimmel 1989; Achsel and Gross 1993). The "discriminator base" at position 73 is a common identity element for many tRNA isoacceptors, and often chemically-similar amino acids share the same discriminator base identity (Crothers *et al.* 1972; Breitschopf and Gross 1996; Hou 1997; reviewed in Giegé and Eriani 2023). In some cases, base modifications represent important identity elements for tRNAs (Senger *et al.* 1997; Madore *et al.* 1999; reviewed in Giegé asnd Lapointe 2009; reviewed in Giegé and Eriani 2023). This is especially true of modifications at positions 34 and 37 as they are important for tRNA stability and decoding potential (Konevega *et al.* 2004; Rogalski *et al.* 2008; Boccaletto *et al.* 2022)

Generally speaking, aaRS enzymes only recognize a single class of tRNA isoacceptors, but there are exceptions. SerRS recognizes selenocysteine tRNAs and charges them with serine, which is necessary for the production of mature Sec-$tRNA^{Sec}$ (Leinfelder *et al.* 1988; Ibba *et al.* 1997; Schmidt and Simonović 2012; Gonzalez-Flores *et al.* 2013). Archaea such as *Methanococcus jannaschii* serylate $tRNA^{Cys}$ and convert it into the cognate cysteine through a similar biochemical process as Ser-$tRNA^{Sec}$ using the enzyme SepCysS (Sauerwald *et al.* 2005; O'Donoghue *et al.* 2005). Some bacteria, such as *Bacillus megaterium*, lack GlnRS and instead use a nondiscriminatory GluRS which is capable of aminoacylating both $tRNA^{Glu}$ and $tRNA^{Gln}$ with glutamic acid (Wilcox and Nirenberg 1968; Salazar *et al.* 2003). After aminoacylation, the glutamic acid is converted into glutamine using Glu-AdT (Curnow *et al.* 1997). A similar process also occurs for conversion of Asp-$tRNA^{Asn}$ to Asn-$tRNA^{Asn}$ (Min *et al.* 2002; Sheppard *et al.* 2007,

2008). Some species such as *D. melanogaster* also have multifunctional *GluProRS* genes which, as the name suggests, is responsible for charging both $tRNA^{Glu}$ and $tRNA^{Pro}$ with their corresponding amino acid by including both aaRS enzymes on the same polypeptide (Cerini *et al.* 1991, 1997).

Some aaRS enzymes across both classes contain editing domains to fix mischarging events with near-cognate amino acids. This editing is proposed to work according to a "double-sieving" model (Fersht and Kaethner 1976; Fersht 1977; Fersht and Dingwall 1979), whereby the catalytic domain excludes large amino acids that do not fit into the enzyme's active site and the editing domain hydrolyzes misactivated isosteric or smaller amino acids (Fersht 1977; Fersht and Dingwall 1979; Ling *et al.* 2009; Rajendran *et al.* 2018). Editing occurs at both the pre-transfer and post-transfer stages of aminoacylation. At the pre-transfer stage, the noncognate aminoacyl-adenylate is recognized through its distinct binding affinity compared the cognate aminoacyl-adenylate and either hydrolyzed by the active site of the aaRS or selectively released into solution to prevent misincorporation (Baldwin and Berg 1966; Fersht 1977; Ling *et al.* 2012; Kuzmishin Nagy *et al.* 2020). If a noncognate amino acid is charged to a tRNA, then post-transfer editing can correct the error. If the aaRSs contains an editing domain, then it transfers the misacylated aa-tRNA there before to hydrolyze the aminoacyl linkage in a process known as *cis*-editing (Hendrickson *et al.* 2002; reviewed in Schimmel 2011; reviewed in Perona and Gruic-Sovulj 2014). Misacylated tRNAs are corrected after release (trans-editing) through hydrolysis by aaRSs or aminoacyl-tRNA deacylases such as AlaXp or YbaK (Ruan and Söll 2005; Chong *et al.* 2008; Kuzmishin Nagy *et al.* 2020).

## 1.4 Translation in Eukaryotes

Translation of mRNA transcripts into protein occurs at the ribosome. The eukaryotic ribosome consists of two subunits, the small 40S subunit and the large 60S subunit (reviewed in Panse and Johnson 2010). These subunits consist not only of protein complexes but also multiple rRNAs which are necessary for ribosome function. The small subunit contains a single 18S rRNA transcript whereas the large subunit contains the 5S, 5.8S, and 28S rRNA (reviewed in Panse and Johnson 2010; Wilson and Cate 2012). The 18S, 5.8S, and 28S rRNA are derived from a larger polycistronic 45S rRNA. (Scherrer

and Darnell 1962; reviewed in Woolford and Baserga 2013; Chaker-Margot and Klinge 2019; reviewed in Baßler and Hurt 2019). Ribosome subunit assembly largely takes place in the nucleolus, but some rRNA maturation takes place in the cytoplasm as well (Udem and Warner 1973; Rouquette *et al.* 2005).

In eukaryotes, translation initiation almost exclusively occurs through the use of an initiator $tRNA^{Met}$ ($tRNA_i^{Met}$, Kozak 1983; reviewed in Kearse and Wilusz 2017). There is a unique methionine tRNA distinct from $tRNA^{Met}$ species used during elongation (Åström *et al.* 1993; Farruggio *et al.* 1996). $tRNA_i^{Met}$ binds to eIF2-GTP and this ternary complex is brought to the 40S ribosomal subunit's peptidyl site (P-site) along with several other eukaryotic translation initiator factors (eIFs) to form the pre-initiation complex (PIC, Figure 1-2A, Jackson *et al.* 2010; Aitken and Lorsch 2012). This PIC attaches to the 5' capped end of mRNA and scans it in the 5' to 3' direction until it finds a start codon (Kozak 1989; Gingras *et al.* 1999; Poulin and Sonenberg 2013). The initiation factors eIF1, which is conserved across bacteria, archaea, and eukaryotes, along with eIF1A play a key role in initiation site scanning (Pestova *et al.* 1998; Basu *et al.* 2022). Upon recognition of the AUG start codon by $tRNA_i^{Met}$, the PIC undergoes a conformational change into a "closed" position and scanning is halted (Maag *et al.* 2005; Saini *et al.* 2010; Jackson *et al.* 2010; Merrick and Pavitt 2018). Ribosomes "commit" to the start codon through hydrolysis of eIF2-GTP to eIF2-GDP by eIF5 (Pestova *et al.* 2000; Unbehaun *et al.* 2004). Hydrolysis of GTP to GDP promotes dissociation of initiation factors, including eIF2, from the 40S subunit (Unbehaun *et al.* 2004; Jackson *et al.* 2010). The dissociation of initiation factors allows the 60S subunit to join the 40S subunit to form the complete ribosome with the Met-$tRNA_i^{Met}$ in the P-site (Pestova *et al.* 2000; Jackson *et al.* 2010; Merrick and Pavitt 2018).

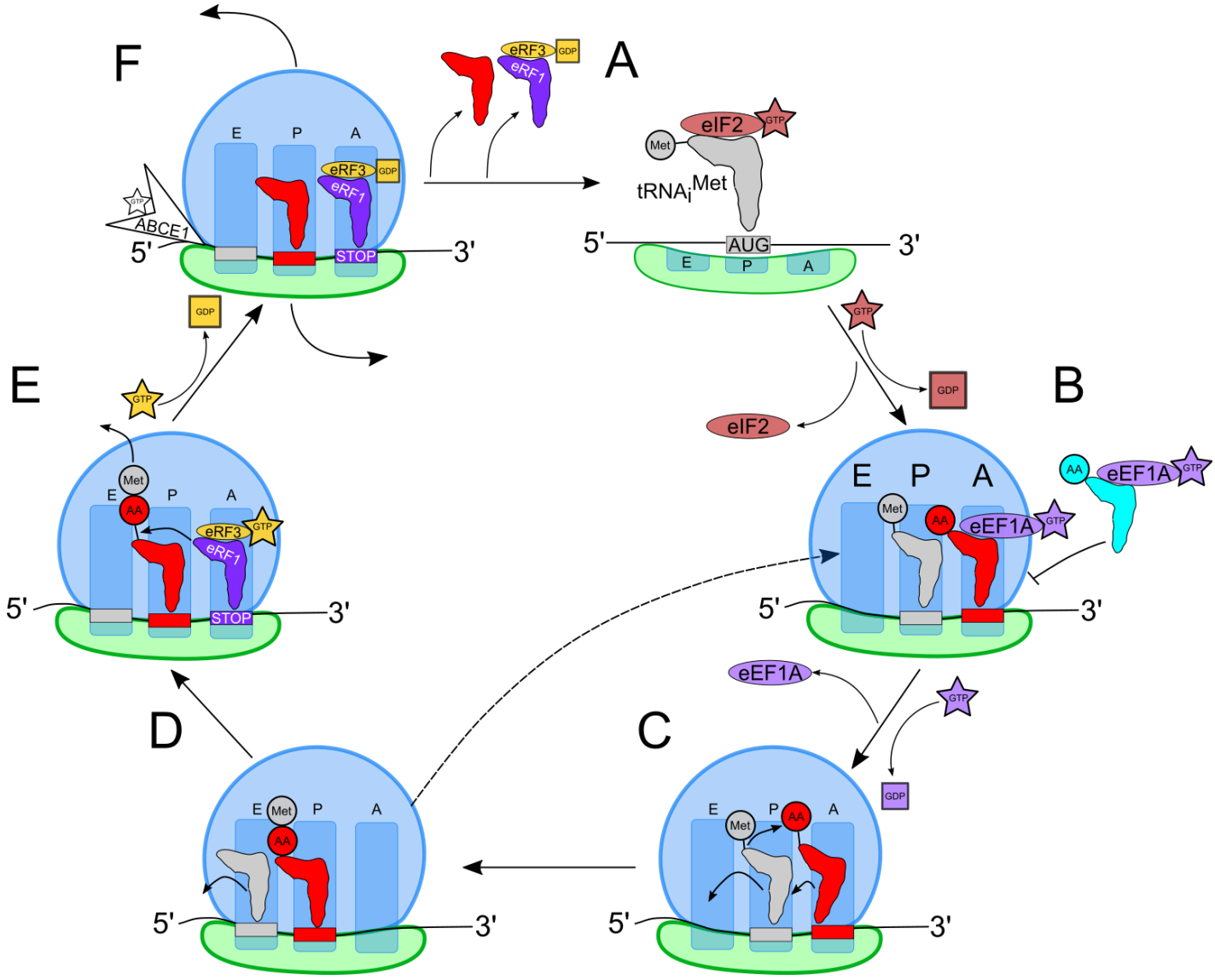


**Figure 1-2.** *Steps of translation in eukaryotes.*

**A)** Translation initiates when the pre-initiation complex composed of the 40S subunit, $tRNA_i^{Met}$/eIF2/GTP ternary complex, and other initiation factors (omitted for clarity) recognize an AUG start codon in the mRNA. This triggers GTP hydrolysis, dissociation of initiation factors, and association of the 60S subunit. **B)** During elongation, aminoacyl-tRNA/eEF1A/GTP ternary complexes are assessed for accuracy at the ribosome's A-site through codon:anticodon base pair stability. Cognate codon:anticodon pairs trigger

hydrolysis of eEF1A-bound GTP. **C)** GTP hydrolysis shifts the position of the aminoacyl-tRNA at the A-site, allowing a peptide bond to form between the P-site peptidyl-tRNA and the aminoacyl-tRNA. **D)** The ribosome ratchets forward, moving the deacylated tRNA into the E-site and the peptidyl-tRNA into the P site. The deacylated tRNA then exits the ribosome. Elongation then continues through steps B-D until a stop codon is reached. **E)** An eRF1/eRF3/GTP ternary complex recognizes the stop codon and hydrolyses its GTP to break the peptidyl-tRNA peptide bond and free the polypeptide. **F)** ABCE1 binds to the post-termination complex and hydrolyses GTP to separate the 60S and 40S ribosomal subunits. Various initiation factors remove the release factors and deacylated tRNA from the 40S subunit, allowing it to bind and translate other mRNA transcripts.

The ribosome contains three tRNA binding sites, the A- (aminoacyl), P- (peptidyl), and E-sites (exit). During translation elongation, aminoacylated tRNAs bound to the eukaryotic elongation factor eEF1A enter the A-site and are evaluated based on codon:anticodon stability (Figure 1-2B, reviewed in Dever and Green 2012). Stability of the codon:anticodon pair is assessed through key residues in the decoding center of the A site (A1492, A1493, and G530 in bacteria and A1755, A1756, and G577 in yeast; Powers and Noller 1994; Ogle *et al.* 2002; Ye and Lehmann 2022; Zhang *et al.* 2023). Noncognate base pairs at the first two positions of the codon prevent stabilization of the minor groove by these key residues and promotes dissociation of the aminoacyl-tRNA/eEF1A-GTP ternary complex from the ribosome (Loveland *et al.* 2017; Ye and Lehmann 2022). In bacteria, cognate codon:anticodon pairings cause the small subunit to adopt a closed conformation and initiate GTP hydrolysis to induce structural changes in the ribosome which accommodate the aminoacyl-tRNA (Loveland *et al.* 2017). Codon:anticodon discrimination is thought to proceed according to similar mechanisms in eukaryotes (Ye and Lehmann 2022).

After accommodation of the tRNA by the ribosome, the growing peptide attached to the tRNA in the P-site is transferred to the aminoacyl-tRNA in the A-site (Figure 1-2C,

Hiller *et al.* 2011). Peptide bond formation takes place in the peptidyl transferase centre (PTC), notable for being composed entirely of RNA (Noller *et al.* 1992; Nissen *et al.* 2000). The PTC accommodates aminoacyl-tRNAs through an induced-fit mechanism, whereby binding of the aminoacyl-tRNA in the A-site reorients the peptidyl-tRNA such that its ester group is exposed and available for peptide bond formation (Schmeing *et al.* 2005a, 2005b). During peptide bond formation, the ribosome reorients to facilitate translocation of the newly deacylated tRNA into the E-site and the peptidyl-tRNA into the P-site (Dever and Green 2012; Behrmann *et al.* 2015). The E-site tRNA then dissociates from the ribosome and the elongation process repeats until a stop codon is reached (Figure 1-2D).

Upon reaching a stop codon (UAG, UGA, or UAA), translation termination occurs. Termination relies on two eukaryotic release factors, eRF1 and eRF3, which slot into the A-site of the ribosome and are functionally similar to a eEF1A/GTP/aminoacyl-tRNA ternary complex (Figure 1-2E, Frolova *et al.* 1994, 1996; Alkalaeva *et al.* 2006; Jackson *et al.* 2012). eRF1 is a tRNA-shaped protein that recognizes all three stop codons (Kisselev *et al.* 2003), and eRF3 is a GTPase that aids in peptide release from the ribosome (Salas-Marco and Bedwell 2004; Taylor *et al.* 2012; Hellen 2018). GTP hydrolysis by eRF3 induces a conformational change in eRF1 that swings part of eRF1 into the PTC to stimulate hydrolysis of the peptidyl-tRNA bond (Taylor *et al.* 2012). While translation has ceased at this stage, the ribosome is still associated with the mRNA, deacylated tRNA is in the P-site, and eRF1 in the A-site (reviewed in Petry *et al.* 2008). Ribosome recycling is the process by which the two subunits of the ribosome split apart, dissociate from deacylated tRNAs and translated mRNA transcripts, and prepare to translate another transcript (reviewed in Petry *et al.* 2008 and Hellen 2018). In eukaryotes, this process is mediated by the ATP-binding cassette protein ABCE1 (Pisarev *et al.* 2010). Ribosome recycling by ABCE1 requires the presence of eRF1 in the ribosome but is inhibited by eRF3, therefore ensuring that ribosomes are only recycled after translation is fully terminated (Shoemaker and Green 2011). ABCE1 hydrolyzes ATP to provide the energy to pry the two ribosomal subunits apart into a free 60S subunit and a 40S subunit still bound to mRNA and a deacylated tRNA (Figure 1-2F). Dissociation of the P-site tRNA from 40S occurs through binding of eIFs 3, 1A, and 1,

and binding of eIF3J induces dissociation of mRNA from the subunit (Pisarev *et al.* 2007). At this point, the two ribosomal subunits are free to participate in translation of other mRNA transcripts.

## 1.5 Mistranslation

Translation is a remarkably accurate process considering the complexity of the genetic code and translational machinery. When the various mechanisms ensuring translational fidelity fail, mistranslation occurs (Ling *et al.* 2007, 2009; Moras 2010; reviewed in Mohler and Ibba 2017). Mistranslation describes the incorporation of an amino acid not specified by the mRNA codon into a nascent polypeptide. Mistranslation naturally occurs at a frequency of once per $10^3$–$10^6$ codons translated (Ellis and Gallant 1982; Joshi *et al.* 2019; Mordret *et al.* 2019), though this rate varies dramatically depending on species, the protein being mistranslated, and environmental conditions (Hanson and Coller 2018; Lant *et al.* 2019; Mordret *et al.* 2019; Samhita *et al.* 2020; Berg *et al.* 2021b; Cozma *et al.* 2023).

Due to the complexity of protein translation, errors at various points in the process can compromise translational fidelity. Mutations within tRNA genes affect both tRNA stability and decoding potential. As mentioned earlier, some tRNA isoacceptors such as $tRNA^{Ser}$ or $tRNA^{Ala}$ do not use the anticodon as identity elements for SerRS or AlaRS recognition (McClain and Foss 1988; Francklyn and Schimmel 1989; Achsel and Gross 1993). Mutations to the anticodon will therefore cause these tRNAs to recognize noncognate codons but still allow them to be efficiently aminoacylated by their corresponding aaRS. The ribosome has a limited ability to identify misacylated tRNAs or those that contain noncognate anticodons (Dale and Uhlenbeck 2005; Dale *et al.* 2009), so these tRNA variants are used during translation and substitute serine or alanine for the amino acid specified by the codon (Figure 1-3A, Berg *et al.* 2017, 2021a; Lant *et al.* 2018, 2021; Isaacson *et al.* 2022; Cozma *et al.* 2023). While tRNA variants with anticodon changes are some of the more obvious mechanisms by which variant tRNAs induce mistranslation, other changes to tRNAs have similarly strong effects. Base changes that recreate strong identity elements, such as the G3:U70 recognized by AlaRS, cause those tRNA variants to be recognized by noncognate aaRSs leading to mischarging

and mistranslation (Figure 1-3B, Hoffman *et al.* 2017; Lant *et al.* 2018, 2021). There also exist some identity elements, including a CNU anticodon, that reliably cause mismethionylation of non-tRNA$^{Met}$ isoacceptors such as tRNA$^{Thr}_{CGU}$ and tRNA$^{Arg}_{CCU}$ (Jones *et al.* 2011). Even without recreating an identity element, base changes can alter tRNA decoding potential by changing their structure or stability. The Hirsh suppressor describes a G24A mutation in the D-arm of tRNA$^{Trp}$ in *E. coli* that causes it to recognize the stop codon UGA (Hirsh 1971). This change creates new hydrogen bonding potential and stabilizes the bent form of the tRNA, making it more likely to misread UGA codons (Schmeing *et al.* 2011). A A9C mutation in the acceptor stem of tRNA$^{Trp}$ enables misreading of UGA codons by increasing tRNA flexibility which lowers the energy penalty of adopting the "active" A/T conformation (Schmeing *et al.* 2011).

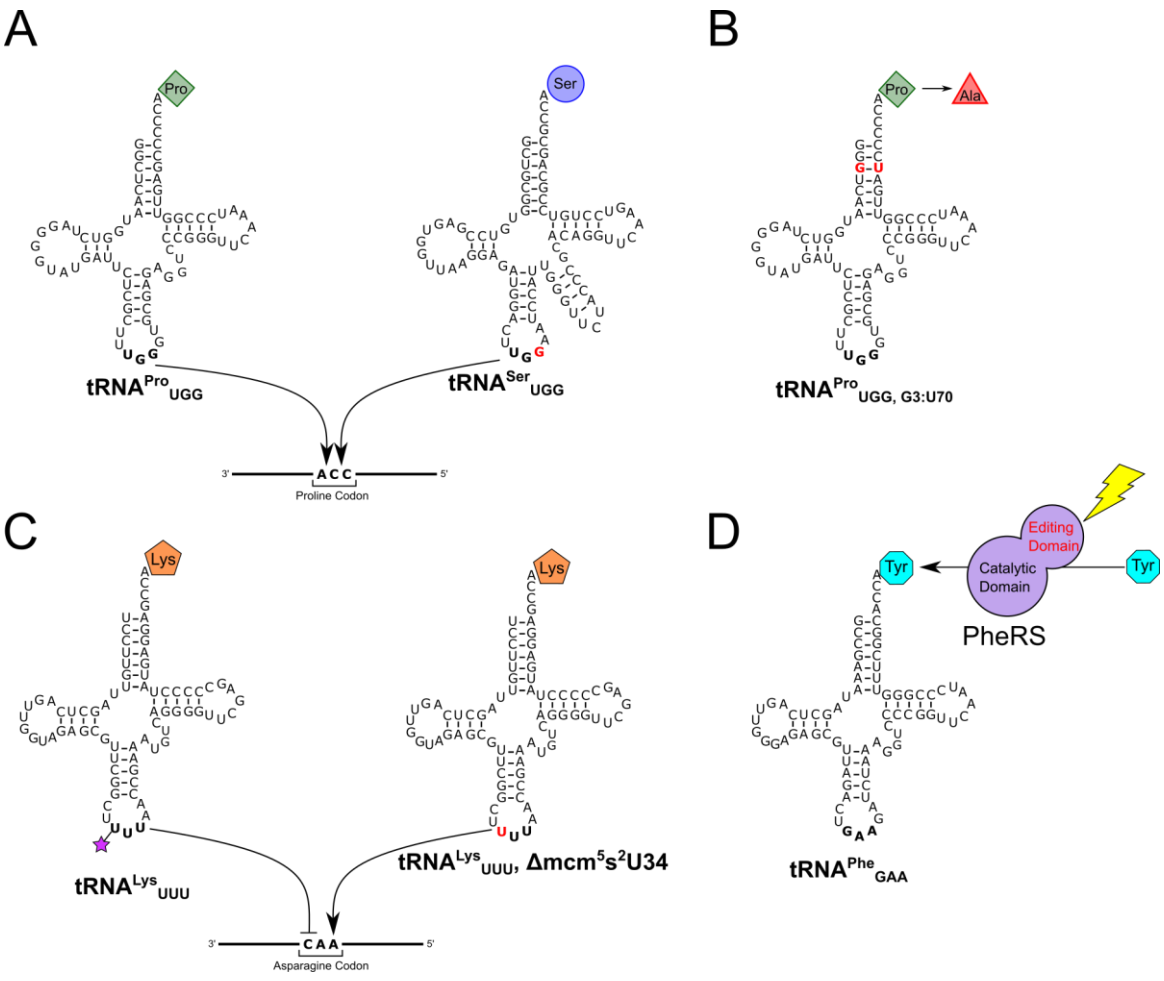


**Figure 1-3.** *Disruptions to translation fidelity that cause mistranslation.*

Base changes and anticodons are bolded. Red text indicates the affected part of the molecule. **A)** tRNA variants containing an anticodon change decode noncognate codons if this change does not disrupt aminoacylation of the tRNA variant. **B)** Base changes that create identity elements for a noncognate aaRS cause the tRNA to be mischarged, leading to mistranslation when the aminoacyl-tRNA is used. **C)** Mutations in tRNA-modifying genes cause tRNAs to lack modifications that help discern between cognate and near-cognate codons. This example was taken from Tavares *et al.* (2021). **D)** Mutations to key regions of aaRS genes, such as the editing domain, reduce selectivity between cognate and near-cognate amino acids and cause mischarging of cognate tRNAs with noncognate amino acids. This example is based on the findings of Lu *et al.* (2014).

Mutations that affect tRNA modifications also influence mistranslation rate. Superwobble by U34 is mitigated through modifications that constrain its ability to decode all four bases at the third position of the codon (Yarian *et al.* 2002; Rozov *et al.* 2016). Tavares *et al.* (2021) identified several tRNA-modification enzymes that cause protein aggregation when deleted. These enzymes modify U34 to improve its discrimination between different wobble pairs. Proteomic analysis of the aggregates revealed increased levels of amino acid misincorporation, and the substitutions identified suggest promiscuity of U34 wobble pairing (Figure 1-3C). Therefore, tRNA-induced mistranslation can occur through a variety of mechanisms including direct anticodon changes, acquisition of a new identity elements, or hypomodification.

Mistranslation is also caused by defective aaRS enzymes. An A158W missense mutation within the editing domain of PheRS in *D. melanogaster* greatly diminishes PheRS discrimination between phenylalanine and the noncognate but similar tyrosine (Figure 1-3D, Lu *et al.* 2014). Some *Mycoplasma* species contain aaRS genes with defective or missing editing domains, leading to misincorporations of tyrosine for phenylalanine and valine for leucine (Li *et al.* 2011). Fidelity of aaRS enzymes can also be compromised due to stressful environmental conditions. *E. coli* exposed to hydrogen

peroxide misacylate $tRNA^{Thr}$ with serine due to oxidation of a key cysteine residue required for ThrRS editing (Ling and Söll 2010).

Low levels of mistranslation have deceptively large effects on the proteome, as global mistranslation rates of once per $10^4$ codons translated would cause ~4% of proteins 400 amino acids long to be translated incorrectly (Ribas de Pouplana *et al.* 2014). Longer proteins would have an even higher prevalence of translation errors. This error rate is not evenly distributed across the entire genome, however. Genes that are conserved or highly expressed experience slower and more accurate translation whereas those expressed less present with a higher error rate (Yang *et al.* 2014; Mordret *et al.* 2019). Slower translation of highly expressed or conserved genes is a mechanism to ensure accurate translation of genes likely necessary for cell function. In addition, "preferred" codons (a member of a synonymous codon group used more often than average) are translated more accurately than nonpreferred synonymous codons (Sun and Zhang 2022).

Levels of mistranslation also vary depending on environmental challenges experienced by cells. Excess carbon in growth media causes *E. coli* cells to experience acidic growth conditions, which in turn promotes stop codon readthrough (Zhang *et al.* 2020b). Prevalence of stop codon readthrough also increases when *E. coli* experience nutrient starvation (Ballesteros *et al.* 2001). Exposure to oxidative stresses such as hydrogen peroxide or hypochlorite promote the mistranslation of serine at threonine codons in *E. coli* (Ling and Söll 2010; Wu *et al.* 2014). Immune response to pathogens also triggers increased levels of mistranslation, as in Netzer *et al.* (2009) who found that HeLa cells greatly increase levels of methionine misincorporation when infected with influenza A or adenovirus 4 viruses.

Mistranslation is often maladaptive and harmful to cells, but there is growing evidence that mistranslation may sometimes help organisms cope with stressful environmental conditions (reviewed in Ribas de Pouplana *et al.* 2014 and Steiner and Ibba 2019). *E. coli* cells missing three of their four copies of $tRNA_i^{Met}$, which causes translation initiation by non-initiator tRNAs, could better withstand exposure to antibiotics, DNA damage, and heat stress when compared to cells without missing copies

of $tRNA_i^{Met}$ (Samhita *et al.* 2020). This group later showed that brief periods of mistranslation through treatment with amino acid analogues improves survival even under optimal conditions (Samhita *et al.* 2021). Mistranslation due to mutations in the ribosomal proteins RpsD and RpsL or exposure to the non-proteinogenic amino acid canavanine improve *E. coli* tolerance to hydrogen peroxide (Fan *et al.* 2015). Strains with an error-prone *RpsD* mutation are also more resistant to heat stress than wild-type bacterial cells, as are wild-type cells grown in media containing canavanine. (Evans *et al.* 2019). Mistranslation-induced resistance to heat stress and oxidative stress are both mediated by the general stress response sigma factor RpoS (Fan *et al.* 2015; Evans *et al.* 2019). Methionine is commonly used to protect cells from reactive oxygen species (Luo and Levine 2009; Kavoor *et al.* 2022). Misincorporation of methionine for other amino acids is therefore thought to help protect key enzymatic residues from oxidative stress, and has been observed in a wide variety of organisms, including *E. coli*, yeast, and human cells (Netzer *et al.* 2009; Jones *et al.* 2011; Wiltrout *et al.* 2012; Lee *et al.* 2014).

An intriguing recent discovery suggests that some species intentionally mistranslate specific codons. Some *Streptomyces* bacterial species encode a unique $tRNA^{Pro}$ that contains an AGC anticodon, which is normally assigned to alanine. This $tRNA^{Pro}$ is recognized by a unique ProRS which charges it with proline, causing these bacteria to substitute alanine with proline (Vargas-Rodriguez *et al.* 2021). Further investigation revealed two other proline tRNAs with non-proline anticodons, namely threonine AGU and aspargine AUU (Schuntermann *et al.* 2023). Like $tRNA^{Pro}_{AGC}$, these tRNAs are functional and substitute proline for threonine or asparagine. These novel $tRNA^{Pro}$ genes impair normal *E. coli* growth but improve their survival when exposed to antibiotics, suggesting that deliberate mistranslation may be a strategy used by pathogenic bacteria such as *Streptomyces* to withstand host defence mechanisms (Schuntermann *et al.* 2023; discussed in Ibba 2023). Deliberate mistranslation is also required by some viruses for normal function, as some key proteins required for infection or multiplication require stop codon readthrough by suppressor tRNAs (Hofstetter *et al.* 1974; Ishikawa *et al.* 1986; reviewed in Beier 2001). Mistranslation could be a strategy to create “statistical proteomes” containing variant proteins slightly different than what is encoded by their

source genes, potentially identifying beneficial variants that help organisms withstand hostile environments (Woese 1965, 2004).

## 1.6 Mistranslation in Eukaryotes

Much of the work investigating the effects of mistranslation in eukaryotes has been conducted in yeast. My lab investigated the effects of mistranslation on *Saccharomyces cerevisiae* and found that various types of amino acid substitutions induce the heat shock response and slow the rate of growth (Berg *et al.* 2017, 2019b; Lant *et al.* 2018; Cozma *et al.* 2023). Research from the Phizicky lab demonstrated that the toxicity of mistranslating $tRNA^{Ser}$ variants does not always correlate well with amount of mistranslation those variants cause, though mistranslation levels of $tRNA^{Ala}$ variants seem to correlate better with toxicity (Zimmerman *et al.* 2018; Cozma *et al.* 2023). Other factors, such as the type of substitution or genetic background, play an important role in cellular response to substitution (Berg *et al.* 2021b, 2022). Two tRNA variants that misincorporate serine at arginine codons or alanine at proline codons affect different cellular processes despite mistranslating at similar rates (Berg *et al.* 2021b). Proteolysis, autophagy, and mitochondrial translation were affected in both mistranslating lines, but other processes including protein localization and purine metabolism were enriched in only one mistranslating line. A recent study from the Brandl lab characterized the effects of $tRNA^{Ala}$ variants containing all 60 non-alanine codons and identified additional factors that influence the impact of tRNA variants (Cozma *et al.* 2023). $tRNA^{Ala}$ variants that contain G/C rich anticodons, such as $tRNA^{Ala}_{CGG}$, tended to reduce growth of yeast cells more than the variants containing A/U-rich variants. In addition, variant $tRNA^{Ala}$ genes that affect synonymous codons, such as the leucine-decoding $tRNA^{Ala}_{CAG}$ and $tRNA^{Ala}_{UAG}$, affect very different sets of peptides with little overlap (Cozma *et al.* 2023). These findings suggest that care should be taken when attempting to generalize the effects of tRNA-induced mistranslation as very similar tRNA variants can have dramatically different effects on eukaryotic biology.

Some yeast species present interesting examples of natural mistranslation. The *Candida* "CTG-clade" is named as such because species within that clade decode the CUG codon as serine instead of leucine or decode it ambiguously as either (Kawaguchi *et*

*al.* 1989; Fitzpatrick *et al.* 2006; Gomes *et al.* 2007), though there is some controversy regarding the prevalence of CUG mistranslation (Mühlhausen *et al.* 2021; Correia *et al.* 2023). While it was initially discovered in *Candida* species, CUG codon ambiguity is also found in other yeast species including *Ascoidea asiatica* and *Pachysolen tannophilus*, where CUG was decoded as alanine rather than serine or leucine (Riley *et al.* 2016; Krassowski *et al.* 2018; Mühlhausen *et al.* 2018). This codon reassignment was thought to occur through the loss of the cognate $tRNA^{Leu}_{CAG}$ which then allowed that codon to be "captured" by other tRNA isoacceptors with mutated anticodons (Mühlhausen *et al.* 2016; Kollmar and Mühlhausen 2017a, 2017b). *Ascoidea asiatica* ambiguously decodes CUG codons as serine or leucine at equal frequencies, which means a 50% mistranslation rate—a staggeringly high level of mistranslation (Mühlhausen *et al.* 2018). To put this in context, misincorporating serine for proline at a frequency of 12% per codon prevented growth of *Saccharomyces cerevisiae* (Berg *et al.* 2021a), so finding an organism that withstands levels of mistranslation fourfold higher is remarkable. *Acsoidea asiatica* can withstand such a high amount of mistranslation by greatly reducing the amount of CUG codons in their genome, especially at conserved positions within proteins (Mühlhausen *et al.* 2018).

Mistranslation research in multicellular eukaryotes presents with additional complexity due to tRNA pools, codon usage, and susceptibility to proteotoxic stress varying between tissues (Dittmar *et al.* 2006; Ishimura *et al.* 2014; Pinkard *et al.* 2020; Allen *et al.* 2022). Postmitotic tissues, such as the heart or nervous system, seem particularly susceptible to translation infidelity (Antonellis *et al.* 2003; Jordanova *et al.* 2006; Lee *et al.* 2006; Liu *et al.* 2014; Vo *et al.* 2018; reviewed in Kapur *et al.* 2017 and Kapur and Ackerman 2018). The "sticky" (*sti*) mutation, which causes hair loss and ataxia in mice, is caused by a C-to-A mutation in the editing domain of AlaRS (Lee *et al.* 2006). This mutation reduced the ability of AlaRS to edit misacylated $tRNA^{Ala}$, causing misincorporation of serine for alanine and visible protein aggregation within cerebellar Purkinje cells. Mice with additional mutations that further impaired AlaRS editing present with reduced body size and cardiac abnormalities (Liu *et al.* 2014). Mice homozygous for a particularly strong editing-deficient AlaRS died during gestation, demonstrating that translation fidelity is required for organism development (Liu *et al.* 2014). Interestingly,

vertebrates have a unique gene (*Ankrd16*) that helps correct AlaRS aminoacylation errors (Vo *et al.* 2018). ANKRD16 associates with the AlaRS catalytic site and enhances pre-transfer editing by binding misactivated serine residues to prevent their misacylation onto $tRNA^{Ala}$, thereby providing an additional mechanism to maintain translation fidelity. While not an example of mistranslation, a mutation of a neuron-specific $tRNA^{Arg}_{UCU}$ that impairs processing by RNAse P also caused neurodegeneration in mice, further demonstrating neuronal sensitivity to translational disruptions (Lai *et al.* 2022).

Research examining the effects of mistranslating tRNA variants on human and mouse cells indicate that they have similar negative effects as mutant aaRSs. Mistranslating tRNA variants suppress translation and impair degradation of protein aggregates (Lant *et al.* 2021; Hasan *et al.* 2023). $tRNA^{Ser}_{AAA}$, which misincorporates serine for phenylalanine, significantly increases cell death compared to cells containing a wild-type $tRNA^{Ser}_{AGA}$ (Hasan *et al.* 2023). Disruptions to proteostasis caused by mistranslation negatively affect the development of multicellular eukaryotes. Zebrafish embryos ectopically expressing mistranslating $tRNA^{Ser}$ variants developed deformities during embryogenesis (Reverendo *et al.* 2014). A double-sieving defective PheRS reduces lifespan, impairs climbing performance, and causes cell death and wing abnormalities when expressed in fruit flies (Lu *et al.* 2014). Variant or mis-processed tRNAs have a particularly strong effect on reproductive processes. Suppressor tRNA variants engineered to read through UAG stop codons cause sterility when integrated into the *D. melanogaster* genome (Laski *et al.* 1989; Garza *et al.* 1990). Mutations to *Rpp30*, a gene encoding a subunit of RNase P, cause an accumulation of improperly processed tRNAs and sterility in female flies (Molla-Herman *et al.* 2015). $tRNA^{Trp}$ variants that induce stop codon readthrough in *Caenorhabditis elegans* lead to death during embryogenesis and adult sterility within a single generation (Waterston 1981; Sagi *et al.* 2016). The effects of tRNA variants on reproduction require additional research, particularly considering that idiopathic recurrent miscarriage in humans is linked to mutations in mt-$tRNA^{Tyr}$ and mt-$tRNA^{Cys}$ (Mojodi *et al.* 2023).

## 1.7 Impaired Translation and Disease

Disruptions to both mitochondrial and cytosolic translation are associated with disease, particularly muscular and neurological disorders (reviewed in Yarham *et al.* 2010; Kapur *et al.* 2017; Kapur and Ackerman 2018; and Lant *et al.* 2019). A>G mutations within mitochondrial tRNAs cause the two mitochondrial encephalomyopathies MELAS and MERRF (Goto *et al.* 1990; Shoffner *et al.* 1990; Yasukawa *et al.* 2001; reviewed in Chujo and Tomizawa 2021). These mutations cause the mitochondrial tRNA to lack a key taurine modification at the wobble position, reducing their ability to form the U:G wobble pair (Figure 1-4A, Kirino *et al.* 2004). Many other harmful mt-tRNA variants are thought to exert their negative effects by preventing key modifications from being added to the mt-tRNA variant (Suzuki *et al.* 2020). A respiratory chain disorder associated with mt-tRNA, notable for being an example of a dominant allele, changes the anticodon of mt-tRNA$^{Trp}$ from ACU to AUU (Sacconi *et al.* 2008). This change allows mt-tRNA$^{Trp}$ to decode threonine UGC and UGU or UAA and UAG stop codons, causing amino acid misincorporation and stop codon read-through. Mitochondria also contain unique aaRS genes which also cause disease when compromised. Mutations in the mitochondrial AspRS that dramatically reduce aminoacylation activity cause the leukoencephalopathy LBSL (Scheper *et al.* 2007). Pathological conditions are associated with mutations to all 19 of the nuclear-encoded mitochondrial aaRS genes (reviewed in Sissler *et al.* 2017). The majority of these conditions affect the central nervous system, but muscular, excretory, and endocrine systems are affected by mt-aaRS mutations as well.

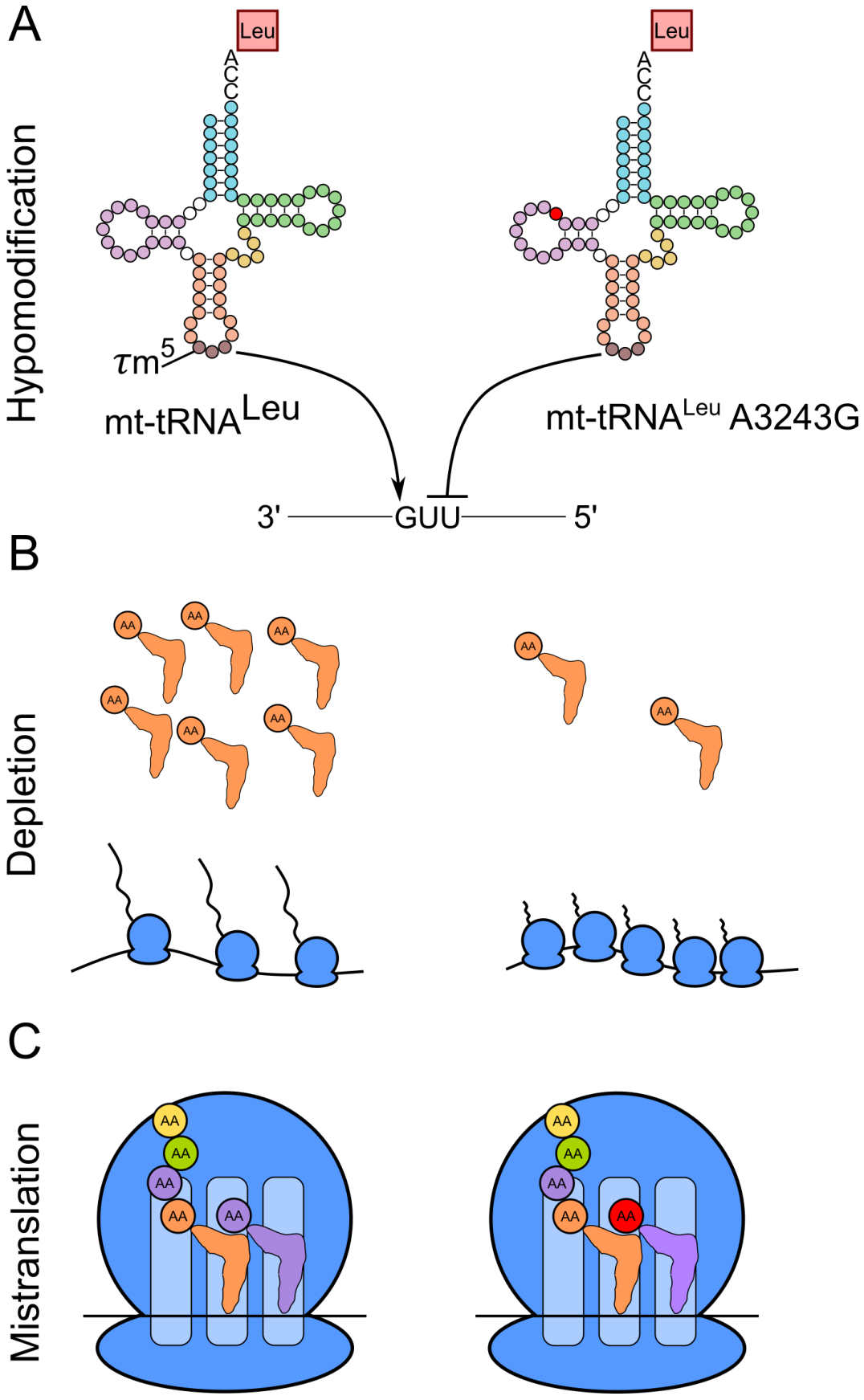
A
Hypomodification
Leu
ACC
τm5
mt-tRNALeu
Leu
ACC
mt-tRNALeu A3243G
3'
GUU
5'
B
Depletion
AA
C
Mistranslation
AA

**Figure 1-4.** *Mechanisms by which impaired translation cause disease.*

**A)** Hypomodification of tRNAs alters their decoding potential, reducing the cell's ability to translate certain codons. In this example, a A3243G mutation removes a $\tau m^5$ modification from U34 of mt-tRNA$^{Leu}_{UAA}$, reducing its ability to wobble pair with UUG codons and causing MELAS (Kirino *et al.* 2004). **B)** Depletion of a tRNA isodecoder due to sequestration, low expression, or high turnover causes ribosome stalling during translation of mRNA transcripts rich in that isodecoder's cognate codon. **C)** Misacylated tRNAs incorporate the wrong amino acid during protein synthesis, leading to mistranslation.

Several neurological disorders are linked to problems with translation. Charcot-Marie-Tooth (CMT) disease type 2D is a neuropathy that causes muscle weakness, and sensory deficits in the extremities, usually starting with the hands (Sivakumar *et al.* 2005) and is caused by mutations to the GlyRS gene *GARS* (Antonellis *et al.* 2003). A mouse model of CMT2D found that mutant GlyRS still efficiently aminoacylated tRNA$^{Gly}$, suggesting that these mutations cause GlyRS to adopt a novel, pathogenic function rather than causing disease through haploinsufficiency or dominant-negative interactions (Seburn *et al.* 2006; reviewed in Burgess and Storkebaum 2023). Further research using mouse models suggest that a pathogenic mechanism of mutant GlyRS is likely impaired release of tRNA$^{Gly}$, thus depleting both tRNA$^{Gly}$ and ribosome rescue factors needed to alleviate ribosome stalling at glycine codons (Figure 1-4B, Zuko *et al.* 2021). However, other studies found that aminoacylation activity of pathogenic *GARS* variants is heavily reduced (Griffin *et al.* 2014), suggesting that different *GARS* mutations may cause CMT2D through a variety of mechanisms. This idea is supported by examining mutations that cause other types of CMT disease. HisRS (HARS) variants are implicated in CMT2W and some pathogenic *HARS* variants cause mistranslation of histidine to glutamine and threonine when expressed in yeast, indicating that misacylation by mutant HARS may be a potential mechanism underlying the pathogenicity of CMT2W (Figure 1-4C, Qiu *et al.* 2023). Some instances of intellectual disability are linked to aberrant tRNA

pools. Loss of *NSUN2*, a gene encoding a cytosine methyltransferase needed to add the common $m^5C$ modification to tRNAs, causes intellectual disability in humans (Abbasi-Moheb *et al.* 2012; Blanco *et al.* 2014). This lack of $m^5C$ reduces expression of all $tRNA^{Gly}$ isodecoders in the forebrain of mice and slows decoding of glycine-rich transcripts, including many involved in synaptic signaling (Blaze *et al.* 2021). Impaired synaptic transmission was observed in these mice along with impaired memory and learning, though these researchers also note that *Nsun2* loss-of-function also reduces depressive and anxious behaviours. Mutations to other tRNA-modifying enzymes also cause intellectual disability, including *TRMT1*, *ADAT3*, and *ELP1–3* (Alazami *et al.* 2013; Zhang *et al.* 2020a; reviewed in Blaze and Akbarian 2022).

There are relatively few diseases known to be caused by cytosolic tRNA variants (reviewed in Abbott *et al.* 2014 and Lant *et al.* 2019). Contributing to this apparent absence is doubtless the difficulty identifying and mapping mutations to a particular tRNA variant, given that there are multiple copies of most cytosolic tRNA isodecoders and many share completely identical sequences (Chan and Lowe 2016). As tRNA sequencing technology improves and becomes more widespread, more cytosolic tRNA variants are likely to be found to influence disease pathology. This is especially true given that a recent tRNA sequencing study found an average of 66 tRNA variants per individual, some of which are expected to mistranslate (Berg *et al.* 2019a). Cytosolic tRNA variants linked to disease tend to affect isoacceptors with few redundancies. One example involves a C65G mutation in $tRNA^{Sec}$, of which there is only a single copy in the human genome (Chan and Lowe 2016). This mutation impairs modification of U34 in the mature $tRNA^{Sec}$, reducing translation of stress-response selenoprotein transcripts (Schoenmakers *et al.* 2016). This $tRNA^{Sec}$ variant caused abdominal pain, fatigue, muscle weakness, and thyroid disfunction in the 8-year-old patient. Mutations to a CNS-specific $tRNA^{Arg}$ causes ribosome stalling and neurodegeneration in mice which also lack the ribosome recycling factor GTPBP2 (Ishimura *et al.* 2014). Expression of mistranslating cytosolic $tRNA^{Ser}$ variants increases tumor cell growth in mice and make cells more likely to become cancerous (Figure 1C, Santos *et al.* 2018).

Disease pathology is also affected by cytosolic tRNA dysregulation and mismatches between tRNA pools and codon usage. A synonymous mutation in CFTR, the gene that causes cystic fibrosis, that alters an mRNA codon from Thr-ACT to Thr-ACG impacts the protein's structure and function. This behaviour is caused by the codon change requiring a less-abundant $tRNA^{Thr}$ for decoding, slowing translation and causing misfolding of the growing CFTR polypeptide (Kirchner *et al.* 2017). Insufficient tRNA levels lead to disease, but overexpressing tRNAs can also induce pathogenic phenotypes. Santos *et al.* (2022) overexpressed a normal $tRNA^{Ser}_{AGA}$ gene in non-tumorous human cell lines and found that tRNA overexpression increases cell proliferation. Mice inoculated with cells overexpressing $tRNA^{Ser}_{AGA}$ were also more likely to present with tumors than control mice, and the tumors overexpressing $tRNA^{Ser}_{AGA}$ were larger than tumors found in mice inoculated with control cells (Santos *et al.* 2022). Taken together, the influence of cytosolic tRNAs on disease is affected by mutations to the tRNA, the modifications it disrupts, the tissue it is expressed in, the level of expression, and the number of competing tRNAs.

While cytosolic tRNA variants sometimes cause disease, they are also being investigated as potential therapeutics, particularly for disease caused by premature stop codons (reviewed in Anastassiadis and Köhrer 2023 and Coller and Ignatova 2023). One large advantage of tRNA-based medicine is that it can alter protein sequence without requiring gene editing, which may itself have negative effects on patient health (reviewed in Guo *et al.* 2023). A recent study used several tRNA variants with an engineered UGA stop anticodon to suppress premature termination of R1162X CFTR mRNA in mammalian cells (Ko *et al.* 2022). These suppressor tRNAs dramatically improve translation of CFTR nonsense mutants when stably integrated into the genome, reaching CFTR activity levels between 75% and 91% of wild-type—well above levels required for therapeutic relevance (Kerem 2004; Ko *et al.* 2022). Other researchers found that suppressor tRNA variants more efficiently read through CFTR premature stop codons than G418, an antibiotic that reduces eukaryotic translation fidelity (Albers *et al.* 2023). These suppressor tRNAs were packaged into lipid nanoparticles and administered to mice intravenously or intratracheally *in vivo*, where they robustly read through stop codons without causing significantly higher readthrough of native UGA stop codons (Albers *et*

*al.* 2023). Engineered tRNA variants could also correct pathogenic missense mutations by decoding the pathogenic mRNA codon while being acylated with the wild-type amino acid, thus rescuing the protein at the translational level. In situations where fewer tRNAs are available than are necessary for translation, cellular function can be restored by supplementing these tissues with additional copies of the needed tRNA. In addition to discovering that $tRNA^{Gly}$ sequestration is the likely mechanism causing CMT2D pathology, Zuko *et al.* (2021) also showed that overexpression of $tRNA^{Gly}$ fully rescues motor performance, neuromuscular transmission, muscle weight, and strongly reduces other negative symptoms in GlyRS mutant mice. Although tRNA-based therapeutics is a new field of medical research, the unique role of tRNAs as adaptor molecules between mRNA and protein position them as strong candidates to treat conditions resistant to conventional approaches.

## 1.8 Summary of Experimental Objectives

This literature review demonstrates that tRNA genes and their variants have large effects on various aspects of eukaryotic biology, including translation, development, stress response, and disease progression. There has been extraordinary work studying the effects of tRNA variants on the biology of eukaryotes such as *Saccharomyces cerevisiae*, but there are aspects of multicellular eukaryote biology that are difficult to study using single-celled organisms. However, there does not currently exist a good model to study the effects of native tRNA variants on multicellular eukaryotes. The goal of my project was to develop a model of tRNA-induced mistranslation in the fruit fly *Drosophila melanogaster*. *Drosophila melanogaster* provides many advantages as a model system, including many available genetic tools, low maintenance cost, and short generation time.

Chapter 2 of my thesis focuses on developing a model of tRNA-induced proline-to-serine (P→S) mistranslation using *D. melanogaster*. I integrated a wild-type $tRNA^{Ser}_{UGA}$ and a P→S mistranslating $tRNA^{Ser}_{UGG,\ G26A}$ variant into the fly genome to determine the effects of mistranslation. The mistranslating $tRNA^{Ser}_{UGG,\ G26A}$ variant caused developmental lethality, morphological deformities, extended development, and impaired climbing performance. I was also surprised to find that females were more susceptible to this mistranslating tRNA variant than male flies. In Chapter 3, I performed

RNA-sequencing on male and female flies from the mistranslating $tRNA^{Ser}_{UGG, G26A}$ and control $tRNA^{Ser}_{UGA}$ lines to identify potential transcriptomic explanations for the sex-specific responses to mistranslation. Males and females showed very different responses to $tRNA^{Ser}_{UGG, G26A}$, though both sexes tended to downregulate metabolic processes. Females upregulated genes associated with DNA damage and cell cycle regulation, suggesting that mistranslation may compromise the genome integrity of female flies. In chapter 4, I tested whether fruit flies respond differently to different types of mistranslation by integrating $tRNA^{Ser}_{AAC, G26A}$ and $tRNA^{Ser}_{AGU, G26A}$ into the fly genome which cause valine-to-serine (V→S) and threonine-to-serine (T→S) substitutions, respectively. These two types of mistranslation broadly replicated my original P→S findings, but there were key differences between each line. Both $tRNA^{Ser}_{AAC, G26A}$ and $tRNA^{Ser}_{AGU, G26A}$ significantly increased female lifespan despite also increasing prevalence of deformities, suggesting mistranslation exerts both positive and negative effects on flies. I have successfully created a model for tRNA-induced mistranslation in the fruit fly *Drosophila melanogaster* and demonstrated that mistranslation has strong, but not universally negative, effects on fly biology.

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# Chapter 2

# 2 A Novel Mistranslating tRNA Model in *Drosophila melanogaster* has Diverse, Sexually Dimorphic Effects

Transfer RNAs (tRNAs) are the adaptor molecules required for reading the genetic code and producing proteins. tRNA variants can lead to genome-wide mistranslation, the misincorporation of amino acids not specified by the standard genetic code into nascent proteins. While genome sequencing has identified putative mistranslating tRNA variants in human populations, little is known regarding how mistranslation affects multicellular organisms. Here, we create a multicellular model of mistranslation by integrating a serine tRNA variant that mistranslates serine for proline ($tRNA^{Ser}_{UGG, G26A}$) into the *Drosophila melanogaster* genome. We confirm mistranslation via mass spectrometry and find that $tRNA^{Ser}_{UGG, G26A}$ misincorporates serine for proline at a frequency of ~ 0.6% per codon. $tRNA^{Ser}_{UGG, G26A}$ extends development time and decreases the number of flies that reach adulthood. While both sexes of adult flies containing $tRNA^{Ser}_{UGG, G26A}$ present with morphological deformities and poor climbing performance, these effects are more pronounced in female flies and the impact on climbing performance is exacerbated by age. This model will enable studies into the synergistic effects of mistranslating tRNA variants and disease-causing alleles.

## 2.1 Introduction

Mistranslation occurs when an amino acid that differs from what is specified by the standard genetic code is incorporated into nascent proteins. Mistranslation is implicated in various disease phenotypes. Editing-defective tRNA synthetases that induce mistranslation cause cardiac abnormalities and neurodegeneration in mice (Lee *et al.* 2006; Liu *et al.* 2014), and impaired locomotion, reduced lifespan, and neurodegeneration in flies (Lu *et al.* 2014). Ectopically expressed mistranslating tRNAs cause developmental deformities in zebrafish (Reverendo *et al.* 2014) and promote tumor growth in mouse cell lines (Santos *et al.* 2018). tRNA variants can cause mistranslation and are also directly linked to human disease, as mitochondrial tRNA variants cause MELAS and MERRF in humans (Goto *et al.* 1990; Shoffner *et al.* 1990). Despite the profound impact of

mistranslation and the prevalence of cytoplasmic tRNA variants with the potential to mistranslate in humans (Berg *et al.* 2019a), the impact of these variants on the biology of multicellular organisms is not well described.

Mutations in tRNAs that cause mistranslation arise spontaneously and were identified initially in *Escherichia coli* as suppressors of nonsense and missense mutations (see for examples; 9–11). Subsequently, mistranslating tRNAs have been identified as suppressors of deleterious phenotypes in other organisms (e.g. 12–16). While no spontaneous tRNA variants have been detected through suppression screens in *Drosophila*, researchers have engineered amber suppressing $tRNA^{Tyr}$ and $tRNA^{Leu}$ variants, respectively, with a low level of amber stop codon suppression activity when integrated into the *Drosophila melanogaster* genome (Laski *et al.* 1989; Garza *et al.* 1990). In both cases sterility was noted.

Translation requires base pairing of the anticodon, the three nucleotides of the tRNA at positions 34, 35 and 36, with complementary codons in mRNA in the A site of the ribosome. Because the anticodon provides a direct link between the tRNA and its amino acid assignment, it is the main identity element for the aminoacylation (the attachment of an amino acid to the 3' end of a tRNA) for most tRNAs (Giegé *et al.* 1998), with the exception of $tRNA^{Ser}$, $tRNA^{Ala}$ and $tRNA^{Leu}$ (McClain and Foss 1988; Hou and Schimmel 1988; Normanly *et al.* 1992; Asahara *et al.* 1993; Achsel and Gross 1993; Breitschopf *et al.* 1995; Himeno *et al.* 1997). Changing the anticodon of the gene expressing a serine tRNA ($tRNA^{Ser}$) does not affect aminoacylation but changes codon recognition (Garza *et al.* 1990; Geslain *et al.* 2010; Reverendo *et al.* 2014; Zimmerman *et al.* 2018; Berg *et al.* 2019b), resulting in mistranslation. In this study, we stably integrate the gene expressing a serine tRNA variant that mistranslates serine at proline codons into the *Drosophila melanogaster* genome. Development time of flies containing the mistranslating tRNA was extended and fewer flies reached adulthood compared to wild type flies. The tRNA variant increased the prevalence of morphological deformities in adult flies, with females being more severely affected than males. Mistranslation also impaired climbing performance. Cytosolic mistranslating tRNA variants thus impact multiple aspects of the biology of a multicellular organism and in a sex-specific manner.

## 2.2 Materials and Methods

*Fly husbandry and stocks*

All fly stocks were obtained from the Bloomington *Drosophila* Stock Centre and maintained on standard Bloomington recipe food medium (BDSC; Bloomington, Indiana) under a 14:10 light:dark cycle at 24°C and 70% relative humidity.

*Creating transgenic stocks*

The gene encoding wild type $tRNA^{Ser}_{UGA}$ (FlyBase ID: FBgn0050201) was amplified from genomic DNA using primers VK3400/VK3401 (primers are listed in Table S1) and cloned into pCFD4, a kind gift from Dr. Simon Bullock (Port *et al.* 2014), as a *Bgl*II/*Xba*I fragment to create pCB4222. The gene encoding $tRNA^{Ser}$ with a proline UGG anticodon and G26A secondary mutation ($tRNA^{Ser}_{UGG, G26A}$) were made by two step mutagenic PCR with primers VK3400/VK3889 and VK3401/VK3890 and pCB4222 as a template. Products from the first round were amplified with primers VK3400/VK3401 and cloned as a *Bgl*II/*Xba*I fragment into pCDF4 to give pCB4250. Sequences of $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG, G26A}$ are found in Figure S2-1.

To create flies containing mistranslating tRNAs, a stock expressing phiC31 (ΦC31) integrase in the germ line and containing an *attP* site in the left arm of the second chromosome was used (BDSC stock # 25709: $y^1$ $v^1$ *P{nos-phiC31\int.NLS}X;* *P{CaryP}attP40*). Plasmids were injected into *D. melanogaster* embryos (Isaacson 2018). Transgenic flies were identified by their wild type eye colour and balanced using stock #3703 ($w^{1118}$*/Dp(1;Y)*$y^+$ *; CyO/nub*$^1$ $b^1$ *sna Sco* $lt^1$ $stw^3$*; MKRS/TM6B,* $Tb^1$) and #76359 ($w^{1118}$*;* $wg^{Sp\text{-}1}$*/CyO, P{*$w^{+mC}$*=2xTb*$^1$*-RFP}CyO; MKRS/TM6B,* $Tb^1$) to create final stocks of the following genotype: $w^{1118}$; *P{CaryP}attP40[*$v^+$*=tRNA]/CyO, P{*$w^+$*mC=2xTb*$^1$*-RFP}CyO*; *MKRS*/*TM6B*, $Tb^1$. This was the genotype of the flies used in all experiments. After producing offspring, DNA was extracted from both parents of the final cross for PCR amplification using the primer set M13R and VK3400 for sequence confirmation.

*Complementation in* Saccharomyces cerevisiae

The *Bgl*II/*Xba*I fragment of pCB422 encoding *Drosophila* $tRNA^{Ser}_{UGG, G26A}$ was cloned into the *Bam*HI/*Xba*I sites of the yeast-*E. coli* shuttle plasmid YEPlac181 (60 pCB4877). pCB4877 and YEPlac181 were transformed into the yeast strain CY9013 (*MATα his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 tti2Δ-met5Δ-mTn10luk* containing pRS313 (Sikorski and Hieter 1989) expressing *tti2-L187P* (Berg *et al.* 2017)) selecting for growth on minimal plates lacking leucine and histidine. Transformants were streaked onto yeast-peptone (YP) plates containing 2% glucose and 5% ethanol and grown at 30°C for 4 days.

*Mass spectrometry*

Detailed mass spectrometry protocols are described in the supplemental information. Briefly, protein was extracted from twenty pupae per sample, reduced, alkylated and digested into peptides following the R2-P1 method as described in (Leutert *et al.* 2019). Peptides were analyzed on a hybrid quadrupole orbitrap mass spectrometer (Orbitrap Exploris 480; Thermo Fisher Scientific). MS/MS spectra were searched against the *D. melanogaster* protein sequence database (downloaded from Uniprot in 2016) using Comet (release 2015.01; 63). Mistranslation frequency was calculated using the unique mistranslated peptides for which the non-mistranslated sibling peptide was also observed and defined as the counts of mistranslated peptides, where serine was inserted for proline, divided by the counts of all peptides containing proline, respectively, expressed as a percentage.

*Scoring deformities*

Virgin, heterozygous flies were collected within ~8 hours of eclosion and scored for deformities in adult legs (limbs gnarled or missing segments), wings (blistered, absent, fluid-filled, or abnormal size), or abdomen (fused or incomplete tergites). Flies collected before wing expansion were excluded. Sex and type of deformity was recorded. Flies with multiple deformities had each recorded. 433 $tRNA^{Ser}_{UGA}$ flies (227 males and 216 females) and 656 $tRNA^{Ser}_{UGG, G26A}$ flies (345 male and 311 female) were scored. All deformities were photographed through the lens of a stereomicroscope using a Samsung Galaxy S8 camera.

*Developmental assays*

Approximately 250 flies of each genotype ($w^{1118}$; *P{CaryP}attP40[v⁺=tRNA$^{Ser}_{UGA}$]/CyO, P{w⁺mC=2xTb$^{1}$-RFP}CyO*; *MKRS/TM6B, Tb$^{1}$* or $w^{1118}$; *P{CaryP}attP40[v⁺=tRNA$^{Ser}_{UGG, G26A}$]/CyO, P{w⁺mC=2xTb$^{1}$-RFP}CyO*; *MKRS/TM6B, Tb$^{1}$)* were placed into fly cages and allowed to lay eggs for one hour. Seven replicates of 30 eggs from each plate were checked every 12 hours to record progress through development. Sex, zygosity, and deformities of adults were recorded.

*Climbing assays*

Virgin adult flies were sorted by sex and scored for deformities. Deformed flies or flies homozygous for the transgenic tRNA were discarded. Equal numbers were collected from each genotype during each collection period. Sixty flies in 11 vials from each genotype were transferred to new food the day before testing. The number of flies that climbed to a 5 cm line in 10 seconds was recorded. Flies were retested every three days until the flies were 51 days old. Each vial was tested three times.

*Statistical analyses*

Statistical analyses were performed using R Studio 1.2.5001. Analyses used for comparisons were: *t*-test (frequency of proline-to-serine misincorporation between tRNA$^{Ser}_{UGA}$ and tRNA$^{Ser}_{UGG, G26A}$); Wilcoxon rank-sum tests (developmental time data, corrected using Holm-Bonferroni's method); Fisher's exact tests (survival between developmental stages and proportion of deformities, corrected using Holm-Bonferroni's method). A generalized linear model was constructed from the climbing assay data and performance was compared using F-tests corrected using Bonferroni's method.

## 2.3 Results

*A tRNA$^{Ser}$ variant induces mistranslation in* Drosophila melanogaster

To characterize mistranslation in a multicellular organism, we integrated genes encoding wild type tRNA$^{Ser}_{UGA}$ as a control and a tRNA$^{Ser}$ variant that mistranslates serine

for proline (Figure 2-1A) into the left arm of the second chromosome of the *D. melanogaster* genome. The tRNA$^{Ser}$ variant has a proline UGG anticodon and G26A secondary mutation (tRNA$^{Ser}_{UGG, G26A}$). The alleles were balanced over a homolog that has serial inversions, preventing recombinant offspring and transgene loss. tRNA insertions were validated with PCR using primers specific to the inserted plasmid and confirmed by sequencing. The secondary G26A mutation was included in the mistranslating tRNA to dampen tRNA function as we have previously found a tRNA$^{Ser}$ variant with a proline anticodon causes lethal levels of mistranslation when expressed in yeast (Berg *et al.* 2017).

Adults homozygous for tRNA$^{Ser}_{UGA}$ or tRNA$^{Ser}_{UGG, G26A}$ can be produced. However, we were unable to propagate the strain homozygous for tRNA$^{Ser}_{UGG, G26A}$ because crosses between male and female tRNA$^{Ser}_{UGG, G26A}$ homozygotes produce no viable offspring. As such, we used heterozygous flies for our experiments with adults. Studying heterozygous flies may be more biologically relevant as mistranslating tRNAs present in populations are likely to arise as single alleles. We determined zygosity by balancing the tRNAs over a *CyO* homolog containing Tubby-linked RFP and *miniwhite* (Pina and Pignoni 2012). Heterozygous larvae and pupae are identified by the presence of RFP and heterozygous adults by their curly wings and non-white eyes.

As an initial test of mistranslation by *Drosophila* tRNA$^{Ser}_{UGG, G26A}$, we determined if the tRNA rescues growth of a *Saccharomyces cerevisiae* strain containing *tti2-L187P* (CY9013). The *tti2-L187P* allele contains a missense mutation converting a CUA codon for leucine to CCA for proline and results in the slow growth of yeast in medium containing 5% ethanol (Hoffman *et al.* 2017). Mistranslation of proline to serine rescues the growth of yeast cells in ethanol medium (Berg *et al.* 2017). The gene encoding *Drosophila* tRNA$^{Ser}_{UGG, G26A}$ was transformed into a yeast strain that contains *tti2-L187P* as the sole copy of *TTI2*. Cells were transformed with plasmid expressing *Drosophila* tRNA$^{Ser}_{UGG, G26A}$ or vector alone. As shown in Figure 2-1B, *Drosophila* tRNA$^{Ser}_{UGG, G26A}$ enabled growth of the strain on medium containing 5% ethanol indicative of mistranslation by *Drosophila* tRNA$^{Ser}_{UGG, G26A}$.

We then analyzed the proteome of *D. melanogaster* pupae by mass spectrometry to determine the mistranslation frequency (Figure 2-1C; Supplemental File S2-1). Pupae were used because of the extensive cellular remodelling and corresponding rapid changes in protein synthesis that occur during this stage (Mitchell *et al.* 1977; Mitchell and Petersen 1981), and the potential of mistranslation during this stage to influence adult traits such as anatomy or neuronal function. The frequency of proline to serine mistranslation, calculated as the ratio of peptides containing the mistranslated serine residue to peptides containing the cognate proline residue, was ~0.6% in flies expressing $tRNA^{Ser}_{UGG,\ G26A}$. In the control strain, the frequency of proline to serine substitutions was 0.1%.

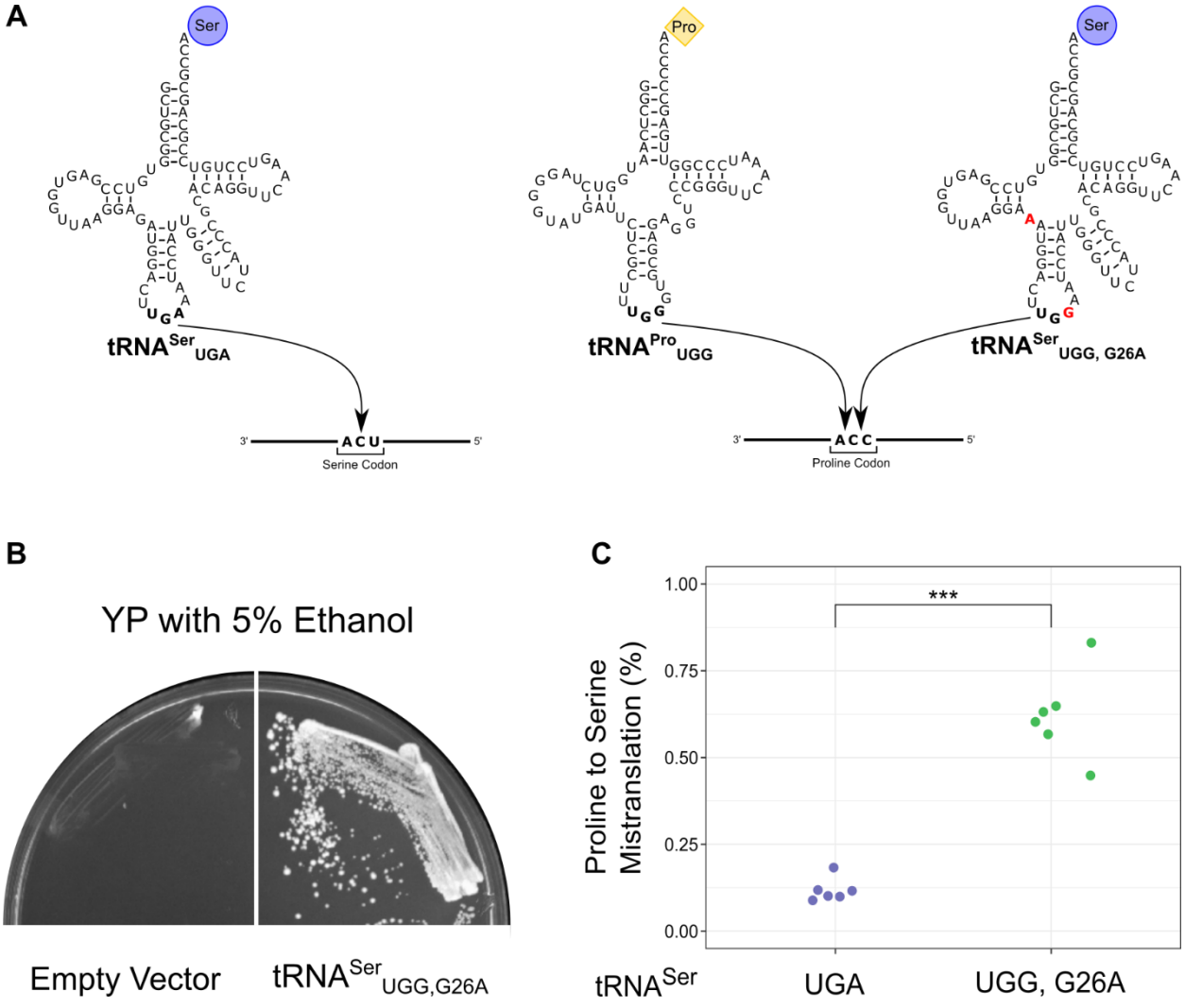


**Figure 2-1.** *$tRNA^{Ser}_{UGG,\ G26A}$ induces mistranslation in D. melanogaster.*

**A)** Wild type serine tRNA base pairs with serine codons, incorporating serine into the growing polypeptide. $tRNA^{Ser}_{UGG,\ G26A}$ competes with $tRNA^{Pro}_{UGG}$ for CCA codons and inserts serine at proline codons. Red bases indicate mutation compared to the wild type $tRNA^{Ser}_{UGA}$. **B)** *D. melanogaster* $tRNA^{Ser}_{UGG,\ G26A}$ transformed into yeast suppresses the ethanol sensitivity caused by *tti2-L187P* in *S. cerevisiae.* Plasmid encoding the vector alone (left) or the gene expressing $tRNA^{Ser}_{UGG,\ G26A}$ (right) were transformed into CY9013 (*tti2-L187P*), streaked onto YP medium containing 5% ethanol and grown at 30°C for 4 days. **C)** Frequency of proline-to-serine mistranslation in $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG,\ G26A}$ pupae (n = 6 replicates of 20 pupae each). Genotypes were compared using a *t*-test. "***" $P < 0.001$.

*$tRNA^{Ser}_{UGG,\ G26A}$ adversely affects* D. melanogaster *development*

To determine if $tRNA^{Ser}_{UGG,\ G26A}$ affects fly development, we collected 210 wild-type $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG,\ G26A}$ one-hour old embryos, comparing survival at twelve-hour intervals through each developmental stage (Figure 2-2A) and time to reach each stage (Figure 2-2B-D): embryos to larvae, larvae to pupae, and pupae to adults. Since the RFP marker used to determine tRNA zygosity is not expressed during early embryonic stages, both homozygotes and heterozygotes were pooled in this assay. While there were fewer female and homozygotic $tRNA^{Ser}_{UGG,\ G26A}$ flies compared to $tRNA^{Ser}_{UGA}$ flies, neither the male bias nor heterozygote bias reached statistical significance (Supplemental file S2-1). Figure 2-2A shows the percentage of individuals who reached a developmental stage relative to those who reached the previous stage (e.g. how many larvae managed to pupate). Of the 210 $tRNA^{Ser}_{UGA}$ embryos collected, 87 hatched into larvae, 47 larvae pupated, and 45 of those pupae reached adulthood. Survival of $tRNA^{Ser}_{UGA}$ was low due to the presence of three balancers (*CyO*, *MKRS*, and *TM6B*) in heterozygous flies and two (*MKRS* and *TM6B*) in homozygous flies. $tRNA^{Ser}_{UGG,\ G26A}$ resulted in reduced viability at each stage as only 66 out of 210 embryos containing $tRNA^{Ser}_{UGG,\ G26A}$ hatched, 32 larvae pupated, and 24 pupae reached adulthood. However, the difference between the proportion of $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG,\ G26A}$ embryos that hatched (41.4% vs. 31.4%, $P = 0.08$, Figure 2-2A) and the proportion of larvae that pupated (54.0% vs. 48.5%, $P = 0.51$) was not statistically significant (Fisher's exact test

corrected using Bonferroni-Holm's method). In contrast, the proportion of $tRNA^{Ser}_{UGA}$ pupae that reached adulthood was significantly higher than $tRNA^{Ser}_{UGG, G26A}$ (95.7% vs. 75.0%, $P = 0.012$). This indicates that flies are particularly susceptible to lethal effects of mistranslating tRNA variants during pupation.

Eggs expressing $tRNA^{Ser}_{UGG, G26A}$ had similar hatching times as eggs expressing wild type $tRNA^{Ser}_{UGA}$ ($P = 0.24$, Wilcoxon rank-sum test corrected using Holm-Bonferroni's method, Figure 2-2B). However, larvae expressing $tRNA^{Ser}_{UGG, G26A}$ pupated significantly slower than the wild type ($P = 0.004$, Figure 2-2C). This trend continued into adulthood, as $tRNA^{Ser}_{UGG, G26A}$ flies eclosed significantly later than control $tRNA^{Ser}_{UGA}$ flies ($P = 0.002$, Figure 2-2D). Median development time of $tRNA^{Ser}_{UGA}$ flies was 288 hours whereas median development time of $tRNA^{Ser}_{UGG, G26A}$ flies was 303 hours. Some extremely late pupation and eclosion events were observed in the mistranslating $tRNA^{Ser}_{UGG, G26A}$ line and were a potential concern as they could have biased the statistical comparisons (Figure 2-2C, D, Supplementary File S2-1). However, $tRNA^{Ser}_{UGG, G26A}$ flies still pupated and eclosed significantly later than $tRNA^{Ser}_{UGA}$ flies even after removing these values ($P = 0.007$ and $P = 0.002$ respectively, Figure S2-1). These results show that flies containing this mistranslating tRNA variant show extended development time and increased developmental lethality.

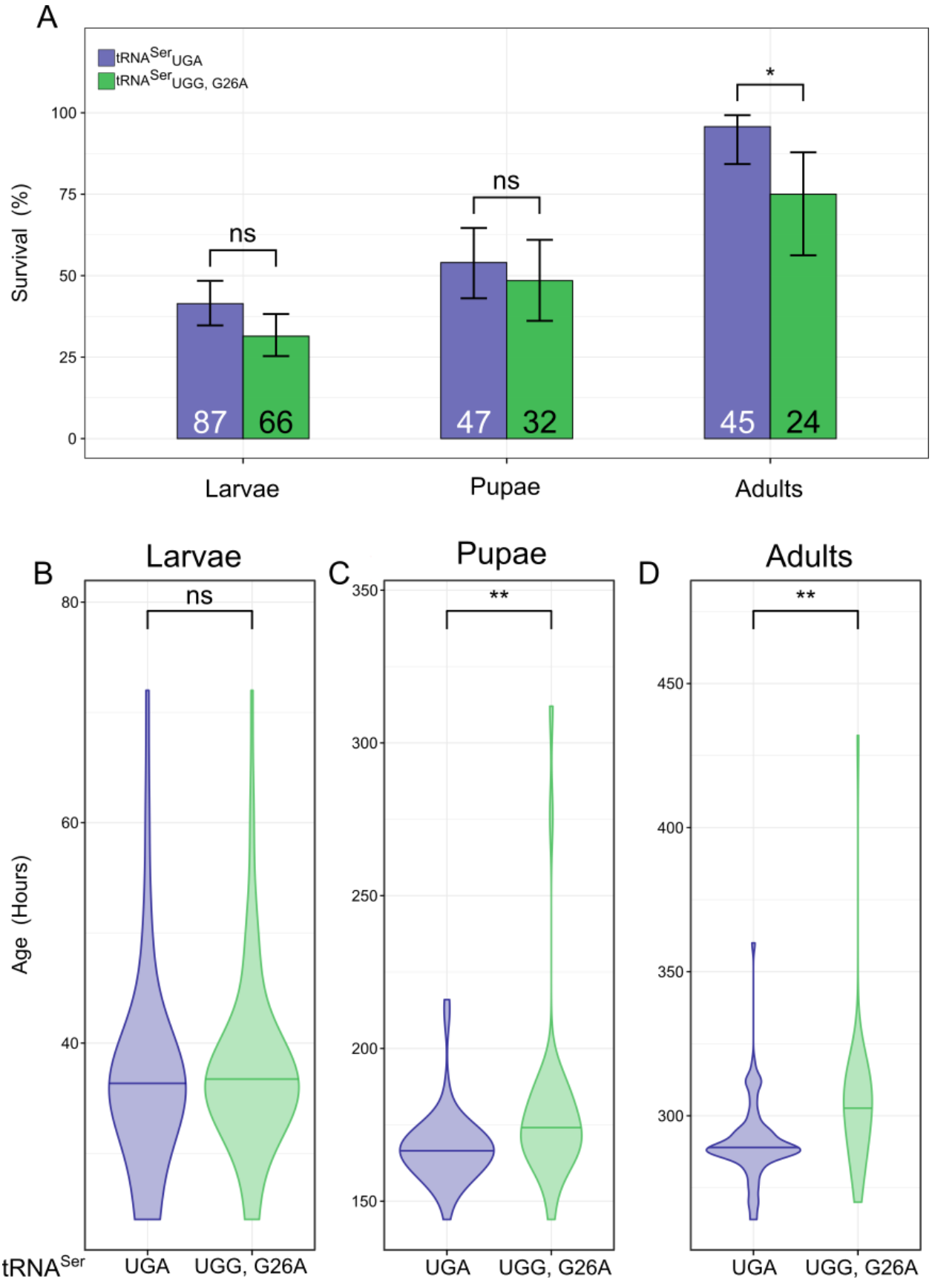
A
tRNA$^{Ser}_{UGA}$
tRNA$^{Ser}_{UGG, G26A}$
Survival (%)
100
75
50
25
0
ns
ns
*
87
66
47
32
45
24
Larvae
Pupae
Adults
B
Larvae
ns
80
60
40
Age (Hours)
tRNA$^{Ser}$
UGA
UGG, G26A
C
Pupae
**
350
300
250
200
150
UGA
UGG, G26A
D
Adults
**
450
400
350
300
UGA
UGG, G26A

Figure 2-2. *A mistranslating tRNA variant impacts development of D. melanogaster*.

**A)** Percentage of the 210 embryos containing $tRNA^{Ser}_{UGA}$ or $tRNA^{Ser}_{UGG, G26A}$ that reached larval, pupal, and adult stages out of survivors from the previous stage. Survival was compared using Fisher's exact test corrected using Holm-Bonferroni's method. Error bars represent the 95% confidence interval of the proportion. Values within bars represent the number of flies that reached that developmental stage. **B)** Violin plot depicting the distribution of times for $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG, G26A}$ embryos to hatch into larva. The horizontal line within the plot represents the median of the distribution. Genotypes were compared using Wilcoxon rank-sum tests corrected using Holm-Bonferroni's method. **C)** Total time until pupation. **D)** Total time until eclosion. "ns" $P \geq 0.05$, "*" $P < 0.05$, "**" $P < 0.01$, "***" $P < 0.001$.

Mutations in genes vital to proteostasis or translation fidelity cause morphological defects (Rutherford and Lindquist 1998; Cui and DiMario 2007; Reverendo *et al.* 2014). We observed that flies containing one copy of the exogenous $tRNA^{Ser}_{UGG, G26A}$ had deformities including gnarled or blistered legs, notched wings, and misfused tergites (Figure 2-3A-D). Other abnormalities (e.g. haltere aberrations or rough eyes) were rarely observed, so only the more common leg, wing, and tergite deformities were scored. To determine if the frequency of deformities was greater than the control, we calculated the proportion of flies that eclosed with at least one deformity. These flies were collected separately from the development assay described above. From a total of 433 $tRNA^{Ser}_{UGA}$ flies (227 males and 216 females) and 656 $tRNA^{Ser}_{UGG, G26A}$ flies (345 male and 311 female) we identified proportionally more deformities in flies containing $tRNA^{Ser}_{UGG, G26A}$ than $tRNA^{Ser}_{UGA}$ (Fisher's exact test corrected using Holm- Bonferroni's method, $P < 0.001$, Figure 2-3E). In addition, female flies containing $tRNA^{Ser}_{UGG, G26A}$ had more deformities than males ($P < 0.001$, Figure 2-3F). Interestingly, flies containing $tRNA^{Ser}_{UGG, G26A}$ presented with disproportionately more tergite deformities than flies with the wild type $tRNA^{Ser}_{UGA}$ (Chi-square test with post-hoc analysis using the method outlined in (Shan and Gerstenberger 2017), $P = 0.03$), indicating that this mistranslating

tRNA$^{Ser}$ variant is particularly deleterious to fly abdominal development. These results suggest that mistranslating tRNA variants can disrupt fly development and that female flies are more sensitive to their effects.

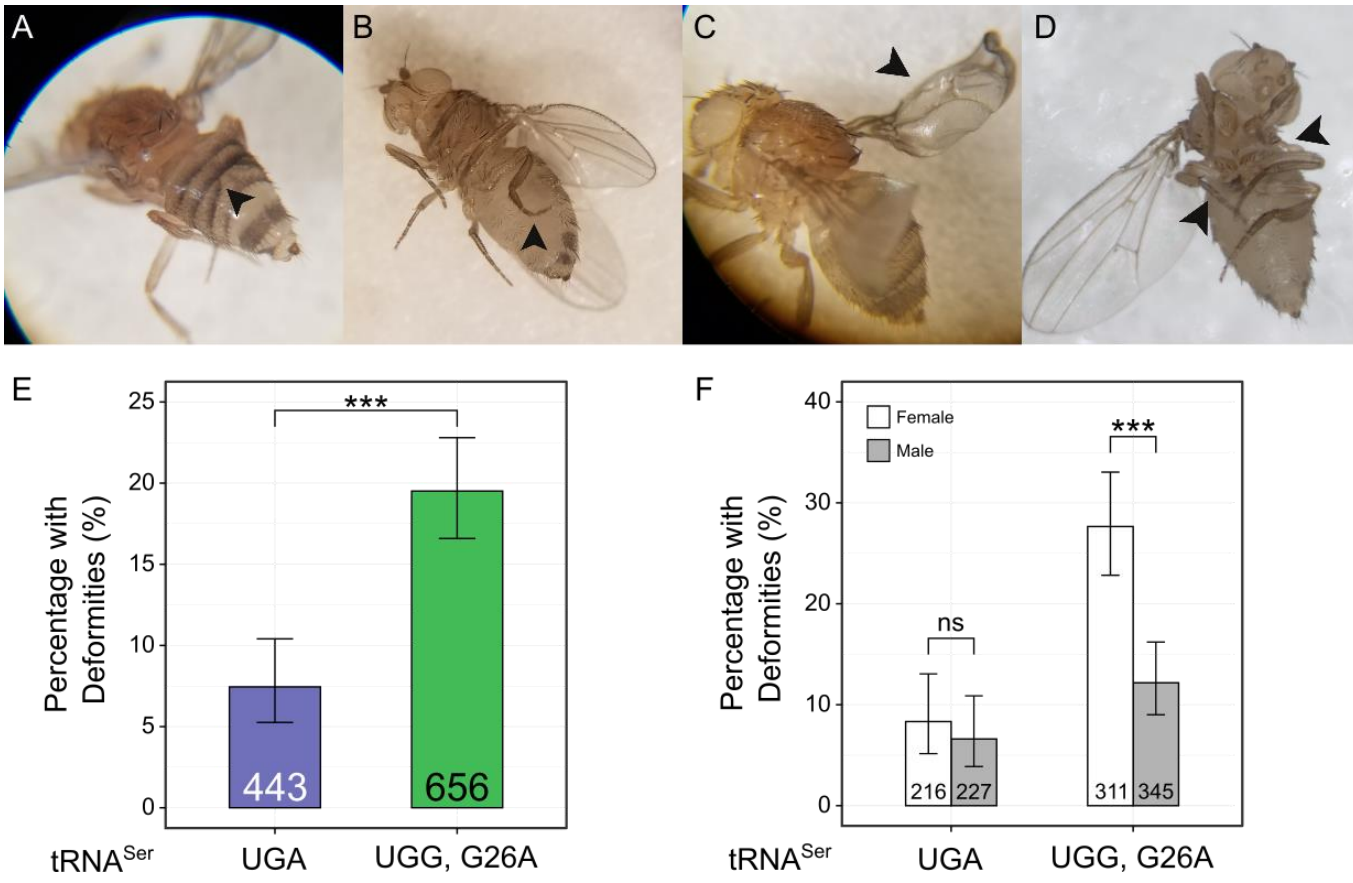


**Figure 2-3.** *The tRNA$^{Ser}_{UGG, G26A}$ variant causes morphological deformities in adults in a sex-specific manner.*

**A)** Examples of flies with misfused tergites, **B)** gnarled hindlegs, **C)** wing blisters, and **D)** missing wings/legs, as indicated by arrowheads. **E)** Percentage of tRNA$^{Ser}_{UGA}$ or tRNA$^{Ser}_{UGG, G26A}$ flies that eclosed with at least one deformity. Groups were compared using Fisher's exact test and corrected using Holm-Bonferroni's method. Bar height represents the percentage of flies of a genotype that had at least one deformity. Error bars represent the 95% confidence interval. Values within bars describe the number of flies examined for deformities. **F)** Same data as E but separated by sex. "ns" $P \geq 0.05$, "*" $P < 0.05$, "**" $P < 0.01$, "***" $P < 0.001$.

### *tRNA$^{Ser}_{UGG, G26A}$ impacts fly motility*

Negative geotaxis assays are often used as an initial test of neurodegeneration in flies (e.g. Warrick *et al.* 1999; Song *et al.* 2017; Aggarwal *et al.* 2019); therefore we determined if tRNA$^{Ser}_{UGG, G26A}$ impaired climbing performance. Sixty virgin, heterozygous flies of the four genotypes (tRNA$^{Ser}_{UGA}$ males and females, and tRNA$^{Ser}_{UGG, G26A}$ males and females) were collected and tested using a climbing assay every three days; flies with deformities were not used in this experiment. Climbing performance of all genotypes decreased with age (F-tests performed on generalized linear models corrected using Bonferroni's method). For both males and females, climbing performance of tRNA$^{Ser}_{UGG, G26A}$ flies was significantly worse than wild type tRNA$^{Ser}_{UGA}$ flies (male: $P$ = 0.001, female: $P < 0.001$, Figure 2-4A, B). Climbing performance was not significantly different when comparing males to females in either the control tRNA$^{Ser}_{UGA}$ ($P = 0.08$) or mistranslating tRNA$^{Ser}_{UGG, G26A}$ flies ($P \rightarrow 1$, Figure 2-4C, D). The climbing ability of male and female flies containing the wild type tRNA$^{Ser}_{UGA}$ declined at similar rates, as evidenced by the parallel performance curves ($P = 0.97$, Figure 2-4C). However, the climbing performance curve of female flies containing tRNA$^{Ser}_{UGG, G26A}$ intersected the male curve, indicating a significant interaction effect between age and sex ($P = 0.038$, Figure 2-4D). Therefore, while overall climbing performance did not differ between tRNA$^{Ser}_{UGG, G26A}$ males and females, rate of performance decline was faster for tRNA$^{Ser}_{UGG, G26A}$ females. These data indicate that the mistranslating tRNA$^{Ser}$ variant negatively affects locomotion and has an accelerated impact on female ability to climb as they age.

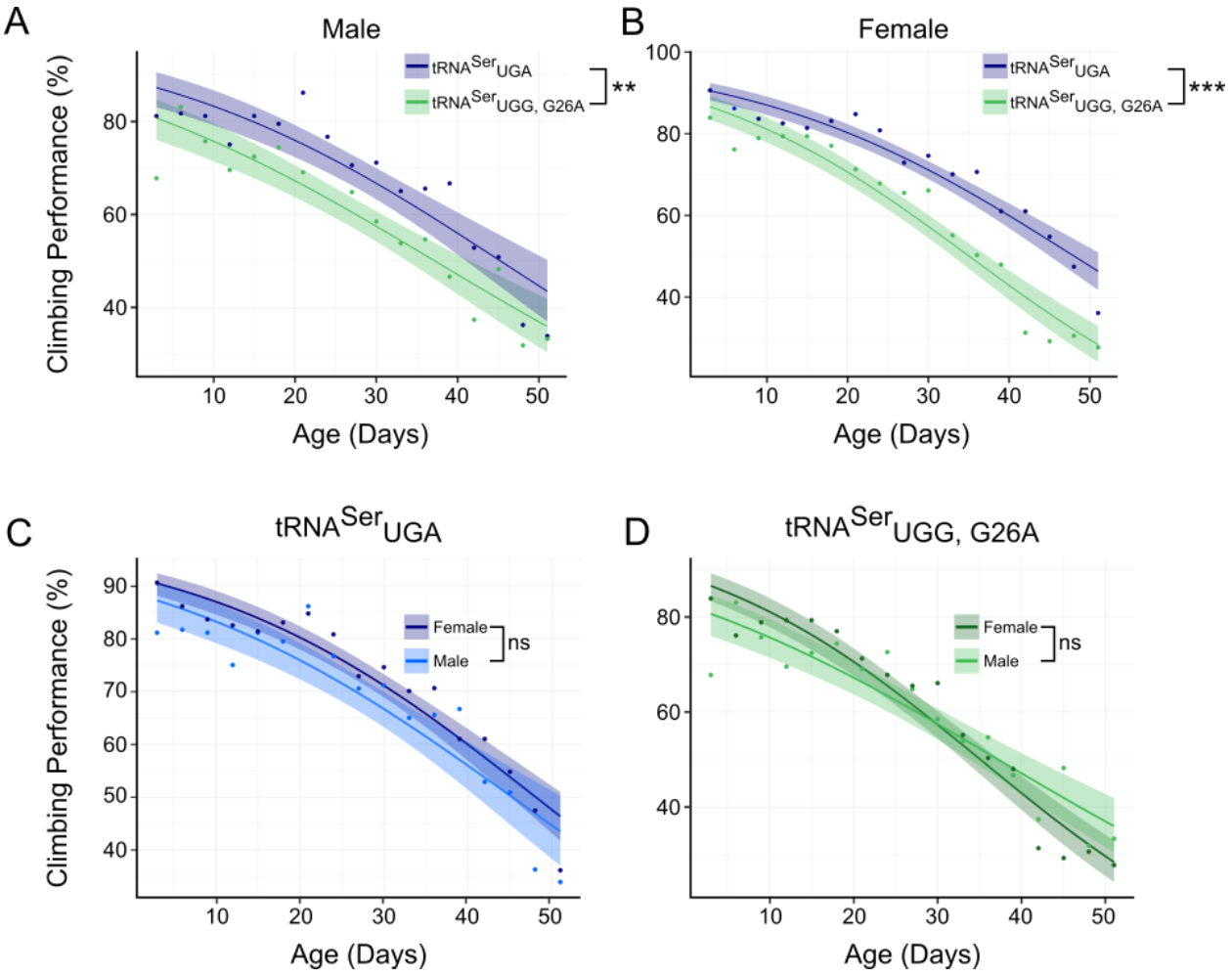


**Figure 2-4.** *Fly locomotion is impacted by a mistranslating tRNA$^{Ser}$ variant.*

Each point represents the percentage of flies (out of 60 individuals from 11 vials) that managed to climb 5 cm in ten seconds averaged over three trials. Generalized linear models were constructed from the performance data and F-tests were performed on the models. P-values were corrected using Bonferroni's method. Shaded region represents the 95% confidence intervals for the fitted performance curves. **A)** Climbing performance of male flies containing tRNA$^{Ser}_{UGA}$ or tRNA$^{Ser}_{UGG, G26A}$. **B)** Climbing performance of female flies containing tRNA$^{Ser}_{UGA}$ or tRNA$^{Ser}_{UGG, G26A}$. **C)** Climbing performance of male and female flies containing tRNA$^{Ser}_{UGA}$ or **D)** tRNA$^{Ser}_{UGG, G26A}$. "ns" $P \geq 0.05$, "*" $P < 0.05$, "**" $P < 0.01$, "***" $P < 0.001$.

## 2.4 Discussion

*A fly model of mistranslation*

We have created a *Drosophila melanogaster* model containing a genomically-integrated cytosolic tRNA that mistranslates serine for proline. The mistranslating fly model allows for studies into sex-specific or tissue-specific effects of mistranslating tRNA variants and the effect of tRNA variants on development and disease. Our method of transgene integration controlled for positional effects by inserting either wild type or mistranslating $tRNA^{Ser}_{UGG,\ G26A}$ into the same locus on chromosome 2L. The fly lines containing $tRNA^{Ser}_{UGG,\ G26A}$ have not lost the transgene for over two years, indicating that mistranslating tRNA variants can be stably maintained in the genome. We observed a proline-to-serine misincorporation rate of ~ 0.6% in the pupae for the genomically integrated $tRNA^{Ser}_{UGG,\ G26A}$ gene. This level of mistranslation was sufficient to cause deleterious phenotypes affecting diverse aspects of fly physiology.

*A mistranslating $tRNA^{Ser}$ variant has diverse and sex-specific effects on flies*

The mistranslating $tRNA^{Ser}_{UGG,\ G26A}$ affects fly physiology consistent with organism-wide loss of proteostasis. Our findings resemble other studies of proteostasis loss in flies. Impaired heat shock response exacerbates neurodegeneration and increases development time (Warrick *et al.* 1999; Gong and Golic 2006), and many of the wing, leg and tergite deformities observed for heterozygous *Heat shock protein 83* (*Hsp83*) mutants look similar to those observed in this study (Rutherford and Lindquist 1998). Developmental and neurodegenerative phenotypes including locomotive defects as measured in a climbing assay were likewise observed in flies containing a misacylation-prone PheRS (Lu *et al.* 2014). It is interesting to note that reduced levels of translation lead to similar deformities as found in mistranslating flies. RNAi knockdown of *Nopp140*, a gene involved in ribosome assembly, causes flies to present with gnarled legs, missing wings, and misfused tergites (Cui and DiMario 2007). *Minute* genes describe a collection of >50 genes required for protein synthesis. Their mutation results in shorter, thinner bristles, delayed development, smaller body size, and anatomical deformities

when mutated (Schultz 1929; Marygold *et al.* 2007), again similar to the developmental and anatomical aberrations seen in flies containing the mistranslating $tRNA^{Ser}$ variant. Though reduced translation and mistranslation are different processes, the similar phenotypes produced demonstrate that development is highly dependent on accurate and efficient translation.

The increased impact of the mistranslating tRNA on female flies was striking. *D. melanogaster* males and females have highly different physiology and experience different developmental challenges. Adult females are larger than males, develop faster, invest more resources into reproduction, and tend to live longer than males (Bonnier 1926; Bakker 1959; Sørensen *et al.* 2007; Ziehm *et al.* 2013). Males and females also display dimorphic responses to proteotoxic stress. Fredriksson *et al.* (2012) examined protein carbonylation in female somatic and germ line cells at different ages to determine how aging affects protein quality control of somatic and reproductive tissues (Fredriksson *et al.* 2012). They found that as females age, there are fewer carbonylated proteins and reduced protein aggregation (both indicators of proteostasis loss) in eggs compared to the soma. Their work shows that females prioritize maintaining proteostasis of their eggs over their somatic cells, even while unmated. This trade-off could exacerbate the stress of mistranslating tRNAs in females, particularly as they experience aging-induced loss of proteostasis, and could contribute to the faster decline of climbing performance observed in female $tRNA^{Ser}_{UGG,\ G26A}$ flies compared to males. Many stress-response pathways affect males and females differently. For example, induction of the heat shock response increases male lifespan whereas female lifespan is unaffected (48, reviewed in 50). Dietary restriction shows the opposite trend, as it increases female lifespan more than male (Nakagawa *et al.* 2012; Regan *et al.* 2016; Garratt 2020). Experiments testing the effects of mistranslating tRNAs on male and female fly longevity are ongoing. It is also possible that expression of the mistranslating tRNA differs between males and females or that the mistranslating tRNA has alternative functions (e.g. tRNA-derived fragments) that differ between males and females.

*Implications for human disease*

Our work suggests that mistranslating tRNA variants have the potential to influence multiple aspects of human physiology. From a development perspective, the alteration in progression through life stages and increased number of deformities suggest that the proteotoxic stress resulting from mistranslating tRNA variants may contribute to congenital or developmental anomalies. Flies expressing $tRNA^{Ser}_{UGG,\ G26A}$ have a pattern of locomotion defects similar to those seen for flies expressing alleles associated with neurodegeneration (Feany and Bender 2000; Song *et al.* 2017; Aggarwal *et al.* 2019). Interestingly, the mistranslating fly model further resembles human neuropathies in that climbing performance declined faster in female compared to male flies, just as some neurodegenerative disorders, such as Alzheimer's and Huntington's Disease, are more common or severe in women compared to men (Viña and Lloret 2010; Zielonka *et al.* 2013).

Given the prevalence of putative mistranslating tRNAs in the human population (Berg *et al.* 2019a) and the potential for mistranslation to disrupt proteostasis, we hypothesize that mistranslating tRNAs can exacerbate diseases characterized by a loss of proteostasis (see also 4), and our results here indicate that these effects may differ in magnitude between sexes. Our previous studies in yeast have shown negative genetic interactions between mistranslation and mutations in genes involved in protein quality control and other pathways that could contribute to disease (Hoffman *et al.* 2017; Berg *et al.* 2020, 2021b). Our *D. melanogaster* model of mistranslation allows for the expansion of these studies into the investigation of mutant tRNA contribution to disease and development.

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**Conflicts of Interest**

The authors declare that there was no conflict of interest while conducting and reporting this research.

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## 2.6 Supplemental Information

*Extended Mass Spectrometry methods*

Liquid chromatography tandem mass spectrometry was performed on strains expressing mistranslating tRNA variants to identify mistranslation. Six replicates of twenty pupae were collected from each genotype and lysed in 8 M urea, 50 mM Tris, 75 mM NaCl, pH 8.2 by grinding with a pestle and with glass beads at 4°C. Protein was reduced with 5 mM dithiothreitol for 30 minutes at 55°C and alkylated with 15 mM iodoacetamine for 30 minutes at room temperature. Robotic purification and digestion of proteins into peptides were performed on the KingFisherTM Flex using LysC and the R2-P1 method as described in Leutert et al. (2019). 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, 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 120 minute acquisition time. The mass spectrometer was operated in data-dependent acquisition mode with a defined cycle time of 3 seconds. 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 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 seconds.

MS/MS spectra were searched against the D. melanogaster protein sequence database (downloaded from Uniprot in 2016) using Comet (release 2015.01; Eng et al. 2013). 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 were allowed per peptide. Search results were filtered to a 1% false discovery rate at the peptide spectrum match level using Percolator (Käll et al. 2007). The mistranslation frequency was calculated using the unique mistranslated peptides for which the non-mistranslated 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, respectively, and expressed as a percentage.

**Table S2-1.** *Primers used in this study.*

| Primer name | Sequence |
|---|---|
| VK3400 | GGGAGATCTGGTATGAAGCATAGATTTCAGC |
| VK3401 | AAATCTAGACCCGCACGGGAAATTCCTAGG |
| VK3889 | AATGGACTTGGAATCCATTGGGTTCTACCCG |
| VK3890 | CCAATGGATTCCAAGTCCATTTCCTTAACCACTC |
| M13R | CAGGAAACAGCTATGACCATG |


**Figure S2-1.** *Sequence alignment of wild type $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG, G26A}$.*

The sequence of the wild type $tRNA^{Ser}_{UGA}$ gene (FlyBase ID: FBgn0050201, top) compared to the engineered $tRNA^{Ser}_{UGG, G26A}$ used in this study (bottom). Highlighted bases represent differences between $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG, G26A}$. Sequences were aligned using Benchling.

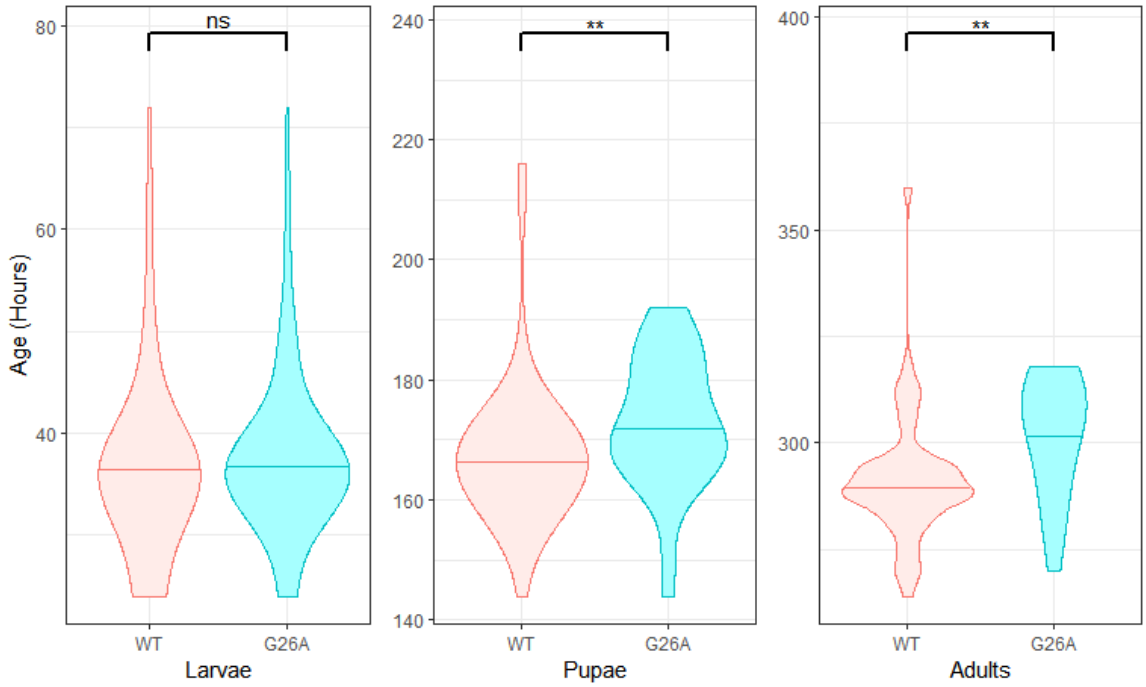


**Figure S2-2.** *Violin plot depicting the distribution of times for $tRNA^{Ser}_{UGA}$ and $tRNA^{SerUGG, G26A}$ embryos to become larvae (left), pupae (middle), or adults (right) excluding very late $tRNA^{Ser}_{UGG, G26A}$ pupation and eclosion events.*

"WT" refers to $tRNA^{Ser}_{UGA}$ and "G26A" refers to $tRNA^{Ser}_{UGG, G26A.}$ The horizontal line within the plot represents the median of the distribution. Genotypes were compared using Wilcoxon rank-sum tests corrected using Holm-Bonferroni's method. "ns" $P \geq 0.05$, "**" $P < 0.01$.

# Chapter 3

# 3 Impact of tRNA-induced proline-to-serine mistranslation on the transcriptome of *Drosophila melanogaster*

Mistranslation is the misincorporation of an amino acid into a nascent polypeptide. Mistranslation has diverse effects on multicellular eukaryotes and is implicated in several human diseases. In *Drosophila melanogaster*, a serine transfer RNA (tRNA) that misincorporates serine at proline codons (P→S) affects male and female flies differently. The mechanisms behind this discrepancy are currently unknown. Here, we compare the transcriptional response of male and female flies to P→S mistranslation to identify genes and cellular processes that underlie sex-specific differences. Both males and females downregulate genes associated with various metabolic processes in response to P→S mistranslation. Males downregulate genes associated with extracellular matrix organization and response to negative stimuli such as wounding, whereas females downregulate aerobic respiration and ATP synthesis genes. Both sexes upregulate genes associated with gametogenesis, but females also upregulate cell cycle and DNA repair genes. These observed differences in the transcriptional response of male and female flies to P→S mistranslation have important implications for the sex-specific impact of mistranslation on disease and tRNA therapeutics.

## 3.1 Introduction

Accurate and efficient translation of mRNA into proteins is required for correct cell function and organism development. Errors during translation can decrease lifespan, induce neurodegeneration, and cause behavioural issues and developmental defects (Lee *et al.* 2006; Lu *et al.* 2014; Reverendo *et al.* 2014; Liu *et al.* 2014). Transfer RNAs (tRNAs) play a major role in determining the fidelity of translation, as do aminoacyl-tRNA-synthetases (aaRSs) which aminoacylate tRNAs with their corresponding amino acid (reviewed in Pang *et al.* 2014). Aminoacyl-tRNA-synthetases recognize specific bases, base pairs, or motifs in their cognate tRNAs to ensure accurate aminoacylation (Hou and Schimmel 1988; Francklyn and Schimmel 1989; Normanly *et al.* 1992; Xue *et al.* 1993; Larkin *et al.* 2002). Because tRNA decoding potential is determined by the

anticodon (the nucleotides at positions 34–36 of the tRNA that base-pair with mRNA codons), the anticodon is an identity element for many tRNAs (Schulman and Pelka 1989; Ruff *et al.* 1991; Jahn *et al.* 1991; Tamura *et al.* 1992; Kholod *et al.* 1997; Giegé *et al.* 1998; Zamudio and José 2018; Giegé and Eriani 2023). However, some aaRSs do not use the anticodon to recognize their cognate tRNA. For example, $tRNA^{Ser}$ and $tRNA^{Ala}$ are recognized through an elongated variable arm and a G3:U70 base pair, respectively (McClain and Foss 1988; Francklyn and Schimmel 1989; Achsel and Gross 1993). Because of this, anticodon mutations in $tRNA^{Ser}$ or $tRNA^{Ala}$ genes cause the tRNA to decode noncognate mRNA codons while still being charged with serine or alanine, thus misincorporating serine or alanine in place of the amino acid normally specified by that codon. This error leads to mistranslation: the incorporation of an amino acid not specified by the standard genetic code. Mistranslation normally occurs at a rate of once per $10^3$–$10^6$ codons (Joshi *et al.* 2019; Mordret *et al.* 2019), but tRNA variants or mutant aaRS genes can dramatically increase mistranslation (Zimmerman *et al.* 2018; Berg *et al.* 2019b; Zhang *et al.* 2021).

Humans have ~66 tRNA variants per person, some of which cause mistranslation (Berg *et al.* 2019a; Lant *et al.* 2021; Hasan *et al.* 2023; Davey-Young *et al.* 2024). Mistranslation induces aberrant phenotypes in a variety of organisms, including slow growth in yeast, deformities and decreased lifespan in flies, and cardiac abnormalities and neurodegeneration in mice (Lu *et al.* 2014; Liu *et al.* 2014; Berg *et al.* 2019b, 2021a; Isaacson *et al.* 2022). Previous work in *Saccharomyces cerevisiae* demonstrated that mistranslation affects various biological processes, including translation, stress response, carbohydrate metabolism, and DNA replication (Paredes *et al.* 2012; Berg *et al.* 2021b). The impact of mistranslation is likely more complex in multicellular organisms as codon usage and gene expression vary by tissue and developmental stage (Moriyama and Powell 1997; Dittmar *et al.* 2006; Vicario *et al.* 2008; Allen *et al.* 2022). Transient expression of mistranslating serine tRNA variants in zebrafish embryos upregulated stress response and DNA repair pathways (Reverendo *et al.* 2014), whereas transfection of human cells with mistranslating tRNAs upregulated protein-folding and small molecule catabolism genes (Hou *et al.* 2024). Some mistranslating tRNA variants reduce overall protein synthesis (Lant *et al.* 2021) and alter expression of other tRNAs (Hou *et al.* 2024). Not surprisingly,

mistranslating tRNAs have been linked to disease (Goto *et al.* 1990; Shoffner *et al.* 1990; reviewed in Abbott *et al.* 2014; Lant *et al.* 2019).

Sex is an understudied but important influence on organismal response to mistranslation, as male and female physiology differ dramatically due to different metabolic and reproductive requirements (reviewed in Millington and Rideout 2018). Supporting this idea, we previously found that a $tRNA^{Ser}$ variant, which causes proline-to-serine (P→S) mistranslation, increased morphological defects and impaired climbing performance in female fruit flies more than males (Isaacson *et al.* 2022). The mechanisms underlying this difference in male and female response to mistranslation are unknown. The goal of this work is to characterize the impact of P→S mistranslation on the transcriptome of male and female *Drosophila melanogaster* to identify genes and cellular processes that are disrupted in one or both sexes. Using a fly line containing a serine tRNA variant ($tRNA^{Ser}_{UGG, G26A}$) that induces P→S mistranslation, we found male mistranslating flies primarily downregulate metabolic, developmental, and extracellular matrix organization genes, and upregulate genes associated with spermatogenesis. Female mistranslating flies downregulate genes associated with metabolism and ATP synthesis, and upregulate genes associated with gametogenesis, cell cycle regulation, and DNA repair. As tRNA variants influence disease and are also being assessed as possible therapeutics (reviewed in Anastassiadis and Köhrer 2023 and Coller and Ignatova 2023; Hou *et al.* 2024), it is vital to understand differences in how males and females respond to mistranslating tRNA variants.

## 3.2 Methods

*Fly stocks and husbandry*

All fly stocks were maintained on standard Bloomington recipe food medium (Bloomington *Drosophila* Stock Center; Bloomington, Indiana) under a 14:10 light:dark cycle at 24° and 70% relative humidity. The tRNA insertion lines used in this study were the same as those described in Isaacson *et al.* (2022). Two fly lines were used: a line containing the wild-type $tRNA^{Ser}_{UGA}$ and a line containing the P→S mistranslating $tRNA^{Ser}_{UGG, G26A}$ (Isaacson *et al.* 2022). The genotype of both lines is as follows:

*$w^{1118}$*; *P{CaryP}attP40[$v^{+}$=tRNA]/CyO, P{$w^{+}$mC=2x$Tb^{1}$-RFP}CyO*; *MKRS/TM6B, $Tb^{1}$*. Note that the lines used in this study are heterozygous for the inserted tRNA. The *attP40* landing site was selected as it is relatively inert while allowing for strong expression of transgenes (Markstein *et al.* 2008).

*RNA extraction, library preparation, and sequencing*

Adult, virgin flies were aged 1–3 days and separated by sex. Ten flies were aspirated into a vial and flash-frozen using liquid nitrogen. Males and females from the $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG,\ G26A}$ (P→S) lines were collected and processed at the same time. Three independent replicates were collected in this manner for each genotype. RNA was extracted from fly tissue using the protocol outlined in Allen (2016), though volumes of all reagents were halved to account for using less tissue than the protocol specified. Following TRIzol extraction, RNA was measured in a Nanophotometer P300 (Implen, Inc.) and concentration, 260/280 ratio, and 260/230 ratio recorded to assess purity (Supplementary Table S1). To ensure RNA was free of genomic DNA, the remaining 25µL of RNA was treated with dsDNAse (New England Biolabs Inc) for 30 minutes at 37°. RNA was recovered through a second TRIzol extraction and samples were assessed again using the Nanophotometer to ensure the RNA remained pure. Up to 20 µg of RNA was loaded into RNA-stabilizing tubes, vacuum-dried, and shipped to GeneWiz (South Plainfield, NJ, USA) for total RNA sequencing. If the total amount of RNA was less than 20 µg, then the entire sample was sequenced. Illumina HiSeq 2x150 bp RNA libraries with polyA selection were prepared from each sample. Number of raw reads obtained from each sample ranged from 12.7 million to 68.7 million.

*RNA sequence data processing*

Analysis of RNA sequencing data was performed using similar methods to those described in Berg *et al.* (2021b). Short and/or low-quality reads were filtered out using a custom bioinformatics pipeline that utilized Trimmomatic v0.39 (Bolger *et al.* 2014) and FASTQC v0.11.9 (Andrews 2010) to produce filtered libraries containing 8.4–35.4 million reads per sample. Reads were aligned to the *Drosophila melanogaster* reference genome (release r6.41_FB2021_04, downloaded from FlyBase.org, Gramates *et al.* 2022)

using STAR v2.7.9a (Dobin *et al.* 2013). Read count data for each gene was summarized using featureCounts v2.0.0 (Liao *et al.* 2014). Only protein-coding genes were included in further analysis. List of protein-coding genes was based on the fly genome assembly BDGP6.46 (Celniker *et al.* 2002; Celniker and Rubin 2003). Parameters and commands used for this pipeline can be found in the extended methods section of supplemental file S2.

*Gene expression and gene ontology analysis*

Statistical tests, principal component analysis (PCA), and RNA-Seq data analyses were conducted using R Studio v1.2.5001. RNA-sequencing sample normalization and differential gene expression analysis was performed using the DESeq2 R package (v1.26.0, Love *et al.* 2014), with a Benjamini-Hochberg false discovery rate (FDR) *P*-value cutoff of $< 0.05$. To control for the batch effect identified by the PCA, we specified sample collection day as a covariate in the statistical model fit by ComBat-seq (Zhang *et al.* 2020). Analysis of differentially-expressed genes was performed using WebGestalt's 2024 release (Liao *et al.* 2019). Lists of down- or upregulated genes were processed by ViSEAGO to produce Gene Ontology (GO) term heatmaps clustered by semantic similarity using Wang's method (Wang *et al.* 2007; Brionne *et al.* 2019; Gene Ontology Consortium *et al.* 2023). Significantly enriched GO terms were identified by ViSEAGO using the "weight01" algorithm and assessed with Fisher's exact test. Background gene lists composed of all genes with non-zero read counts for a given sample set (e.g. all female $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG,\ G26A}$ samples) were provided to WebGestalt and ViSEAGO during enrichment analysis of that sample set as recommended by Timmons *et al.* (2015) and Wijesooriya *et al.* (2022). Figures were produced using RStudio and Inkscape v1.0.1.

*Validation of RNA-Sequencing results using RT-qPCR*

RNA from three new replicates of ten male or female virgin flies containing $tRNA^{Ser}_{UGA}$ or $tRNA^{Ser}_{UGG,\ G26A}$ (P→S) aged 1–3 days were extracted using the protocol described above. cDNA was synthesized from RNA using a Maxima H- First Strand cDNA Synthesis Kit (Thermo Scientific™). Quantitative PCRs were performed on three

independent replicates in duplicate using 10 ng/µL cDNA template, 500 ng/µL primers, and 1x PowerUp™ SYBR™ Green Master Mix for qPCR (Applied Biosystems™) in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The Ct values of experimental genes were compared to the Ct values of αTub84B (FBgn0003884) for normalization and statistical analysis, which was performed by the Bio-Rad CFX Manager 3.0 software (Bio-Rad Laboratories, Inc.). A full list of qPCR primers can be found in Supplemental Table S2.

*Clustering analysis*

Clustering analysis was performed on the relative fold change of gene expression for $tRNA^{Ser}_{UGG,\ G26A}$ (P→S) compared to control $tRNA^{Ser}_{UGA}$ lines and the relative fold change of gene expression between treatment lines and controls within the microarray data described in Zhou *et al.* (2012). Normalized count data were obtained for all samples and relative fold change compared to controls were calculated for each gene within each treatment. Duplicate genes, genes with < 10 normalized reads, or genes with a relative fold change > |5| were excluded from analysis as Z-score transformation is sensitive to outliers. Relative expression fold change values within each sample were Z-transformed and clustered using the ComplexHeatmap package in RStudio (Gu *et al.* 2016) using Ward's method (Ward 1963). Male and female data were clustered separately.

*Data Availability*

Fly lines are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and supplemental material. A full list of all differentially expressed genes and all significantly enriched GO terms can be found in Supplemental file S1. Supplemental file S2 contains all supplementary figures and tables. Supplemental file S3 contains R code used to analyze RNA-sequencing data and perform clustering and ViSEAGO analysis. All raw and processed data can be found at the NCBI GEO database using the accession number GSE256332.

## 3.3 Results

*Identifying mistranslation-induced differentially expressed genes*

To analyze the transcriptomic response in *Drosophila melanogaster* to serine mistranslation at proline codons, we sequenced polyA-enriched RNA from 1–3 day old virgin adult male and female flies containing a single copy of either a wild-type $tRNA^{Ser}_{UGA}$ or a $tRNA^{Ser}_{UGG, G26A}$ variant that mistranslates proline to serine at a frequency of ~0.6% per codon. The secondary G26A mutation was included in the mistranslating $tRNA^{Ser}$ variant as it disrupts a key modification in $tRNA^{Ser}$ species, reducing mistranslation to tolerable levels based on work in yeast (Berg *et al.* 2021a; Boccaletto *et al.* 2022). tRNA insertion lines were maintained as heterozygotes because naturally-occurring mistranslating tRNA variants are likely to arise as single alleles. Principal component analysis (PCA) was performed on the male and female $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG, G26A}$ (P→S) transcriptomic data (Supplemental Figure S3-1). The first two principal components (PC1 and PC2) summarize ~55% of the variance of both male and female data. The variation in PC1 captures the batch effect related to the day each sample was collected, as RNA from replicate 1 was harvested a day before replicates 2 and 3. Samples belonging to $tRNA^{Ser}_{UGA}$ or $tRNA^{Ser}_{UGG, G26A}$ (P→S) cluster together along the PC2 axis, indicating that the variance explained by PC2 likely represents differences due to the mistranslating tRNA (Figure S3-1A, B). We corrected the batch effect using ComBat-seq (Zhang *et al.* 2020), and the resulting PCA plots show that samples cluster well and PC1 represents the variance explained by mistranslation (Figure S3-1 C, D). These plots indicate considerable mitigation of the batch effect.

Differentially expressed genes between $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG, G26A}$ (P→S) were identified using the R package DESeq2 (Love *et al.* 2014). Male and female samples were analysed separately to determine the effects of $tRNA^{Ser}_{UGG, G26A}$ (P→S) on each sex. 13202 genes with nonzero total read counts were evaluated in male samples, whereas 12893 genes were evaluated in female samples. RNA sequencing revealed substantial sex-specific alterations to gene expression, as 426 genes were downregulated and 566 genes were upregulated uniquely in males, whereas 507 genes were downregulated and 432 genes upregulated uniquely in females (Wald test performed by DEseq2, Benjamini-

Hochberg adjusted *P*-values < 0.05, Figure 1A, B). Only 20 genes were upregulated in both male and female flies containing $tRNA^{Ser}_{UGG,\ G26A}$ (P→S) (Figure 3-1A), whereas 340 genes were downregulated in both sexes in the mistranslating line (Figure 3-1B). When comparing male and female expression of differentially-expressed genes, we observed that relative expression of these genes often differed substantially between the sexes (Figure 3-1C). MA plots constructed from male or female RNA-sequencing data show that the majority of genes have a $\log_2$ fold change near zero, as expected (Figure 3-1D, E). These results show that P→S mistranslation disrupts expression of different sets of genes in males and females.

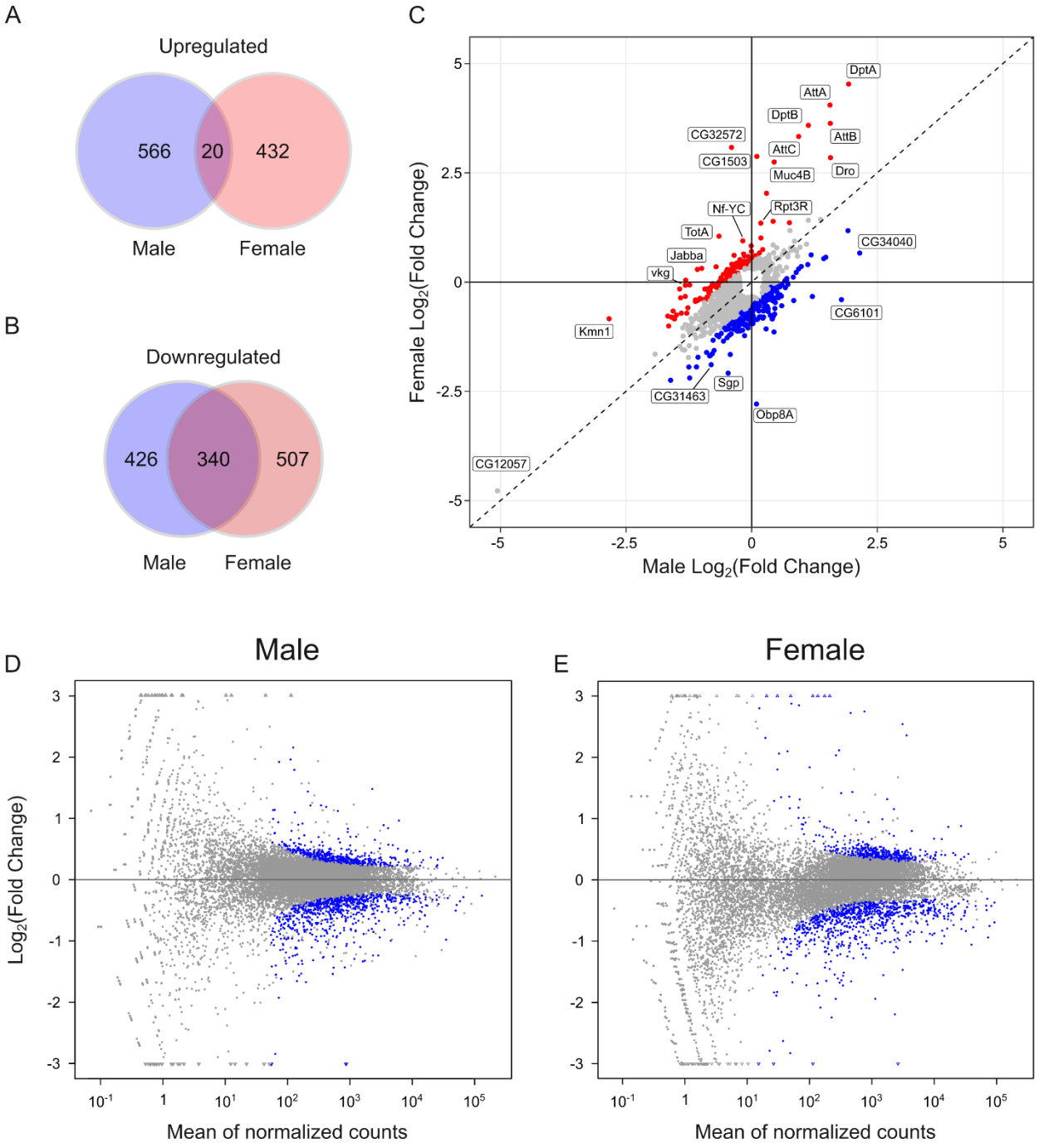


**Figure 3-1.** *Differentially expressed genes in male or female flies containing tRNA$^{Ser}_{UGG, G26A}$ (P→S).*

**A)** Venn diagram showing the number of significantly upregulated (FDR adjusted $P < 0.05$) genes unique to tRNA$^{Ser}_{UGG, G26A}$ (P→S) males, females, or genes upregulated in both sexes. **B)** Venn diagram showing the number of significantly downregulated (FDR

adjusted $P < 0.05$) genes unique to $tRNA^{Ser}_{UGG, G26A}$ (P→S) males, females, or genes downregulated in both sexes. **C)** Scatterplot showing male *vs.* female relative expression for all 1705 genes that were identified as differentially expressed and not filtered out from analysis in either sex. Blue points represent genes that have higher relative expression in mistranslating males compared to females ($log_2$ fold change difference > 0.5); red points represent genes with higher relative expression in mistranslating females compared to males. Genes that demonstrate highly sexually-dimorphic patterns of relative expression ($log_2$ fold change difference > 1) in response to $tRNA^{Ser}_{UGG, G26A}$ (P→S) are labelled. *CG12057* is also labelled due to its strong downregulation in both sexes. The dashed line represents identical fold changes in expression for both males and females. **D)** MA plot visualizing the relationship between transcript abundance and the difference in fold change of expression between male $tRNA^{Ser}_{UGG, G26A}$ (P→S) and control $tRNA^{Ser}_{UGA}$ samples. Blue points represent genes that are significantly differentially expressed between mistranslating $tRNA^{Ser}_{UGG, G26A}$ and control $tRNA^{Ser}_{UGA}$ samples, whereas grey points represent genes where the expression change was not statistically significant. Triangular points at the edge of the y-axis indicate genes that have a fold change exceeding the limits of the y-axis. **E)** MA plot visualizing the relationship between transcript abundance and fold change of expression difference between female $tRNA^{Ser}_{UGG, G26A}$ (P→S) and control $tRNA^{Ser}_{UGA}$ samples.

To provide further support for the transcriptomic data, we confirmed expression level of six genes using RT-qPCR with RNA extracted from three independent replicates of both male and female flies. We analyzed three genes that were downregulated in both sexes (*CG12057, CG11911, and fiz*), one gene that was upregulated in both sexes (*CG4650*), one gene significantly upregulated in males (*Pif1A*), and one gene that was differentially expressed between males and females (*CG1503*). All genes showed the same pattern of expression in both qPCR and RNA-sequencing analysis for both sexes except for *CG11911*, where the difference between flies containing $tRNA^{Ser}_{UGA}$ or $tRNA^{Ser}_{UGG, G26A}$ (P→S) was nonsignificant (Supplemental Figure S3-2). This rate of non-concordance matches the non-concordance rate of 15–19% between RNA-sequencing and

RT-qPCR analysis observed by Everaert *et al.* (2017), who also found non-concordance was more common for short one-exon genes such as *CG11911*. Overall, our RT-qPCR results indicate that RNA-sequencing accurately captures sex-specific differences in transcriptomic response to P→S mistranslation.

*Proline-to-serine mistranslation causes sex-specific transcriptional responses*

We analysed the lists of differentially-expressed genes using two different tools to identify cellular processes affected by the presence of $tRNA^{Ser}_{UGG, G26A}$ (P→S). WebGestalt (Liao *et al.* 2019) was used to identify the ten most enriched Gene Ontology (GO) terms in the list of genes affected by $tRNA^{Ser}_{UGG, G26A}$ (P→S). We also used ViSEAGO (Brionne *et al.* 2019) to construct a heatmap of enriched (GO) terms for males and females, allowing for visualization of sex differences in the fly response to P→S mistranslation. All enriched GO terms, their associated *P*-values, and the genes identified in our analysis that belong to those categories are reported in Supplemental file S1. The list of GO terms produced by WebGestalt showed similarities and differences between male and female response to P→S mistranslation. Both males and females downregulated various metabolic processes (Figure 3-2A), with females primarily downregulating aerobic respiration (e.g., *ox*, *ND-23*, *ND-24*, *UQCR-6.4*, *Cyt-C1*, *COX4*) and males downregulating lipid and fatty acid metabolism (e.g., *Lip4*, *Lsd-1*, *Hacl*, *FASN1*, *CDase*).

There was limited overlap in the biological processes enriched in the upregulated genes shared between males and females, consistent with our observation that relatively few genes were upregulated in both sexes (Figure 3-2B). Females upregulated genes associated with cell cycle regulation and cell division (e.g. *CycA*, *CycB*, *Cdc16*, *APC7*, and *Mink*) as well as genes involved in response to DNA replication (e.g., *DNAlig1*, *PolA1*, *Prim1*, *RecQ4*, and *Fen1*). Only three biological processes were significantly enriched in the list of upregulated genes in male $tRNA^{Ser}_{UGG, G26A}$ (P→S) flies. This may arise because most upregulated genes (438 of 586) are uncharacterized (Supplemental file S1). The three enriched male terms all correspond to male gamete generation and development (e.g., *fan*, *ProtA*, *Pif1A*, and *ntc*).

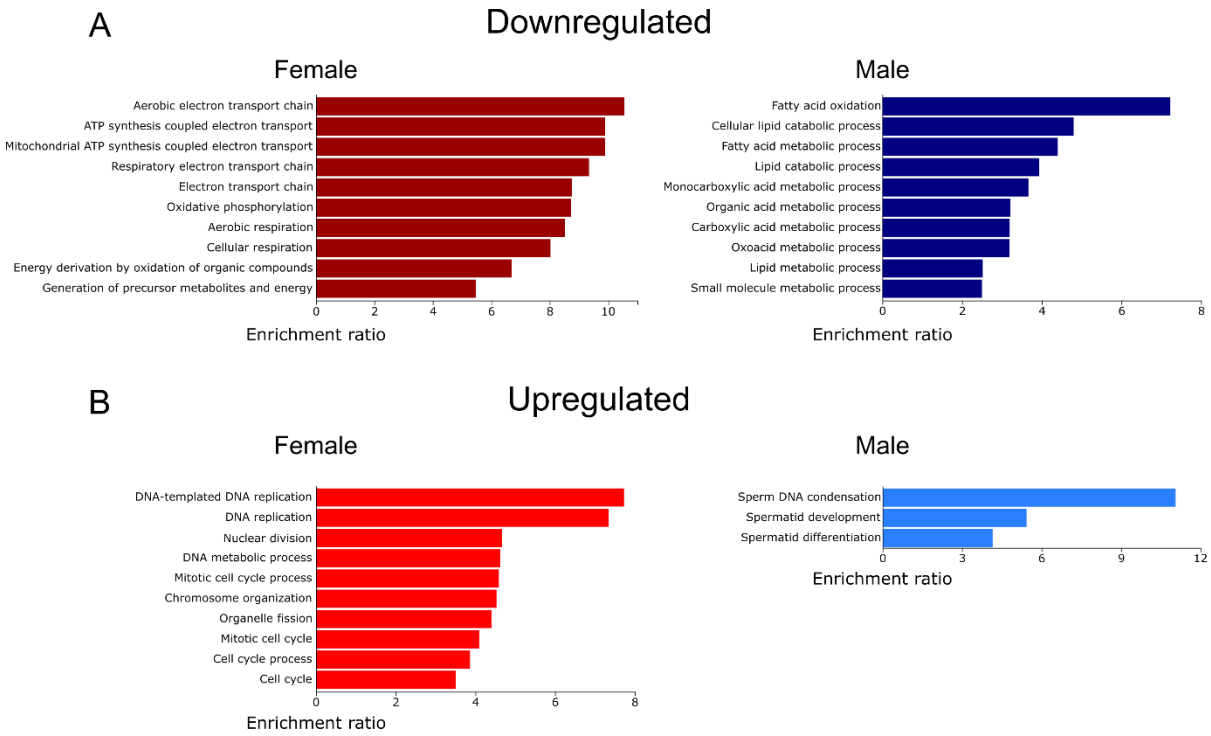


**Figure 3-2.** *The top 10 significantly enriched gene ontology (GO) terms in the list of genes.*

**A)** downregulated or **B)** upregulated in male or female flies containing $tRNA^{Ser}_{UGG,\ G26A}$ (P→S) compared to control $tRNA^{Ser}_{UGA}$ flies. Higher enrichment ratios indicate that the set of genes associated with that GO term were more highly represented in our gene set. Note the differences in scale. Lists were produced using WebGestalt (Liao *et al.* 2019). A list of significantly enriched GO terms and associated statistics is found in Supplemental file S1.

We next used ViSEAGO to construct heatmaps of GO terms enriched in the set of genes down- or upregulated gene in $tRNA^{Ser}_{UGG,\ G26A}$ (P→S). ViSEAGO clusters GO terms by semantic similarity, so GO terms corresponding to similar biological processes are near each other in the dendrogram (Brionne *et al.* 2019). Functional enrichment was determined using the Fisher's exact test. Figure 3-3A further emphasizes the downregulation of genes involved in metabolic processes in response to P→S

mistranslation, with different aspects of metabolism being affected in each sex (Figure 3-3A). In agreement with the WebGestalt results, females downregulated genes associated with oxidative phosphorylation and ATP synthesis whereas males downregulated genes involved in fatty acid and carboxylic acid catabolism. In addition, both males and females downregulated genes involved in chemical or ion transport (e.g., *nrv2*, *blw*, *rumpel*, *snu*). In contrast, biological processes such as response to negative stimuli such as wounding (e.g., *Atg2*, *PPO2*, *Hml*, *Tg*) and extracellular structure organization (*Cad99C, LanA, LanB1*, *LanB2, Col4a1, vkg*) were downregulated only in males. Females uniquely downregulated genes associated with muscle function and development, such as myosin (*Mhc, Mlc1, Mlc2*), troponin (*up*, *wupA*), and tropomyosin (*Tm1* and *Tm2*) genes.

When examining the lists of genes upregulated in male or female flies containing $tRNA^{Ser}_{UGG,\ G26A}$ (P→S), ViSEAGO did not identify any GO terms that were significantly enriched in both males and females though we note that gametogenesis and metabolic processes were affected in both sexes (Figure 3-3B). Of the upregulated genes with identified function, only genes associated with spermatogenesis, protein localization to microtubules, the electron transport chain, and maltose metabolism were enriched in males. For females, in addition to genes associated with cell cycle regulation and DNA repair (discussed above), genes associated with protein and mRNA localization (e.g., *Nup154*, *Elys*, *Fmr1*), development (e.g., *glu*, *mor,* and *fz*), and regulation of gene expression (e.g., *bcd*, *Marf1*, *pum*) were upregulated. Genes involved in antibacterial immune response (e.g., *DptA*, *Dro*, *AttA*, and *BomS5*) were also upregulated in females but not males. These results emphasize that the cellular response to P→S mistranslation differs between male and female flies, and that the difference is particularly pronounced when comparing upregulated genes.

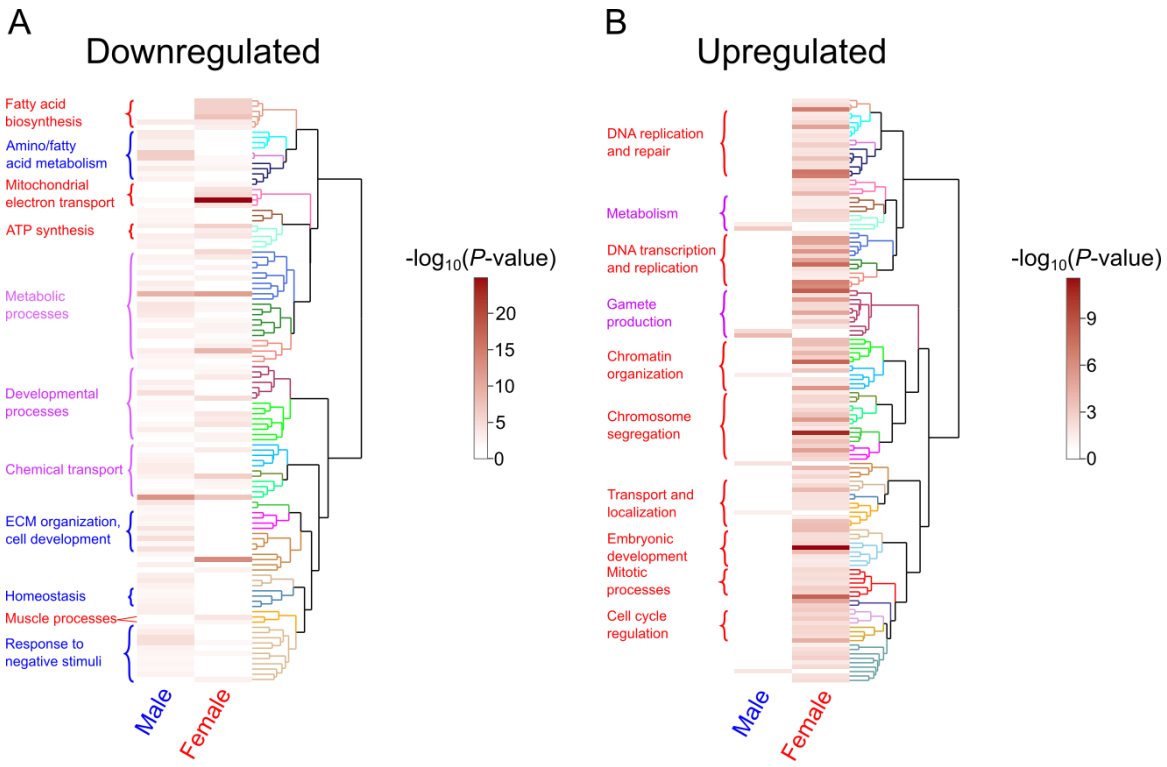


**Figure 3-3.** *Heatmap of enriched gene ontology (GO) terms from the differentially-expressed genes in male or female flies containing tRNA$^{Ser}_{UGG,\ G26A}$ (P→S).*

**A)** Heatmap of enriched GO terms in the list of downregulated genes in male and female flies containing tRNA$^{Ser}_{UGG,\ G26A}$ (P→S). Each horizontal bar represents a GO term identified as significantly enriched in male and/or female data. GO terms were clustered by semantic similarity according to ViSEAGO using Wang's method (Wang *et al.* 2007; Brionne *et al.* 2019). Dendrogram clades of the same colour represent semantically similar GO terms. Darker bars within the heatmap represent lower *P*-values as determined through Fisher's exact test. Notable groups of enriched processes are labelled in blue if enriched in males, red if enriched in females, or purple if enriched in both sexes. **B)** Same as **A)** but using the list of upregulated genes. A full list of enriched GO terms and their associated genes can be found in Supplemental File S1.

*tRNA-induced P→S mistranslation clusters with heat shock and nutrient stress*

Clustering analysis groups genes or treatments based on similarity and is useful to predict functions of uncharacterized genes or identify treatments that produce similar cellular effects (reviewed in Oyelade *et al.* 2016). To identify which environmental or physiological conditions resemble tRNA-induced P→S mistranslation in flies, we clustered the gene expression data from male and female flies containing $tRNA^{Ser}_{UGG,\ G26A}$ with the microarray gene expression data from (Zhou *et al.* 2012), containing the transcriptional response of male and female flies from the same genetic background exposed to 20 different nutritional, chemical, and physiological conditions (Figure 3-4).

As the transcriptomic data acquisition method differed between this study and Zhou *et al.* (2012), we used Z-transformed relative fold changes to compare these datasets. Clustering analysis of male data revealed that tRNA-induced P→S mistranslation induced a transcriptional response most resembling starvation (Figure 3-4A). Mistranslating males also clustered with temperature or chemical stressors such as heat shock, chill coma, and ethanol exposure. In females, the transcriptional response of tRNA-induced P→S mistranslation most resembled flies reared on high yeast or high sugar and high yeast diets (Figure 3-4B). Both male and female flies containing $tRNA^{Ser}_{UGG,\ G26A}$ (P→S) clustered with treatments affecting nutrition, which aligns with our observations that various metabolic processes are affected by $tRNA^{Ser}_{UGG,\ G26A}$ (Figure 3-2A).

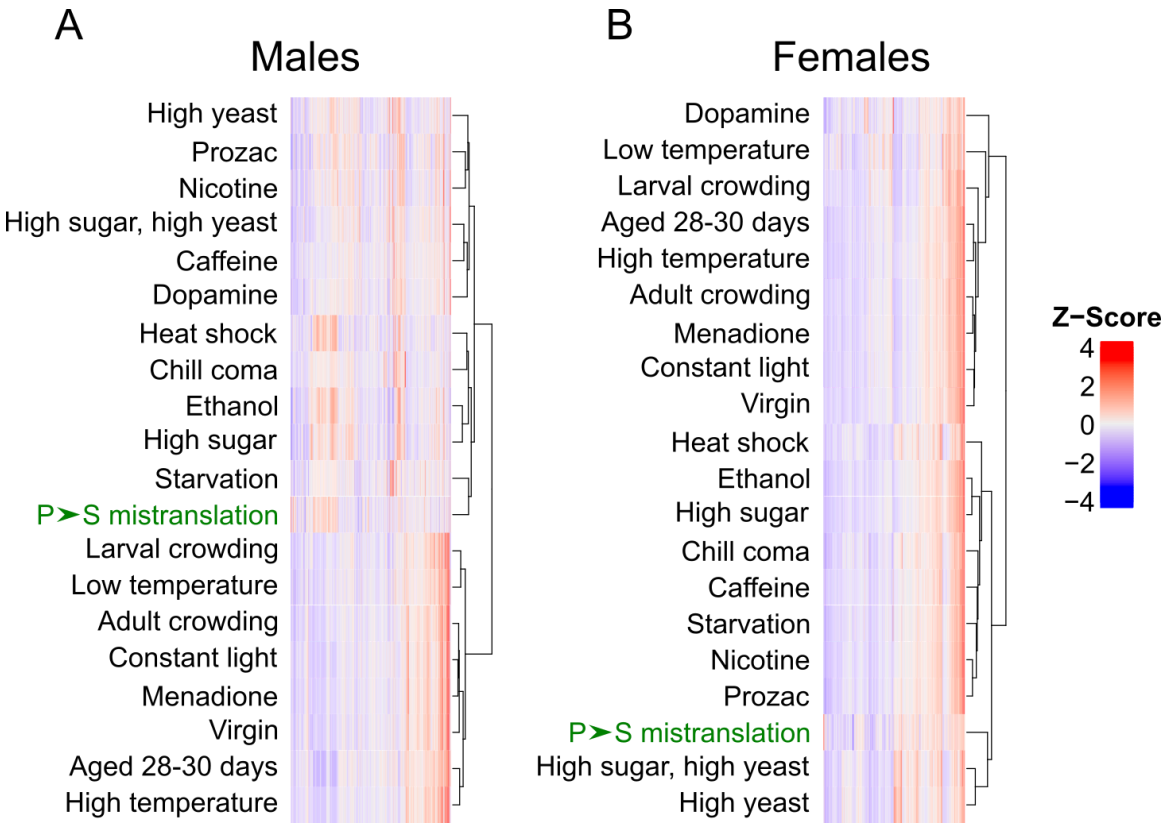


**Figure 3-4.** *Clustering proline-to-serine mistranslation-induced transcriptome changes with transcriptome changes due to various other physiological or environmental conditions.*

**A)** Z-score normalized gene expression changes in $tRNA^{Ser}_{UGG,\ G26A}$ (P→S) males relative to $tRNA^{Ser}_{UGA}$ (wild-type) males clustered with normalized male gene expression changes from Zhou *et al.* (2012). Genes with fewer than 10 normalized reads or fold changes > |5| for any condition were excluded from analysis. Clustering was performed using the "ComplexHeatmap" R package using Ward's method (Ward 1963; Gu *et al.* 2016). The P→S mistranslation condition is highlighted in green. **B)** same as **A)** but clustering female data.

## 3.4 Discussion

*Proline-to-serine mistranslation exerts sex-specific transcriptomic effects*

In this study, we examined how *Drosophila melanogaster* males and females alter their transcriptome when exposed to a mistranslating $tRNA^{Ser}_{UGG, G26A}$ variant that causes P→S mistranslation. While some biological processes such as carboxylic acid metabolism, chemical transport, and germ cell production were affected in both sexes, we observed a disparity between male and female transcriptional response to P→S mistranslation. This result is consistent with the different physiological and nutritional requirements of male and female flies. Female flies are larger, require a greater quantity and variety of nutrients, and store more triglycerides and glycogen than male flies (Bakker 1959; Wu *et al.* 2020, reviewed in Millington and Rideout 2018;). These requirements are largely due to the increased cost of gamete production in females, which also affects virgin flies as they still devote resources to egg production and laying (Partridge *et al.* 1986; Wu *et al.* 2020). Disruptions to proteostasis, such as mistranslation, would exacerbate this discrepancy between males and females, as maintaining proteostasis requires a substantial proportion of all energy produced by the cell (Buttgereit and Brand 1995; Lahtvee *et al.* 2014). The relatively mild phenotypes previously observed in male flies containing $tRNA^{Ser}_{UGG, G26A}$ (P→S) compared to females may in part be due to having more cellular resources available to maintain homeostasis (Isaacson *et al.* 2022).

One notable group of sex-specific upregulated genes were associated with DNA repair and cell cycle regulation. Genes involved with DNA repair are often upregulated in response to cellular stress, and vice versa (Mendez *et al.* 2000; Pregi *et al.* 2017; Sottile and Nadin 2018; Clementi *et al.* 2020). Our observation that DNA repair and cell cycle genes are disrupted in mistranslating flies is consistent with the genetic instability observed by Kalapis *et al.* (2015) in response to mistranslation in yeast. Genetic interactions with mistranslation in yeast and transcriptional responses to mistranslation in human cells also identified the importance of genes involved in cell cycle and DNA damage response (Shcherbakov *et al.* 2019; Berg *et al.* 2021b). Furthermore, mistranslation causes aneuploidy and aberrant nuclear division in yeast species and increases mutation rate in *Escherichia coli* (Balashov and Humayun 2002; Al Mamun *et al.* 2002; Kimata and Yanagida 2004; Silva *et al.* 2007). Mistranslation caused by $tRNA^{Ser}_{UGG, G26A}$ may be exerting similar effects in female flies. Interestingly, female flies

are less susceptible to sources of DNA damage such as oxidative stress or radiation and are better able to decompose reactive oxygen species than male flies (Parashar *et al.* 2008; Edman *et al.* 2009; Moskalev *et al.* 2011; Niveditha *et al.* 2017). The upregulation of DNA repair genes in mistranslating females may result from their observed increased resistance to stress and DNA damage relative to male flies (reviewed in Pomatto *et al.* 2018). Future studies should examine if flies containing $tRNA^{Ser}_{UGG,\ G26A}$ (P→S) show similar genetic instability as mistranslating yeast or *E. coli*.

*Similarity to other transcriptomic studies of tRNA-induced mistranslation*

Other studies have examined the transcriptomic effects of tRNA-induced mistranslation on organisms including yeast (Paredes *et al.* 2012; Berg *et al.* 2021b), zebrafish (Reverendo *et al.* 2014), and human cells (HEK293, Hou *et al.* 2024), though none investigated how males and females differ in their response to mistranslation. Paredes *et al.* (2012) engineered a $tRNA^{Ser}$ variant that mistranslates leucine-to-serine in yeast and observed upregulation of stress response chaperone genes and downregulation of protein synthesis. In agreement with our results, the mistranslating yeast transcriptome clusters with the transcriptome of yeast experiencing nutrient stresses, particularly nitrogen deprivation and amino acid starvation. Zebrafish embryos transiently expressing mistranslating $tRNA^{Ser}$ variants similarly downregulate protein synthesis and upregulate stress response genes and genes associated with DNA damage and repair (Reverendo *et al.* 2014). Human cells transfected with mistranslating $tRNA^{Arg}$ variants upregulate genes involved in protein folding and endoplasmic reticulum stress (Hou *et al.* 2024). Interestingly, some mistranslating $tRNA^{Arg}$ variants have minimal effects on the transcriptome. While we did not observe significant downregulation of genes involved in protein synthesis in males or females containing $tRNA^{Ser}_{UGG,\ G26A}$ (P→S), female flies containing $tRNA^{Ser}_{UGG,\ G26A}$ upregulated genes involved in DNA damage and repair, which aligns with the previous studies. Our transcriptomic data also clusters with data derived from organisms exposed to nutrient stress. Overall, our data is consistent with previous work characterizing the transcriptomic effects of mistranslation in other organisms while uncovering novel sex-specific differences in these general responses.

*Future work and conclusions*

These transcriptomic results provide intriguing avenues for future research. *D. melanogaster* tissues have different codon usages and tRNA expression profiles and thus might be differently susceptible to $tRNA^{Ser}$ variants that cause P→S mistranslation (Dittmar *et al.* 2006; Allen *et al.* 2022). A focused transcriptomic approach centered on specific cell types, such as neurons or muscle, could reveal trends that are difficult to observe from whole-fly transcriptomics. Testing other life stages could also reveal stage-specific transcriptomic responses to mistranslating tRNA variants. Different types of mistranslation exert unique cellular effects (Berg *et al.* 2021b; Cozma *et al.* 2023; Hou *et al.* 2024; Davey-Young *et al.* 2024), so testing other amino acid substitutions will uncover which cellular responses are common to mistranslation and which are unique to proline-to-serine substitutions.

The differentially-expressed genes identified in this analysis can be targeted using available *D. melanogaster* knockout lines to determine which are necessary for the fly response to mistranslation. The uncharacterized gene *CG12057* in particular is worthy of further investigation as its expression was reduced >25-fold in both male and female $tRNA^{Ser}_{UGG,\ G26A}$ (P→S) flies. *CG12057* is primarily expressed in the midgut and its expression is impacted by various stresses, including hypoxia, infection, and mitochondrial dysfunction (Carpenter *et al.* 2009; Fernández-Ayala *et al.* 2010; Mosqueira *et al.* 2010; Moskalev *et al.* 2015; Krause *et al.* 2022). Determining the function of *CG12057* would provide insight into how flies cope with cellular stress. Further investigation into the cellular processes disrupted by P→S mistranslation may elucidate the genetic and physiological mechanisms behind sex-specific response to mistranslation and the striking phenotypes observed in mistranslating adult flies (Isaacson *et al.* 2022). Overall, this study demonstrates that sex strongly affects response to mistranslation and must be considered when studying mistranslation in sexually-dimorphic organisms.

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## 3.6 Supplemental Information

**Extended Methods**

Parameters and commands specified for the programs used as part of the RNA-sequencing pipeline are as follows:

*Trimmomatic v0.39*

Remove poor-quality bases and Illumina adapter sequences

```
java -jar trimmomatic-0.39.jar PE input_forward.fq.gz
input_reverse.fq.gz output_forward_paired.fq.gz
output_forward_unpaired.fq.gz output_reverse_paired.fq.gz
output_reverse_unpaired.fq.gz ILLUMINACLIP:TruSeq3-
PE.fa:2:30:10:2:keepBothReads LEADING:3 TRAILING:3 MINLEN:36
```

*STAR v2.7.9a*

Create genome index:

```
STAR --runThreadN 4 --runMode genomeGenerate --genomeDir
./DrosophilaGenome --genomeFastaFiles ./dmel-all-chromosome-
r6.41.fasta --sjdbGTFfile ./dmel-all-r6.41.gtf --sjdbOverhang
149
```

Align reads to genome:

```
STAR --runThreadN 4 --genomeDir ./DrosophilaGenome/Index --
readFilesIn ./Trimmed/Reads_filtered_1P
./Trimmed/Reads_filtered_2P --outFileNamePrefix
./BAMfiles/Reads_paired --outSAMtype BAM Unsorted

STAR --runThreadN 4 --genomeDir ./DrosophilaGenome/Index --
readFilesIn ./Trimmed/READS_filtered_1U --outFileNamePrefix
./BAMfiles/READS_oneU --outSAMtype BAM Unsorted
```

*FeatureCounts v2.0.0*

Count reads that map to each gene

```
featureCounts -T 4 -p -B -a
./DrosophilaGenome/dmel-all-r6.41.gtf -o ./RNAseq_output.txt
./BAMfiles/PairedAlignedReads.bam
./Bamiles/UnpairedAlignedReads.bam
```

The featureCounts output was then used for DEseq2 analysis in RStudio.

**Table S3-1.** *Concentration and absorbance ratios of RNA extracted from all three replicates of ten 1–3-day old virgin male and female $tRNA^{Ser}_{UGA}$ and $tRNA^{Ser}_{UGG, G26A}$ flies.*

The second value in the replicate column refers to the first or second round of RNA extractions performed on those samples (the extraction before DNase treatment or the extraction following DNase treatment).

| Sample | Replicate | Concentration (ng/µL) | A260/A280 | A260/A230 |
|---|---|---|---|---|
| $tRNA^{Ser}_{UGA}$ -Male | 1-1 | 984 | 2.016 | 2.196 |
| $tRNA^{Ser}_{UGA}$ -Female | 1-1 | 1346 | 2.071 | 2.199 |
| $tRNA^{Ser}_{UGG, G26A}$ -Male | 1-1 | 986 | 2.029 | 2.241 |
| $tRNA^{Ser}_{UGG, G26A}$ -Female | 1-1 | 1604 | 2.062 | 2.197 |
| $tRNA^{Ser}_{UGA}$ -Male | 2-1 | 804 | 2.051 | 2.083 |
| $tRNA^{Ser}_{UGA}$ -Female | 2-1 | 1486 | 2.105 | 2.366 |
| $tRNA^{Ser}_{UGG, G26A}$ -Male | 2-1 | 536 | 2.015 | 2.015 |
| $tRNA^{Ser}_{UGG, G26A}$ -Female | 2-1 | 1664 | 2.096 | 2.514 |
| $tRNA^{Ser}_{UGA}$ -Male | 3-1 | 496 | 2.033 | 2.138 |
| $tRNA^{Ser}_{UGA}$ -Female | 3-1 | 1322 | 2.072 | 2.241 |
| $tRNA^{Ser}_{UGG, G26A}$ -Male | 3-1 | 204 | 2.04 | 1.925 |

| | | | | |
|---|---|---|---|---|
| $tRNA^{Ser}_{UGG, G26A}$ -Female | 3-1 | 1360 | 2.092 | 2.297 |
| $tRNA^{Ser}_{UGA}$ -Male | 1-2 | 520 | 2.000 | 2.047 |
| $tRNA^{Ser}_{UGA}$ -Female | 1-2 | 516 | 2.000 | 2.263 |
| $tRNA^{Ser}_{UGG, G26A}$ -Male | 1-2 | 358 | 1.989 | 1.967 |
| $tRNA^{Ser}_{UGG, G26A}$ -Female | 1-2 | 662 | 2.018 | 2.489 |
| $tRNA^{Ser}_{UGA}$ -Male | 2-2 | 608 | 1.987 | 2.068 |
| $tRNA^{Ser}_{UGA}$ -Female | 2-2 | 1100 | 2.022 | 2.321 |
| $tRNA^{Ser}_{UGG, G26A}$ -Male | 2-2 | 348 | 2.000 | 1.596 |
| $tRNA^{Ser}_{UGG, G26A}$ -Female | 2-2 | 1144 | 2.021 | 2.444 |
| $tRNA^{Ser}_{UGA}$ -Male | 3-2 | 266 | 1.985 | 1.511 |
| $tRNA^{Ser}_{UGA}$ -Female | 3-2 | 972 | 2.042 | 1.869 |
| $tRNA^{Ser}_{UGG, G26A}$ -Male | 3-2 | 172 | 1.870 | 1.458 |
| $tRNA^{Ser}_{UGG, G26A}$ -Female | 3-2 | 998 | 2.037 | 2.189 |

**Table S3-2.** RT-qPCR primers used in this study

| Primer name | Sequence |
|---|---|
| CG12057_qPCR_F | CGCTCCTCCATCAAGACCAT |
| CG12057_qPCR_R | ACAAGCAACACTAGCGACGA |
| fiz_qPCR_F | ACCCGTCGAATCTGAGTTGC |
| fiz_qPCR_R | CCCGATCCTCCCAGCATTTT |
| CG4650_qPCR_F | CGGACTTCTGACGAATGGGA |
| CG4650_qPCR_R | CGCTGCAGTCAGAACTAATTTTTCA |
| Pif1A_qPCR_F | GCCAAGTCGAAGGATCCCAA |

| | |
|---|---|
| Pif1A_qPCR_R | GTCCAGGTCCTGCAGTGTTT |
| CG1503_qPCR_F | TTTCCACCCATCCAAGACCC |
| CG1503_qPCR_R | GCAAAGTTTCCGACACCGAG |
| CG11911_qPCR_F | GTTGAGTTCACAAACGCCCC |
| CG11911_qPCR_R | AATGTAGGCCGACACCTTGG |

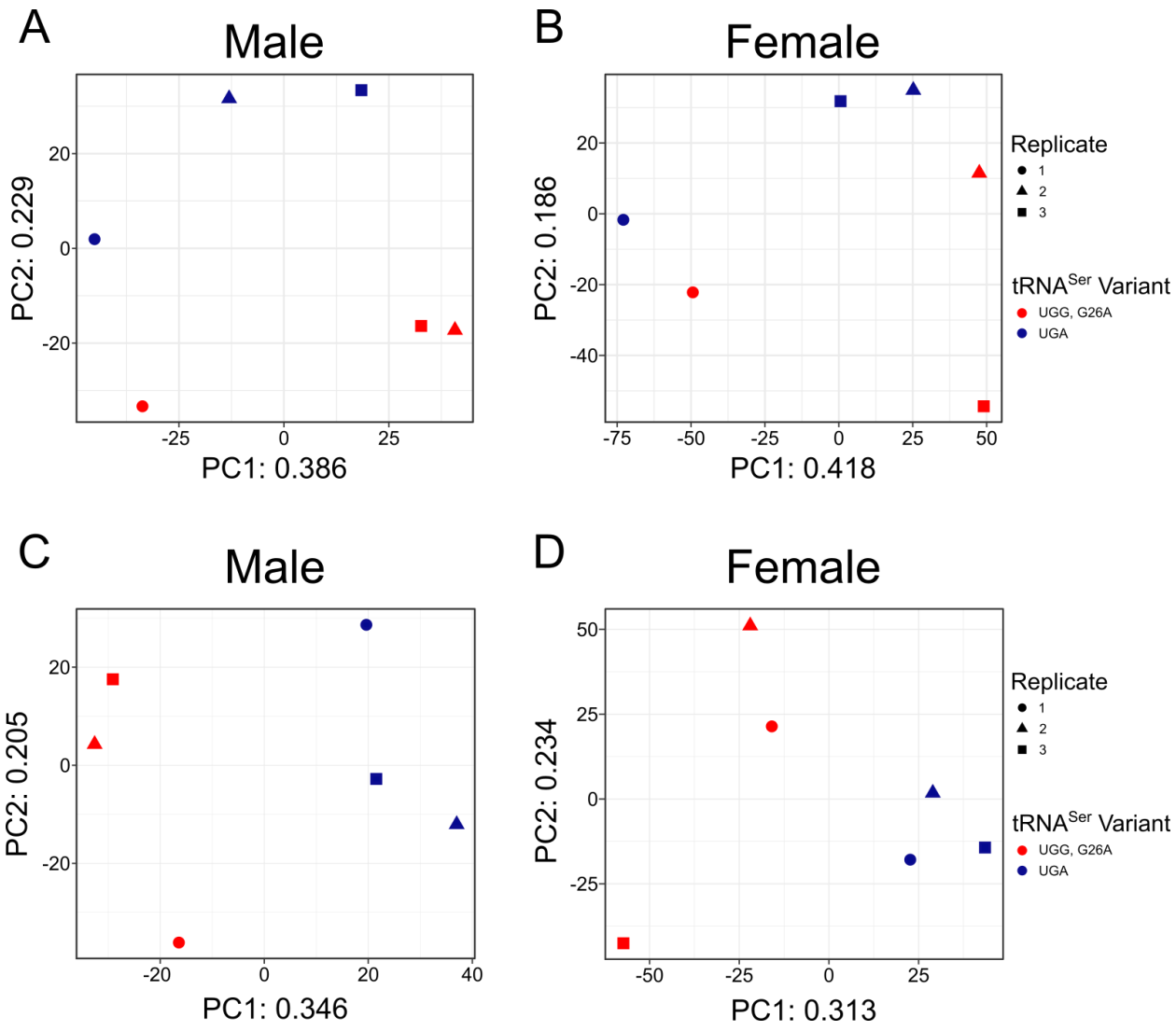

**Figure S3-1.** *Principal component analysis (PCA) of all three replicates of tRNA$^{Ser}_{UGA}$ and tRNA$^{Ser}_{UGG, G26A}$ (P→S) centered log ratio transformed RNA sequencing data.*

**A)** PCA of male RNA sequencing data prior to batch correction. **B)** PCA of female RNA sequencing data prior to batch correction. **C)** and **D)** are the same as **A)** and **B)** but after batch correction using ComBat-seq (Zhang *et al.* 2020). RNA from replicate 1 was extracted on a different day than RNA from replicates 2 and 3. Each point represents one replicate of 10 flies.

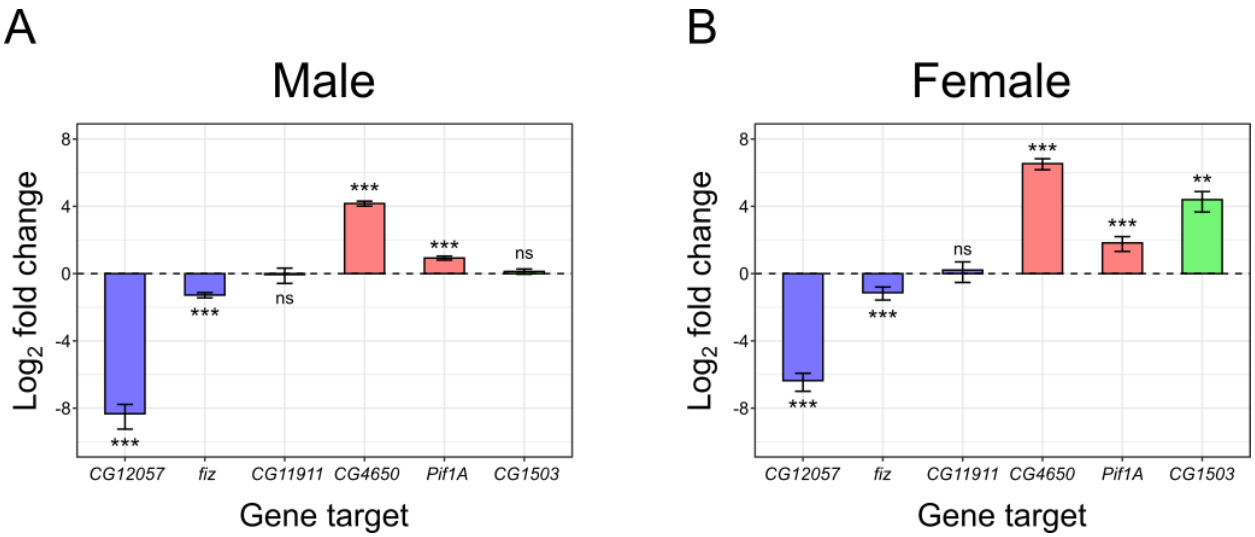


**Figure S3-2.** *RT-qPCR quantification of expression changes for genes identified as differentially expressed from RNA-sequencing.*

Relative expression of the listed gene in **A)** male or **B)** female flies containing tRNA$^{Ser}_{UGG, G26A}$ (P→S) compared to flies containing tRNA$^{Ser}_{UGA}$. Expression changes depicted in blue, red, and green were identified as downregulated, upregulated, or differentially regulated only one sex according to RNA-sequencing, respectively. Significance was calculated using an unpaired *t*-test in the Bio-Rad CFX Manager 3.0 software. Error bars represent the mean ± SEM. "ns" $P \geq 0.05$, "**" $P < 0.01$, "***" $P < 0.001$.

# Chapter 4

# 4 Female fruit flies (*Drosophila melanogaster*) experience neuroprotective effects and lifespan extension when exposed to mistranslating tRNA variants

Transfer RNAs (tRNAs) are vital in determining the specificity of translation. Variant tRNAs can result in the misincorporation of amino acids into nascent polypeptides in a process known as mistranslation. We previously created a model of tRNA-induced mistranslation in the fruit fly *Drosophila melanogaster* and found that mistranslation has diverse and sex-specific effects. Since impact on the proteome depends on the type of amino acid substitution, here we characterize the effects on fruit fly development, lifespan, and behaviour of two mistranslating $tRNA^{Ser}$ variants that misincorporate serine at either valine codons (V→S) or at threonine codons (T→S). While both variant tRNAs increased development time and developmental lethality, these manifest differently depending on the amino acid substitution and sex of the fly. The V→S variant extended embryonic, larval, and pupal development whereas the T→S only extended larval development. Females, but not males, containing either mistranslating variant presented with significantly more anatomical deformities than control lines and had an extended lifespan. In addition, males and females from both mistranslating lines climbed as well or better than male or female control flies. These results show that the impact of mistranslation on fruit flies varies with type of amino acid substitution. We also demonstrate that mistranslation can have positive effects on complex traits such as lifespan and locomotion, which has important implications for human health given the prevalence of tRNA variants in humans.

## 4.1 Introduction

The translation of nucleotide sequence into protein is a fundamental cellular process that requires a high degree of accuracy. By delivering the correct amino acid to the nascent peptide chain at the ribosome, aminoacylated transfer RNAs have a principal role in translation fidelity. Aminoacyl-tRNA-synthetases (aaRSs), one for each of the twenty amino acids in eucaryotes, aminoacylate their tRNA substrates with their cognate

amino acid (reviewed in Pang *et al.* 2014). Specific bases and motifs within tRNAs, known as identity elements, are used by aaRSs to identify their cognate tRNA for accurate aminoacylation (Hou and Schimmel 1988; Normanly *et al.* 1992; Xue *et al.* 1993). The anticodon, spanning bases 34–36 and which base-pairs with the mRNA codon, is an identity element for many aaRSs as it can be a direct link to both the aaRS and the codon assignment (Schulman and Pelka 1989; Ruff *et al.* 1991; Jahn *et al.* 1991; Tamura *et al.* 1992; Kholod *et al.* 1997; Giegé *et al.* 1998; Zamudio and José 2018; Giegé and Eriani 2023). If mischarging of a tRNA does occur, some aaRS molecules also contain editing domains that deacylate the tRNA (Dock-Bregeon *et al.* 2000; Perona and Gruic-Sovulj 2014; Kuzmishin Nagy *et al.* 2020).

The anticodon is not an identity element for eukaryotic $tRNA^{Ser}$, $tRNA^{Leu}$, and $tRNA^{Ala}$ (McClain and Foss 1988; Hou and Schimmel 1988; Asahara *et al.* 1993; Achsel and Gross 1993; Breitschopf *et al.* 1995; Himeno *et al.* 1997; reviewed in Giegé and Eriani 2023). For $tRNA^{Ser}$, the extended variable arm is the principal identity element (Normanly *et al.* 1992; Achsel and Gross 1993; Lenhard *et al.* 1999). Because only the extended variable arm is required for aminoacylation by SerRS, $tRNA^{Ser}$ variants that contain non-serine anticodons will be serylated and result in the incorporation of serine in place of the anticodon-designated amino acid (Garza *et al.* 1990; Reverendo *et al.* 2014; Berg *et al.* 2017, 2019b; Lant *et al.* 2018; Zimmerman *et al.* 2018; Isaacson *et al.* 2022). In addition, since the ribosome has a limited ability to screen for misacylated tRNAs (Dale *et al.* 2009), increased levels of mistranslation can be achieved by $tRNA^{Ser}$ anticodon variants.

Mistranslation has diverse effects on an organism. An editing-deficient PheRS reduces lifespan, impairs locomotion, and causes neurodegeneration in *Drosophila melanogaster* (Lu *et al.* 2014), promotes cardiac abnormalities, neurodegeneration and tumor growth in mice (Lee *et al.* 2006; Liu *et al.* 2014; Santos *et al.* 2018), and causes developmental deformities in zebrafish (Reverendo *et al.* 2014). In human cells, mistranslating tRNAs reduce translation rate and impair clearing of polyQ protein aggregates (Lant *et al.* 2021). Interestingly, mistranslation can also have a positive impact (Ribas de Pouplana *et al.* 2014). For example, mistranslation acts as a stress-response

mechanism, used by bacterial, yeast, and human cells to withstand oxidative stress (Santos *et al.* 1999; Netzer *et al.* 2009; Fan *et al.* 2015; Evans *et al.* 2019; Samhita *et al.* 2020).

Despite the effects of mistranslation on cell biology and a recent sequencing study estimating that humans contain ~66 cytoplasmic tRNA variants per individual (Berg *et al.* 2019a), the effects of mistranslating tRNAs on multicellular organisms are poorly understood. To address this, we previously created a model of cytoplasmic tRNA mistranslation in the fruit fly *Drosophila melanogaster* that misincorporates serine at proline codons (Isaacson *et al.* 2022). Flies containing the mistranslating tRNA variant had increased development time and developmental lethality, more anatomical deformities, and worse climbing performance than flies containing a wild-type serine tRNA. Consistent with differences of the serine at proline mistranslation on the transcriptomic profile of males versus females (see Chapter 3), females presented with more deformities and faster climbing performance decline than males. Since previous work in yeast demonstrated that the effects of mistranslation vary with type of amino acid substitution (Berg *et al.* 2021b), it is important to determine how different mistranslating $tRNA^{Ser}$ variants impact the physiology of flies. In this study, we generate $tRNA^{Ser}$ variants that substitute serine at either valine (V→S) or threonine codons (T→S) and compare how these two different types of mistranslation affect flies. Both substitutions extended development time, reduced survival through development, and significantly increased the prevalence of deformities in females. Females from both $tRNA^{Ser}$ lines experienced an increase in lifespan whereas male lifespan was unaffected. Variant $tRNA^{Ser}$ genes improved climbing performance in both males and females. Thus, mistranslating tRNA genes exert strong positive and negative effects on fruit flies that differ by sex and type of amino acid substitution.

## 4.2 Materials and Methods

*Fly husbandry and stocks*

All fly stocks were obtained from the Bloomington *Drosophila* Stock Center and maintained on standard Bloomington recipe food medium (BDSC; Bloomington, Indiana) under a 14:10 light:dark cycle at 24°C and 70% relative humidity.

*Plasmid construction*

The shuttle vector used to integrate tRNAs into the *D. melanogaster* genome is pattB, which was a kind gift from Bischof *et al.* (2012, DGRC # 1420). The *Not*I site within pattB was removed through digestion and blunting with the Klenow fragment of DNA polymerase, creating pattB-*Not*IΔ. A $tRNA^{Ser}_{UGG,\ G26A}$ gene (a variant of FlyBase ID: FBgn0050201), along with ~300bp of upstream and downstream sequence (Figure S4-1), was flanked with FRT sites and synthesized by Integrated DNA Technologies, Inc. The tRNA sequence within the FRT sites was bookended by *Not*I sites, and the entire FRT-tRNA-FRT fragment was flanked by *Eco*RI and *Bam*HI sites, enabling swapping the tRNA by cloning in a new tRNA gene as a *Not*I fragment. The synthesized fragment was cloned into pattB-*Not*IΔ as an *Eco*RI/*Bam*HI fragment, creating pattB-*Not*IΔ/pUCIDT.

The serine tRNA variant that contains a valine AAC anticodon and G26A mutation ($tRNA^{Ser}_{AAC}$) was made through two-step PCR using $tRNA^{Ser}_{UGA}$ (FlyBase ID: FBgn0050201) from genomic DNA as a template. The primers tSerAAC_F/tSerDS and tSerAAC_R/tSerUS were used in the first round, and products from the first round were amplified using outside primers tSerUS/tSerDS during the second round (all primer sequences are listed in Table S1). Second round PCR products were cloned into pGEM®-T Easy (Promega) and sequenced. Correct plasmids were digested with *Not*I and the tRNA fragment cloned into pattB-*Not*IΔ/pUCIDT to flank the tRNA with FRT sites. An identical procedure was used to create the serine tRNA variant that contains a threonine AGU anticodon and G26A mutation ($tRNA^{Ser}_{AGU}$), instead using primers tSerAGU_F/tSerDS and tSerAGU_R/tSerUS in the first round.

*Creating mistranslating stocks*

Mistranslating tRNAs were integrated into flies by injecting plasmids into *D. melanogaster* embryos from BDSC stock # 24872 (*$y^1$ M{RFP[3xP3.PB]*

*GFP[E.3xP3]=vas-int.Dm}ZH-2A w*$^{*}$*; PBac{y*$^{+}$*-attP-3B}VK00037*), which expresses phiC31 (ΦC31) in the germ line and contains an *attP* site in the left arm of the second chromosome. The injection protocol has been described (Isaacson 2018). Transgenic flies were identified through their mini-white eye colour and balanced using BDSC stock # 3703 (*w*$^{1118}$*/Dp(1;Y)y*$^{+}$ *; CyO/nub*$^{1}$ *b*$^{1}$ *sna Sco lt*$^{1}$ *stw*$^{3}$*; MKRS/TM6B, Tb*$^{1}$) to create stocks of the genotype *w*$^{1118}$; *P{CaryP}-attP40[w*$^{mw+}$*=pattB-tRNA]*/*CyO*; *MKRS*/*TM6B*. DNA was extracted from parents of the final cross, PCR amplified using the primer set pattB-tRNA-Ver_F/pattB-tRNA-Ver_R, and sequenced to confirm accuracy of the inserted tRNA.

*Creating FLP-out controls*

Flanking the inserted tRNA with FRT sites oriented in the same direction allowed removal of the inserted tRNA in the presence of flippase (Gronostajski and Sadowski 1985). To ensure complete removal of the tRNA in control lines, flies containing $tRNA^{Ser}_{AAC}$ or $tRNA^{Ser}_{AGU}$ were crossed to a UAS-FLP line (BDSC stock # 4540: *w*$^{*}$*; P{w*$^{+mC}$*=UAS-FLP.D}JD2*) and a germ-line specific *nanos*-Gal4 line (BDSC stock # 4937: *w*$^{1118}$*; P{w*$^{+mC}$*=GAL4::VP16-nanos.UTR}CG6325*$^{MVD1}$). Offspring were crossed to each other and removal of the tRNA in both parents was confirmed by PCR using primer set FRT-tRNA-Ver_F/FRT-tRNA-Ver_R (Figure S4-2). Successful tRNA FLP-out lines were then crossed back to stock # 3703 to create control lines of the following genotype: *w*$^{1118}$; *P{CaryP}-attP40[w*$^{mw+}$*=pattB-FLP-out]*/*CyO*; *MKRS*/*TM6B*. Control lines for $tRNA^{Ser}_{AAC}$ are referred to as $tRNA^{Ser}_{AAC}$-FLP and control lines for $tRNA^{Ser}_{AGU}$ are referred to as $tRNA^{Ser}_{AGU}$-FLP.

*Mass spectrometry*

Five replicates of twenty pupae or ten adults were collected from each genotype and lysed in 8 M urea, 50 mM Tris, 75 mM NaCl, pH 8.2 by beating with 0.5 mm glass beads at 4°C and protein concentration was determined by bicinchoninic acid assay (Pierce, ThermoFisher Scientific). Protein was reduced with 5 mM dithiothreitol for 30 minutes at 55°C, alkylated with 15 mM iodoacetamine for 30 minutes at room temperature in the dark and the alkylation was quenched with an additional 5 mM

dithiothreitol for 30 minutes at room temperature. For each sample, 50 µg of protein was diluted four-fold with 50 mM Tris pH 8.9 and digested for 4 hours at 37°C with 1.0 µg LysC (Wako Chemicals). Digestions were acidified to pH 2 with trifluoro-acetic acid and desalted over Empore C18 stage tips (Rappsilber *et al.* 2007) .

Peptides were resuspended in 4% acetonitrile, 3% formic acid and subject to liquid chromatography couple to tandem mass spectrometry on a tribrid quadrupole Orbitrap mass spectrometry (Orbitrap Eclipse; ThermoFisher Scientific) operated in data dependent acquisition mode as described in Cozma *et al.* (2023).

MS/MS spectra were searched against the *D. melanogaster* protein sequence database (downloaded from Uniprot in 2016) using Comet (release 2015.01; Eng *et al.* 2013). 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) were used for all searches. A variable modification of valine to serine (-12.0364 Da) or threonine to serine (-14.0156 Da) were used for the respective mistranslating tRNA and control samples. A maximum of two of each variable modification were allowed per peptide. Search results were filtered to a 1% false discovery rate at the peptide spectrum match level using Percolator (Käll *et al.* 2007). The mistranslation frequency was calculated using the unique mistranslated peptides for which the non-mistranslated sibling peptide was also observed. The frequency is defined as the counts of mistranslated peptides, where serine was inserted for valine or threonine, divided by the counts of all peptides containing valine or threonine, respectively, and expressed as a percentage.

*Development assay*

Approximately 250 flies from each of the four genotypes were placed into fly cages and allowed to lay eggs for one hour. Equal numbers of eggs were collected from each plate and checked every 12 hours to record progress through each of the following developmental stages: egg hatching into larva, larva pupating into pupa, and adult eclosing from pupa. In total, 200 eggs from each genotype were collected. Sex and zygosity of adults were recorded.

*Scoring for deformities*

Virgin, heterozygous flies from the two mistranslating lines and their corresponding controls were collected within ~8 hours of eclosion and scored for deformities in adult legs (limbs gnarled or missing segments), wings (blistered, absent, fluid-filled, or abnormal size), or abdomen (fused or incomplete tergites). Flies collected before wing expansion were excluded. Sex and type of deformity was recorded. Flies that had multiple deformities had each recorded. For the valine lines, 723 $tRNA^{Ser}_{AAC}$ V→S (373 males and 350 females) and 552 $tRNA^{Ser}_{AAC}$-FLP (282 males and 270 females) flies were scored. For the threonine lines, 591 $tRNA^{Ser}_{AGU}$ T→S (287 males and 304 females) and 550 $tRNA^{Ser}_{AGU}$-FLP (276 males and 274 females) flies were scored. Deformities were photographed through the lens of a stereomicroscope using a Samsung Galaxy S8 camera.

*Longevity assays*

Equal numbers of adult, virgin flies of each sex were collected from all lines within 8 hours of eclosion and placed in new food vials (119 flies for each valine line and 101 flies for each threonine line). Flies with deformities were noted but still used in the assay. Flies were transferred to new food every three days and deaths were recorded. If dead flies were found in a vial known to contain a fly with a deformity, the dead fly was examined for deformities.

*Climbing assays*

Climbing assays were conducted on the flies in the longevity assay. The day before testing, flies were transferred to fresh food. The number of flies that reached a goal line 5 cm above the surface of the food within 10 seconds were recorded. Each vial was tested three times. Climbing performance calculated as the percentage of successful flies out of the total number of flies in the vial. Flies were tested 30, 51, and 72 days after eclosion. Only nondeformed flies were considered when recording the total number of flies (e.g. a vial with six flies but one deformed fly was treated as containing five flies).

*Scoring for eye degeneration*

Equal numbers of male and female adults containing a mistranslating $tRNA^{Ser}$ variant or its corresponding control were collected within 8 hours of eclosion and aged 30 days. Flies were transferred to new food every three days. Upon reaching 30 days of age, fly heads were removed and immobilized in Blu-Tack (Bostik, Ltd.) with their left eye pointing upwards. The left eye was imaged at 10x magnification on a Zeiss Axio Imager Z1 Fluorescent microscope using ZEN Blue Pro software (v3.1, Zeiss Inc.) and ~25 images 4.08 µm apart were combined using focus stacking to produce the final image. A circle 15 ommatidia in diameter was drawn on the centre of the eye and the number of pixels within recorded. The number of pixels within that region corresponding to degenerated regions of the eye were recorded and used to calculate the percentage of the eye that showed signs of neurodegeneration.

*Statistical analyses*

Statistical analyses were performed using R Studio v1.2.5001. Analyses used for comparisons were: *t*-test (frequency of V→S misincorporation between $tRNA^{Ser}_{AAC}$ and $tRNA^{Ser}_{AAC}$-FLP, or T→S misincorporation between $tRNA^{Ser}_{AGU}$ and $tRNA^{Ser}_{AGU}$-FLP); Wilcoxon rank-sum tests (developmental time data and eye degeneration data, corrected using Holm-Bonferroni's method); and Fisher's exact tests (survival between developmental stages, proportion of deformities, and climbing performance, all corrected using Holm-Bonferroni's method). Fly longevity was quantified and compared using the "survminer" R package (Kassambara *et al.* 2017) and log-rank tests corrected using Holm-Bonferroni's method. Survival *P*-values were corrected using Holm-Bonferroni's method. Because the climbing assays were performed on flies undergoing the longevity assay, climbing assay and longevity assay *P*-values were corrected together. All raw data can be found in Supplemental file S1.

*Data Availability*

Fly lines and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and supplemental material. Supplemental File S1 contains supplemental figures

and tables. Supplemental File S2 contains all raw data. Supplemental File S3 contains R code used to analyze all raw data.

## 4.3 Results

*Creating mistranslating fly lines*

We altered the anticodon region of the gene encoding *Drosophilia* $tRNA^{Ser}_{UGA}$ (FlyBase ID: FBgn0050201) with AAC and AGT to allow expression of $tRNA^{Ser}$ variants that misincorporate serine at valine (V→S) or serine at threonine codons (T→S), respectively. The tRNA sequence also included a G26A base change to remove a key modification site in $tRNA^{Ser}$ (Boccaletto *et al.* 2022). The G26A change causes increased degradation of the $tRNA^{Ser}$ variants through the rapid tRNA decay pathway and ensured that mistranslation would occur at tolerable levels based on work in yeast (Dewe *et al.* 2012; Berg *et al.* 2021a). We flanked tRNA variant constructs with FRT sites to allow flippase driven in the germ line to excise the tRNA from germ cells and produce control offspring with no copies of the inserted tRNA. The control lines ($tRNA^{Ser}_{AAC}$-FLP and $tRNA^{Ser}_{AGU}$-FLP) share a genetic background with their corresponding mistranslating line ($tRNA^{Ser}_{AAC}$ and $tRNA^{Ser}_{AGU}$). The presence of the variants in the experimental lines and the absence of inserted tRNAs in the controls was confirmed through PCR and sequencing (Figure S4-1).

To determine frequency of V→S or T→S mistranslation in the $tRNA^{Ser}_{AAC}$ and $tRNA^{Ser}_{AGU}$ containing lines, we compared the proteome of pupae and adults from mistranslating lines and with their respective control using mass spectrometry. We define mistranslation as the number of unique peptides observed where valine or threonine is replaced with serine relative to the total number of peptides observed with valine or threonine. Both tRNA variants contain A at base 34, and Since $tRNA^{Ser}$ undergoes modification of adenine at base 34 to inosine, both tRNA variants should decode codons ending in U, A and C (Crick 1966; reviewed in Agris *et al.* 2018; Boccaletto *et al.* 2022). Pupae were analysed first as levels of translation are relatively high during this

developmental stage and pupation determines adult neuronal and skeletomuscular structures (Mitchell *et al.* 1977; Mitchell and Petersen 1981; Truman and Bate 1988; Truman 1990). Both lines had significantly higher mistranslation frequencies as pupae than control lines, though frequency of V→S mistranslation was less than T→S mistranslation (Figure 4-1A, B; for $tRNA^{Ser}_{AAC}$ V→S: 0.03% vs 0.08%, $P < 0.001$; for $tRNA^{Ser}_{AGU}$ T→S: 0.25% vs. 2.01%, $P < 0.001$, Student's *t*-test).

Adults were then analyzed to identify potential sex-specific differences in mistranslation frequency and to ensure that mistranslation persisted into adulthood. Frequency of mistranslation decreased in adulthood for both $tRNA^{Ser}_{AAC}$ V→S and $tRNA^{Ser}_{AGU}$ T→S lines compared to frequencies observed during pupation (Figure 4-1C, D). The frequency of T→S mistranslation was significantly higher for both female and male adults containing $tRNA^{Ser}_{AGU}$ compared to $tRNA^{Ser}_{AGU}$-FLP (Figure 4-1D; females: 0.23% vs. 1.00%, $P < 0.001$; males: 0.23% vs. 0.73%, $P < 0.001$). Interestingly, female flies containing $tRNA^{Ser}_{AGU}$ mistranslated T→S significantly more often than male flies containing the same variant (1.00% vs. 0.73%, $P = 0.001$). These results show that we have successfully created new mistranslating fly lines and that mistranslation frequency varies by developmental stage and sex. Observed frequencies of V→S mistranslation for adult female and adult male flies containing $tRNA^{Ser}_{AAC}$ were not significantly different than female or male adults containing control $tRNA^{Ser}_{AAC}$-FLP (Figure 4-1C, $P > 0.05$ for both). However, given the low frequency of mistranslation observed in $tRNA^{Ser}_{AAC}$ pupae and the mistranslation in the T→S adults, we predict that mistranslation is occurring in $tRNA^{Ser}_{AAC}$ adults but at a frequency below the threshold of detection.

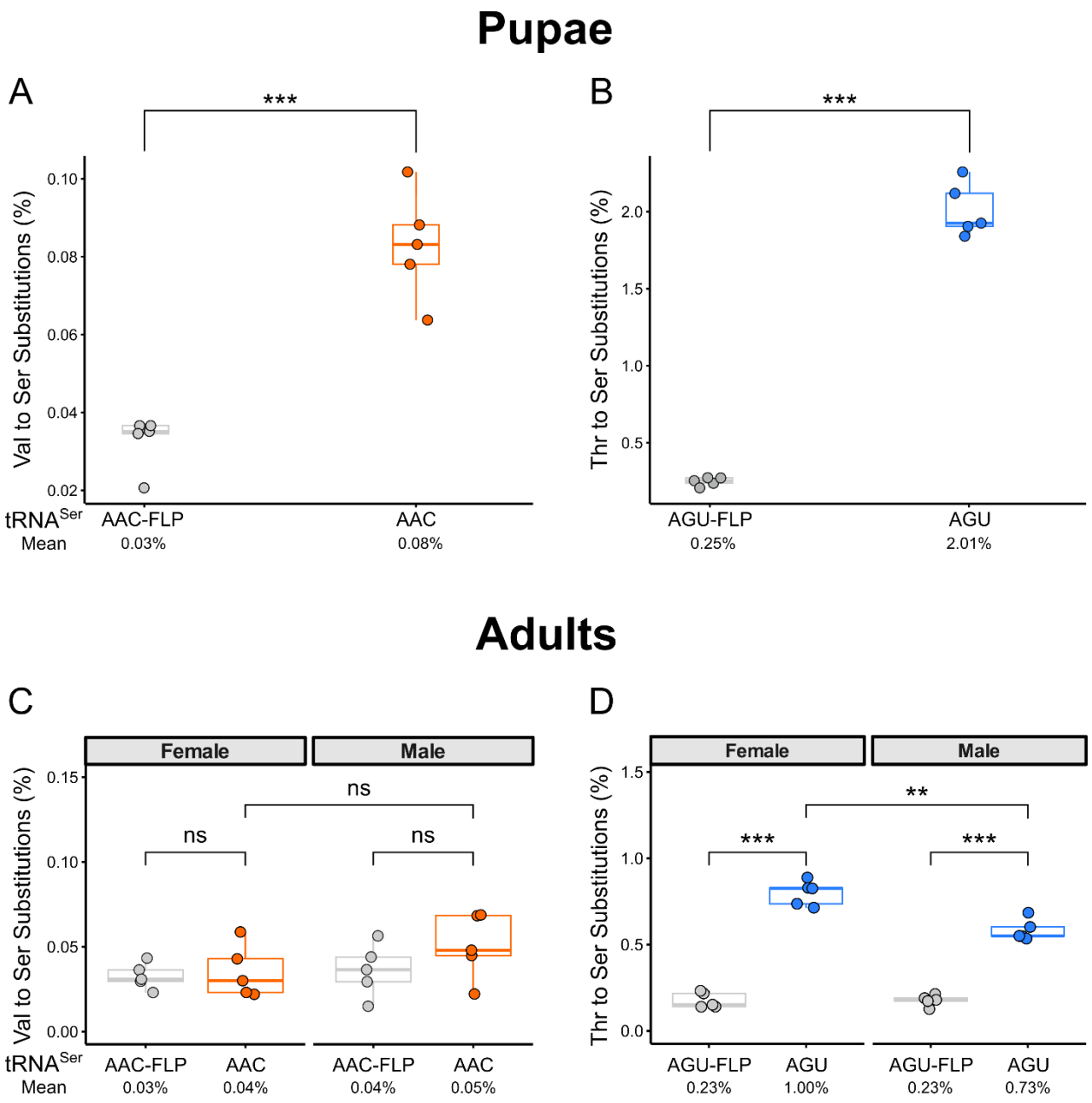


**Figure 4-1.** *Misincorporation frequency of pupae or adults containing $tRNA^{Ser}_{AAC}$ V→S and $tRNA^{Ser}_{AGU}$ T→S as determined from whole-proteome mass spectrometry.*

**A)** Frequency of V→S mistranslation in $tRNA^{Ser}_{AAC}$-FLP and $tRNA^{Ser}_{AAC}$ pupae (n = 5 replicates of 20 pupae each). Numbers under the x-axis represent average misincorporation frequency. Genotypes were compared with a *t*-test. **B)** Frequency of T→S mistranslation in $tRNA^{Ser}_{AGU}$-FLP and $tRNA^{Ser}_{AGU}$ pupae. **C)** Frequency of V→S mistranslation in 1–3-day old female or male adult flies containing $tRNA^{Ser}_{AAC}$-FLP or

$tRNA^{Ser}_{AAC}$ (n = 5 replicates of 10 flies each). **D)** Frequency of T→S mistranslation in female or male adult flies containing $tRNA^{Ser}_{AGU}$-FLP or $tRNA^{Ser}_{AGU}$. Note the difference in y-axis scale**.** "ns" $P > 0.05$, "**" $P < 0.01$; "***" $P < 0.001$.

*Mistranslation extends developmental time and causes lethality*

To determine how V→S and T→S substitutions affect development, we collected 200 eggs from each of the mistranslating lines and their controls and checked them every 12 hours to measure the time until eggs hatch into larvae, larvae pupate into pupae, and pupae eclose into adults. The number of survivors beyond each life stage transition was recorded. Survival of $tRNA^{Ser}_{AAC}$ V→S individuals through embryonic and larval development was not significantly different than the control, but significantly more mistranslating pupae died compared to non-mistranslating pupae (Figure 4-2A, $P < 0.001$). Notably, all control pupae survived until the adult stage whereas only 54% of $tRNA^{Ser}_{AAC}$ pupae reached the adult stage. In contrast, flies with $tRNA^{Ser}_{AGU}$ T→S experienced significantly decreased survival rates compared to the control during both embryonic and pupal development (Figure 4-2B, $P = 0.003$ and $P = 0.004$ respectively). Neither mistranslating line experienced statistically significant lethality compared to controls during larval development.

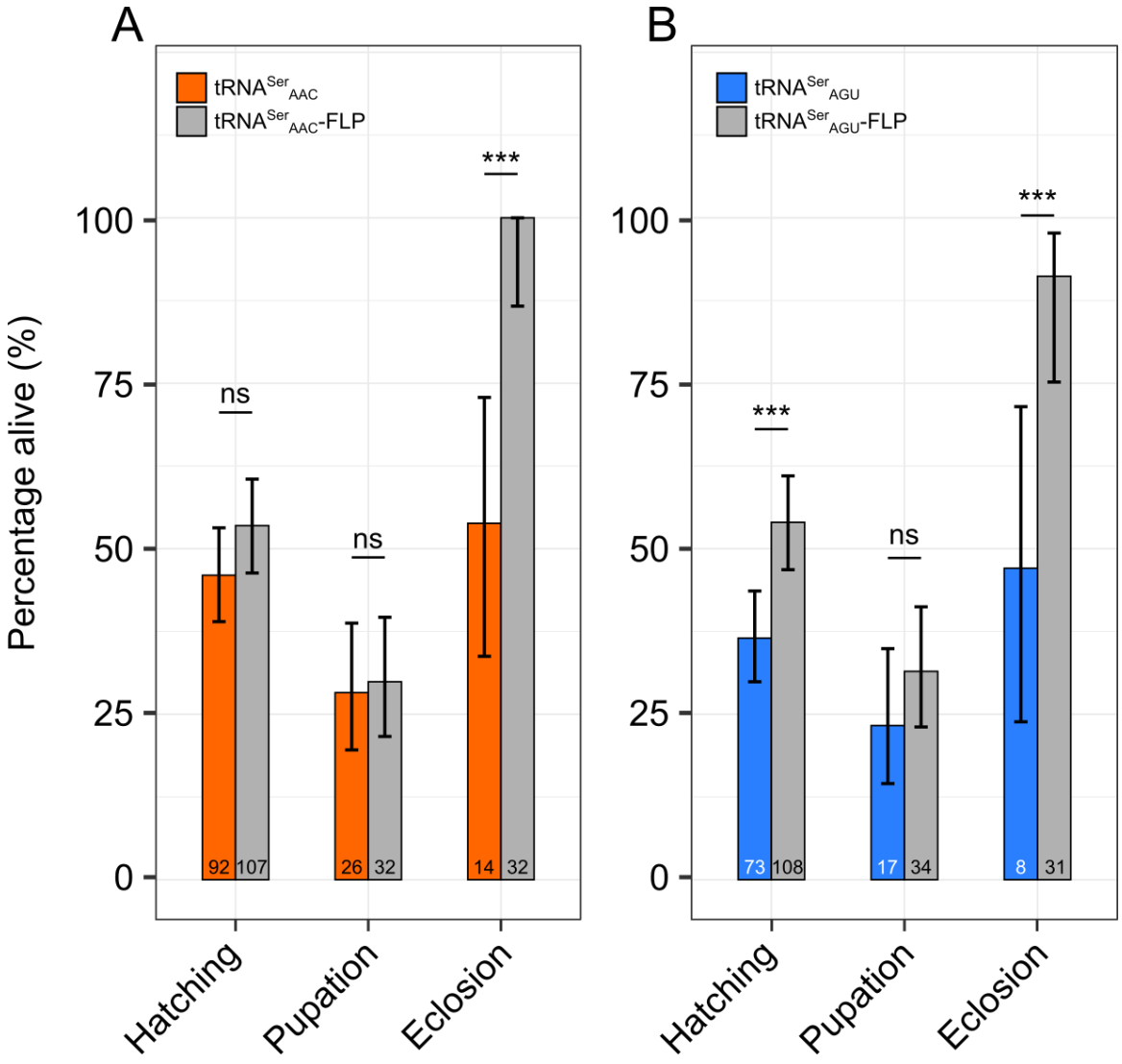


**Figure 4-2.** *Flies containing tRNA$^{Ser}_{AAC}$ V→S and tRNA$^{Ser}_{AGU}$ T→S experience developmental lethality.*

Bars show the percentage of 200 embryos from **A)** tRNA$^{Ser}_{AAC}$ V→S and tRNA$^{Ser}_{AAC}$-FLP or **B)** tRNA$^{Ser}_{AGU}$ T→S and tRNA$^{Ser}_{AGU}$-FLP that successfully hatched, pupated, and eclosed. Numbers within the bars indicate the number of embryos that survived beyond that life stage transition, and percentages describe the number of survivors from the previous stage that survived beyond the current transition. Error bars represent the 95% confidence interval of the proportion. Survival rates were compared using Fisher's exact test corrected using Holm-Bonferroni's method. "ns" $P \geq 0.05$; "***" $P < 0.001$.

Offspring from the $tRNA^{Ser}_{AAC}$ V→S line took significantly longer to hatch (Figure 4-3A; $P = 0.018$), pupate (Figure 4-3B; $P < 0.001$) and eclose (Figure 4-3C; $P = 0.002$) compared to control flies. Eggs from the $tRNA^{Ser}_{AGU}$ T→S line took significantly longer to pupate and eclose compared to the control $tRNA^{Ser}_{AGU}$-FLP line (Figure 4-3E, F; $P < 0.001$ and $P = 0.026$), whereas the difference in hatching time was not statistically significant (Figure 4-3D, $P > 0.05$). Development time and survival did not differ between the two control lines at any developmental stage ($P > 0.05$ for all comparisons).

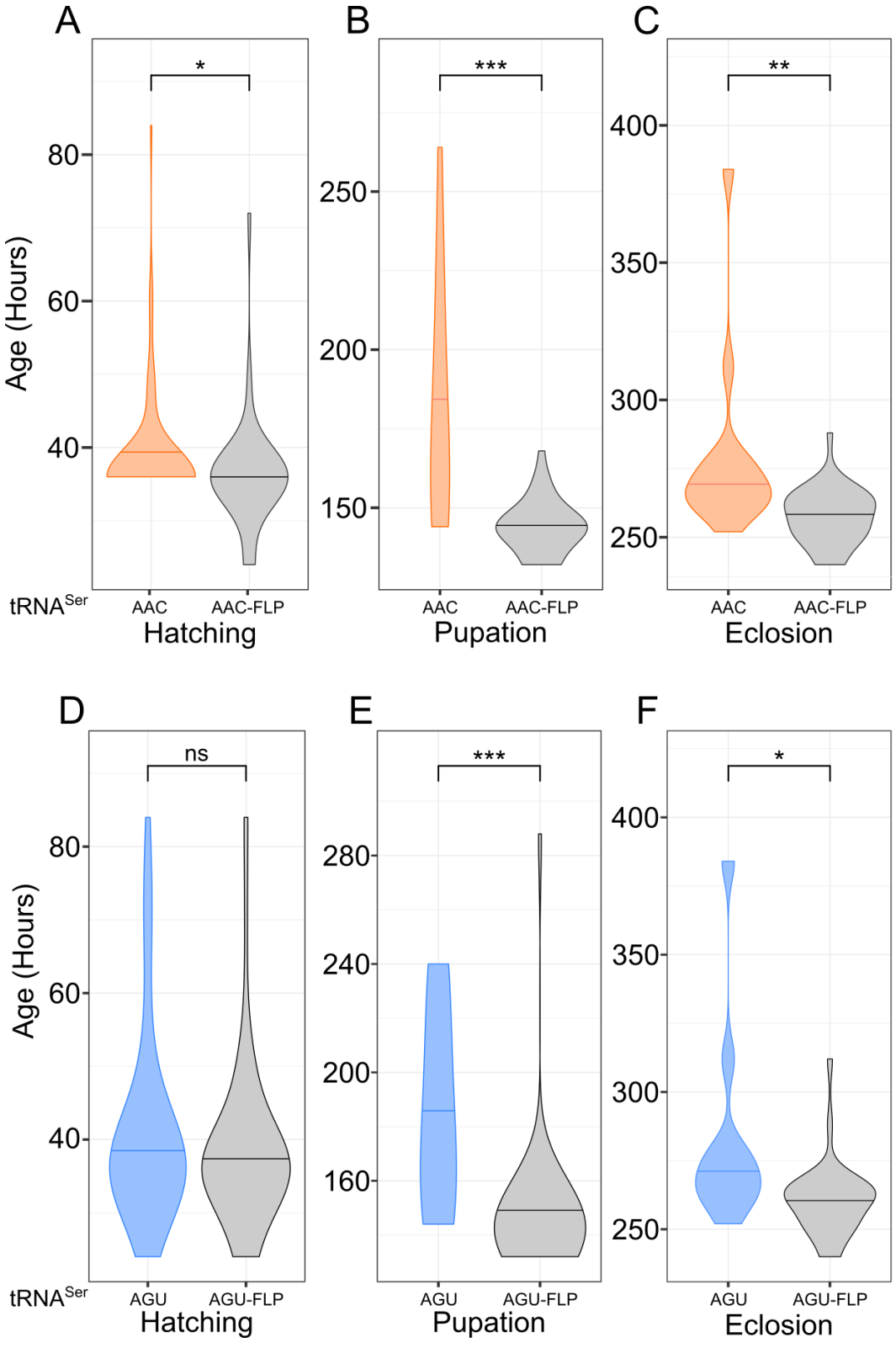


**Figure 4-3.** *Flies containing $tRNA^{Ser}_{AAC}$ V→S and $tRNA^{Ser}_{AGU}$ T→S experience extended development.*

**A)** Violin plot representing the distribution of total time it took $tRNA^{Ser}_{AAC}$ V→S or $tRNA^{Ser}_{AAC}$-FLP one-hour old embryos to hatch into larvae, **B)** pupate into pupae, and **C)** eclose into adult flies. **D)** Violin plot representing total time it took $tRNA^{Ser}_{AGU}$ T→S or $tRNA^{Ser}_{AGU}$-FLP one-hour old embryos to hatch into larvae, **E)** pupate into pupae, and **F)** eclose into adult flies. Horizontal bars within the plot represents the median of the distribution. Sample size is identical to the values within the corresponding bars in Figure 2. Genotypes were compared using Wilcoxon rank-sum tests corrected using Holm-Bonferroni's method. "ns" $P \geq 0.05$; "*" $P < 0.05$; "**" $P < 0.01$; "***" $P < 0.001$.

Next, we determined if there was a difference in sex or zygosity distribution among flies that reached the adult stage. Because there were only 23 surviving adults available to score from the $tRNA^{Ser}$ variant lines (one adult was lost during transfer), data from the mistranslating lines were pooled to assess if mistranslation caused any general trends. Sex distribution of surviving adults was roughly 50% for both $tRNA^{Ser}$ variant lines and their controls (variant lines: 56.5% male; controls: 50.7% male, Table 1). Interestingly, 91% of adult flies containing a $tRNA^{Ser}$ variant were heterozygotic in comparison to 66% for the control flies, the latter matching the 2:1 heterozygote:homozygote ratio expected. This suggests that two copies of the $tRNA^{Ser}$ variant are poorly tolerated by flies and thus few homozygous flies reach the adult stage.

**Table 4-1.** *Categorization of adult flies that survived the developmental assay by sex and zygosity.*

| | $tRNA^{Ser}$ variant[a] | $tRNA^{Ser}$-FLP control |
|---|---|---|
| Male | 13 | 32 |
| Female | 10 | 31 |
| Heterozygote | 21 | 42 |
| Homozygote | 2 | 21 |

[a]Flies belonging to both $tRNA^{Ser}$ variant lines and both control lines were pooled.

*Mistranslation causes deformities in adult female flies*

We previously showed that flies containing a $tRNA^{Ser}$ variant that causes P→S mistranslation increases the prevalence of anatomical deformities with female flies containing this tRNA variant are twice as likely to present with at least one deformity compared to males (Isaacson *et al.* 2022). We therefore wanted to determine if other mistranslating tRNA variants cause deformities in flies and if the greater female susceptibility was found for other amino acid substitutions. Adult heterozygous flies from all four lines were separated by sex and scored for leg, wing, and tergite defects (Figure 4-4A-D). Female flies containing $tRNA^{Ser}_{AGU}$ T→S presented with deformities greater than twice as often as control females (Figure 4-4F, 17.1% vs 8.0%, $P = 0.001$) and mistranslating males (17.1 % vs 7.0%, $P < 0.001$). In contrast, male $tRNA^{Ser}_{AGU}$ T→S flies eclosed with a similar number of deformities as male control flies (7.0% vs 6.2%, $P = 0.74$). Females containing $tRNA^{Ser}_{AAC}$ V→S also presented with significantly more deformities than control females (Figure 4-4E, 10.6% vs. 5.2%, $P = 0.036$, Fisher's exact test corrected using Holm-Bonferroni's method). There was an increased fraction of male flies containing $tRNA^{Ser}_{AAC}$ V→S that presented with a deformity compared to male control $tRNA^{Ser}_{AAC}$-FLP flies but this difference was not statistically significant (7.2% vs. 3.5%, $P = 0.12$). Though mistranslating females containing $tRNA^{Ser}_{AAC}$ V→S had a greater tendency toward deformities than males but the difference was not statistically significant (10.6% vs. 7.2%, $P = 0.12$). The increased prevalence of deformities in mistranslating females compared to control females replicates our previous findings that females are particularly susceptible to mistranslating tRNA variants during development (Isaacson *et al.* 2022). Since female $tRNA^{Ser}_{AAC}$ V→S flies did not present with a statistically significant increase in deformities compared to males containing $tRNA^{Ser}_{AAC}$ V→S, we suggest that the sex-specific effects of V→S mistranslation may be weaker than P→S or T→S mistranslation.

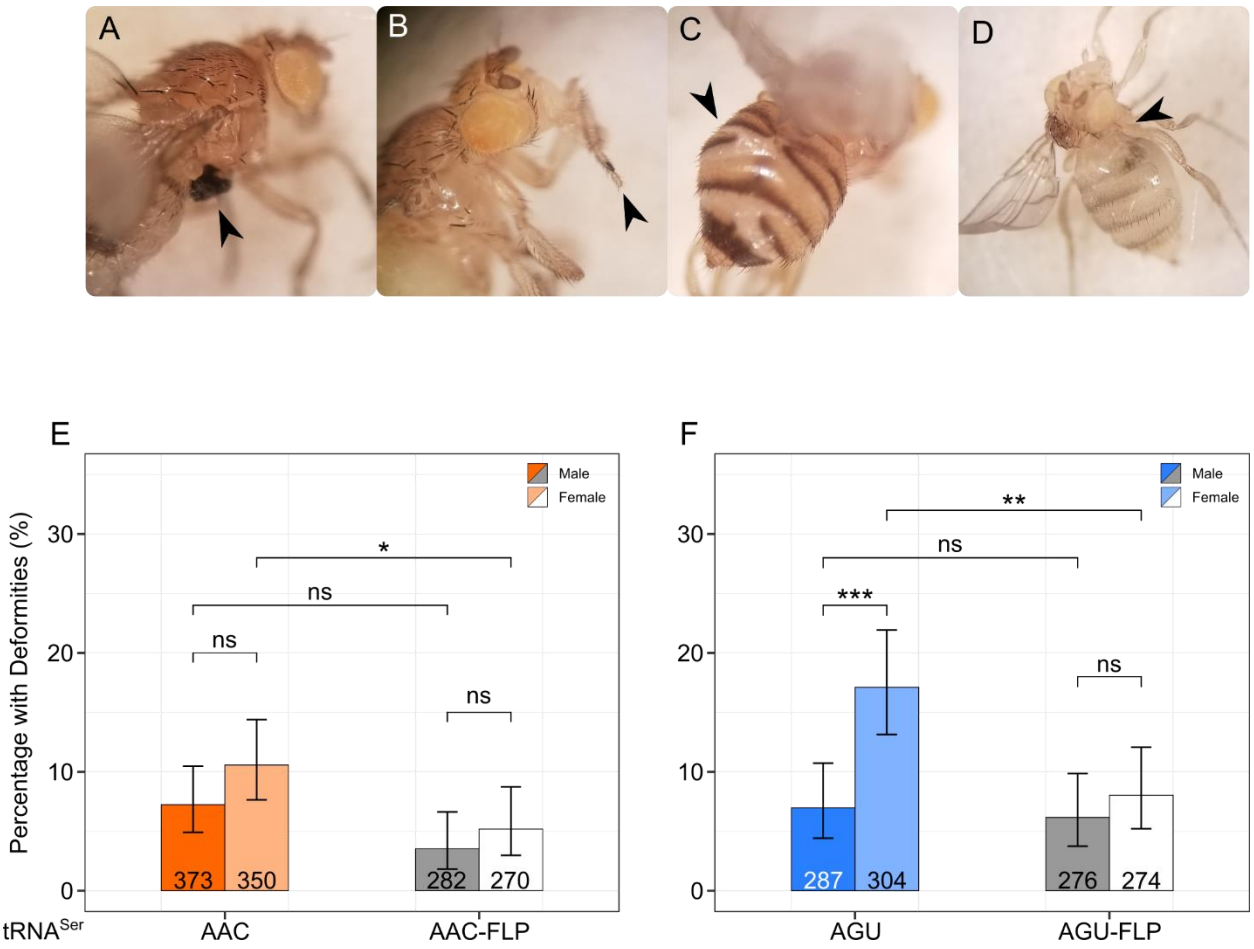


**Figure 4-4.** *A tRNA$^{Ser}_{AGU}$ T→S variant, but not tRNA$^{Ser}_{AAC}$ V→S, increases prevalence of deformities in adult female flies.*

**A)** Example of a fly with a malformed leg, **B)** missing metatarsal, **C)** misfused tergites and **D)** a missing wing. **E)** Percentage of male or female tRNA$^{Ser}_{AAC}$ V→S or tRNA$^{Ser}_{AAC}$-FLP flies presenting with at least one deformity. Groups were compared using Fisher's exact test and corrected for multiple comparisons using Holm-Bonferroni's method. Error bars represent the 95% confidence interval of the proportion. Values within bars describe the number of flies examined for deformities. **F)** Percentage of male or female tRNA$^{Ser}_{AGU}$ T→S or tRNA$^{Ser}_{AGU}$-FLP flies presenting with at least one deformity. "ns" $P \geq 0.05$; "*" $P < 0.05$; "**" $P < 0.01$; "***" $P < 0.001$.

*Mistranslating tRNA variants increase female fly lifespan*

Given the dramatic effects of mistranslation on development time and survival, we next tested whether mistranslation affects the lifespan of adult flies. Equal numbers of heterozygous virgin males and females from each mistranslating $tRNA^{Ser}$ variant line and its control were collected and transferred to new food vials every three days. Dead flies were recorded and removed during transfer, and survival curves were calculated. In total, 119 male and female $tRNA^{Ser}_{AAC}$ V→S and $tRNA^{Ser}_{AAC}$-FLP and 101 $tRNA^{Ser}_{AGU}$ T→S and $tRNA^{Ser}_{AGU}$-FLP males and females were analyzed. Virgin female lifespan was longer than male lifespan for both $tRNA^{Ser}_{AAC}$ V→S (Figure 4-5A, $P < 0.001$) and $tRNA^{Ser}_{AGU}$ T→S flies (Figure 4-5B, $P = 0.022$). Virgin female flies tend to live longer than virgin males (Ziehm *et al.* 2013), so this was an expected result. Neither male $tRNA^{Ser}_{AAC}$ V→S nor $tRNA^{Ser}_{AGU}$ T→S flies experienced a change in lifespan compared to control male flies ($tRNA^{Ser}_{AAC}$ V→S: Figure 4-5C, $P \approx 1$ after correction; $tRNA^{Ser}_{AGU}$ T→S: Figure 4-5D, $P \approx 1$ after correction). Interestingly, female $tRNA^{Ser}_{AAC}$ V→S flies lived longer than control female $tRNA^{Ser}_{AAC}$-FLP flies (Figure 4-5E, $P < 0.001$). Increased survival was also observed between female $tRNA^{Ser}_{AGU}$ T→S and $tRNA^{Ser}_{AGU}$-FLP flies (Figure 4-5F, $P < 0.001$). Our results demonstrate that two different mistranslating $tRNA^{Ser}$ variants extend female *D. melanogaster* lifespan without impacting male longevity.

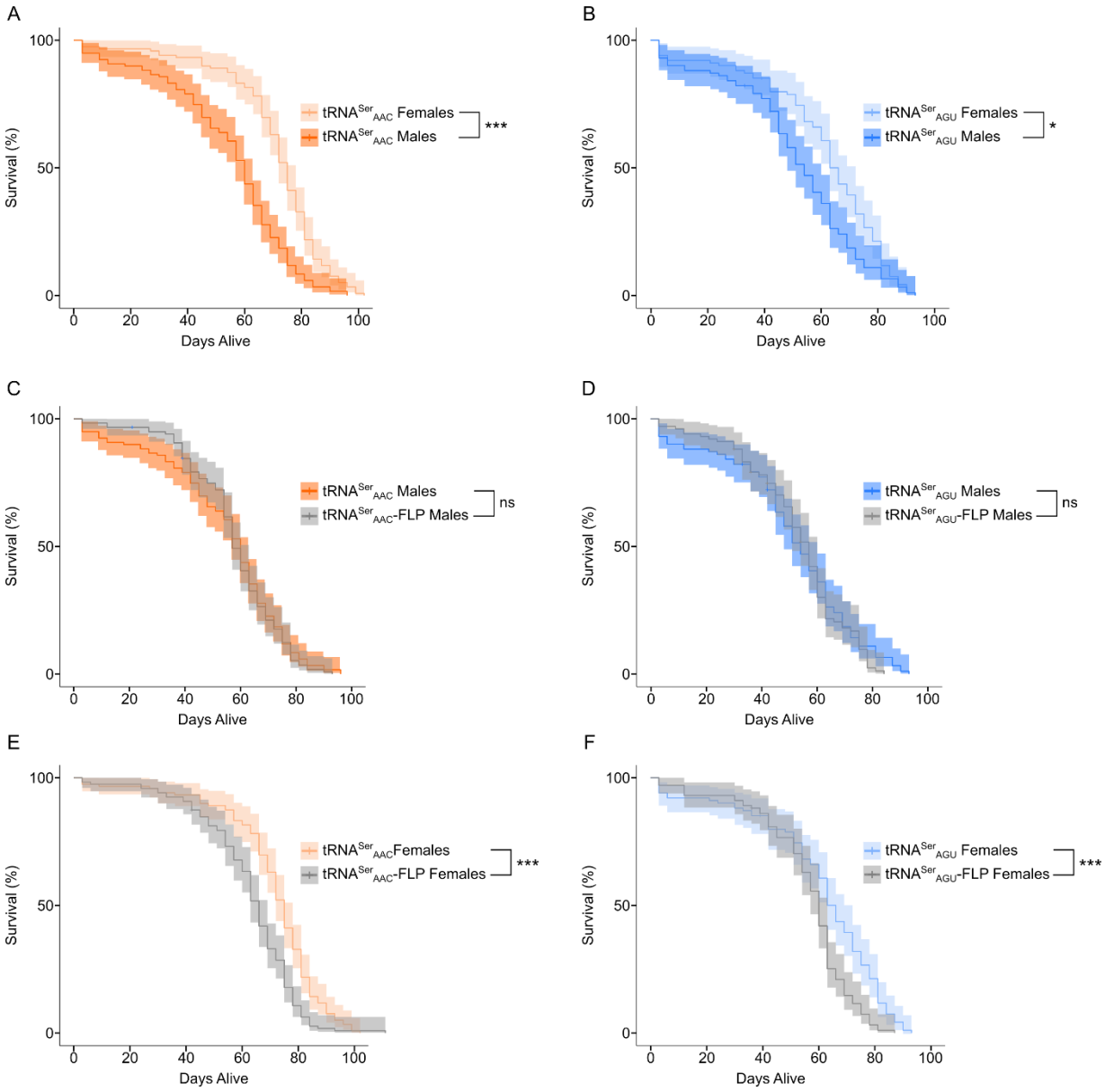


**Figure 4-5.** *Mistranslating tRNA variants increase female Drosophila melanogaster lifespan.*

Adult, virgin flies were collected within eight hours of eclosion and transferred to new food and scored for survival every three days. 119 male and female tRNA$^{Ser}_{AAC}$ V→S and tRNA$^{Ser}_{AAC}$-FLP flies were collected, and 101 male and female tRNA$^{Ser}_{AGU}$ T→S and tRNA$^{Ser}_{AGU}$-FLP flies were collected. Kaplan-Meier survival curves are shown with the shaded region representing the 95% confidence interval of survival probability. Vertical ticks along the line represent censored observations (e.g escaped flies). Survival curves

were statistically compared using log-rank tests corrected using Holm-Bonferroni's method. "ns" $P \geq 0.05$; "*" $P < 0.05$; "***" $P < 0.001$.

*Mistranslating tRNA$^{Ser}$ variants improve fly climbing performance*

Fly performance in negative geotaxis assays, also known as climbing assays, is commonly used as an indicator of neurodegeneration (e.g., Feany and Bender 2000; Song *et al.* 2017; Aggarwal *et al.* 2019). We conducted climbing assays on 30, 51, and 72-day old adult virgin flies that were undergoing the longevity assay (Figure 4-6). Flies were tested for their ability to climb 5 cm in 10 seconds with each vial tested three times. Both male and female flies from the tRNA$^{Ser}_{AGU}$ T→S line climbed significantly better than their corresponding control flies at 30 days of age ($P < 0.001$ for both, Figure 4-6B). At 30 days of age, female flies containing tRNA$^{Ser}_{AAC}$ V→S climbed significantly better than control females (Figure 4-6A, $P = 0.005$). Male flies containing tRNA$^{Ser}_{AAC}$ V→S had statistically similar climbing performance to control males ($P \approx 1$ after correction). Female climbing performance tended to be better than male performance for both tRNA$^{Ser}_{AAC}$ V→S and tRNA$^{Ser}_{AGU}$ T→S lines. This difference was statistically significant for tRNA$^{Ser}_{AAC}$ V→S at 30 days of age (Figure 4-6A, B; tRNA$^{Ser}_{AAC}$ V→S $P < 0.001$). The climbing performance of 30-day old females containing the control tRNA$^{Ser}_{AGU}$-FLP was also significantly better than males containing the control tRNA$^{Ser}_{AGU}$-FLP at this time point ($P = 0.006$).

There was substantial die-off in all lines by day 51 which affected statistical power. This decrease in sample size was most pronounced in male flies containing tRNA$^{Ser}_{AGU}$ T→S and tRNA$^{Ser}_{AGU}$-FLP, as nearly half of the flies alive at day 30 had died by day 51. Despite this die-off, there were still significant differences between groups at this time point. At 51 days, males from the tRNA$^{Ser}_{AAC}$ V→S line climbed significantly better than control males ($P = 0.01$, Figure 4-6A) and female flies containing tRNA$^{Ser}_{AGU}$ T→S climbed significantly better than control females ($P < 0.001$, Figure 4-6B). Females containing tRNA$^{Ser}_{AGU}$ T→S also tended to climb better than male tRNA$^{Ser}_{AGU}$ T→S flies, though this difference was not statistically significant after *P*-value correction. At 72 days, no groups showed significantly different performance after *P*-value correction,

though we note that no control $tRNA^{Ser}_{AGU}$-FLP males or females successfully reached the 5 cm goal line whereas ~15% of male and females from the $tRNA^{Ser}_{AGU}$ T→S line reached the goal (Figure 4-6B). Together these results show that both mistranslating $tRNA^{Ser}$ variant lines climbed as well or better than their corresponding control at all time points measured.

Thirty-day old flies were also scored for a rough-eye phenotype, which is a common method to quantify neurodegeneration (Sang and Jackson 2005; Prüßing *et al.* 2013). We observed no difference in the number of disrupted ommatidia between mistranslating and control flies (Figure S4-3). While it is possible that measuring eye degeneration in older flies may reveal a difference between mistranslating and control lines, the effect of mistranslation at thirty days of age is evidently not substantial enough to be visible. These findings suggests that V→S and T→S mistranslation do not cause neurodegeneration and may instead confer neuroprotective effects.

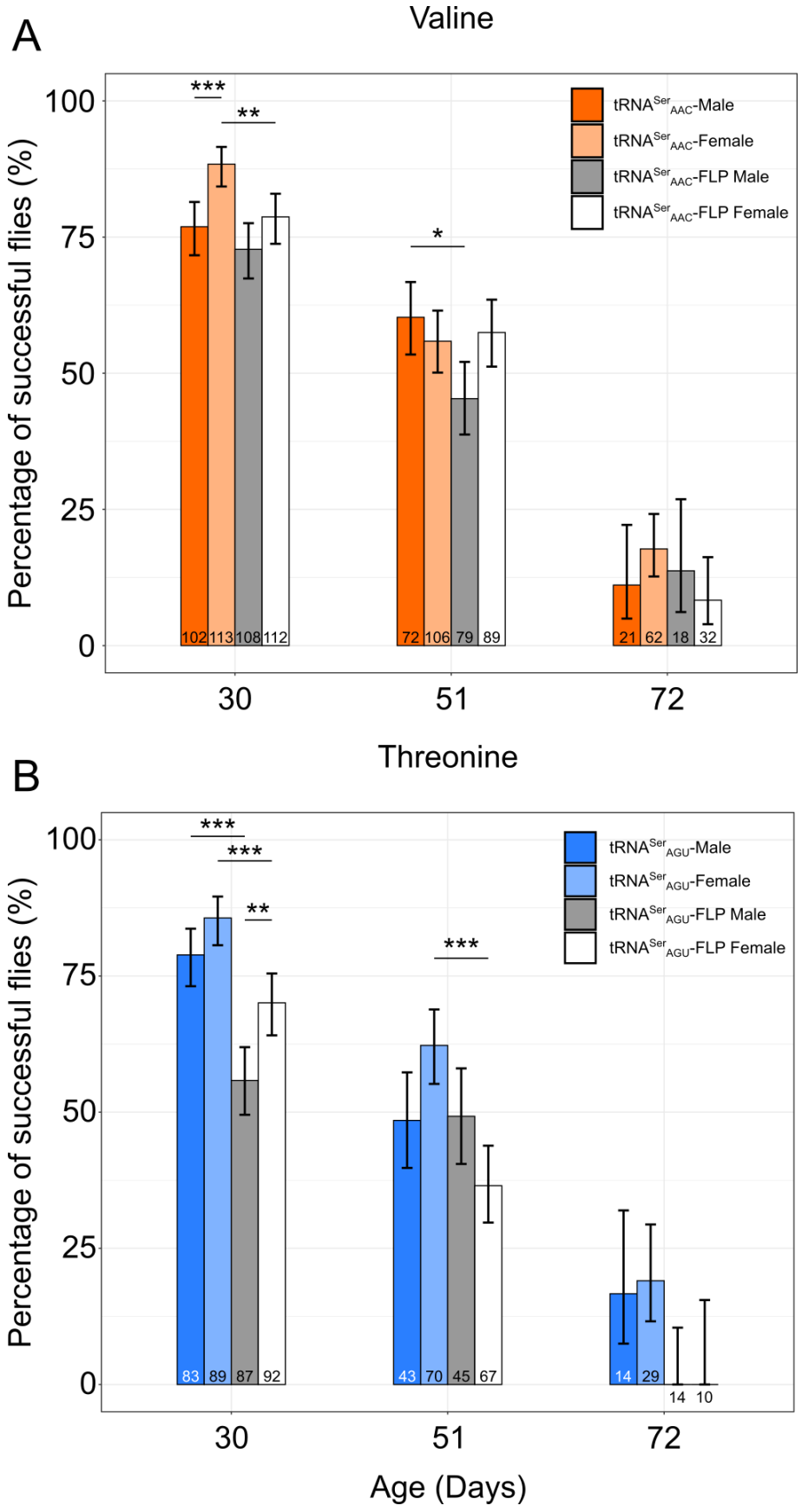
A
Valine
Percentage of successful flies (%)
100
75
50
25
0
***
**
*
tRNA^Ser_AAC-Male
tRNA^Ser_AAC-Female
tRNA^Ser_AAC-FLP Male
tRNA^Ser_AAC-FLP Female
102 113 108 112
72 106 79 89
21 62 18 32
30
51
72
Threonine
B
Percentage of successful flies (%)
100
75
50
25
0
***
***
**
***
tRNA^Ser_AGU-Male
tRNA^Ser_AGU-Female
tRNA^Ser_AGU-FLP Male
tRNA^Ser_AGU-FLP Female
83 89 87 92
43 70 45 67
14 29 14 10
30
51
72
Age (Days)

Figure 4-6. *Adult flies containing mistranslating tRNA$^{Ser}$ variants have improved climbing performance.*

Each bar represents the percentage of flies from the specified genotype that successfully reached a 5 cm goal line in 10 seconds. All flies were tested three times. Performance was compared between groups using Fisher's exact test and *P*-values were corrected for multiple comparisons using Holm-Bonferroni's method. Error bars represent the 95% confidence interval of the proportion. The numbers within or below bars represent the number of flies of that genotype and age that were tested. **A)** Climbing performance of male and female tRNA$^{Ser}_{AAC}$ V→S or tRNA$^{Ser}_{AAC}$-FLP flies at 30, 51, and 72 days of age. **B)** Climbing performance of male and female tRNA$^{Ser}_{AGU}$ T→S and tRNA$^{Ser}_{AGU}$-FLP flies at 30, 51, and 72 days of age. Only significant comparisons are shown. "*" $P < 0.05$; "**" $P < 0.01$; "***" $P < 0.001$.

## 4.4 Discussion

This study investigated how tRNA-induced V→S and T→S mistranslation impacts *Drosophila melanogaster*. The two tRNA$^{Ser}$ variants, tRNA$^{Ser}_{AAC}$ V→S and tRNA$^{Ser}_{AGU}$ T→S, resulted in detectable mistranslation in pupae though only T→S mistranslation was detectable during adulthood. Mistranslation caused developmental lethality, extended development, and increased the prevalence of deformities in female flies in both the tRNA$^{Ser}_{AAC}$ V→S and tRNA$^{Ser}_{AGU}$ T→S lines. Both T→S and V→S tRNA$^{Ser}$ variants extended female lifespan without impacting male longevity and improved climbing performance in both sexes compared to control lines. Taken together, these results demonstrate that mistranslating tRNA$^{Ser}$ variants have complex effects on eukaryotic biology that are influenced by sex and the anticodon substitution.

*Impact of different mistranslating serine tRNA variants on fruit flies*

The tRNA$^{Ser}_{AAC}$ V→S variants caused 0.04–0.08% of identified valines to be mistranslated as serine, depending on sex and developmental stage. Mistranslation by the tRNA$^{Ser}_{AGU}$ T→S variant was greater: 0.73–2.01% of identified threonines mistranslated as serine,. Both tRNA$^{Ser}$ variants were integrated into the same location on chromosome

2L and maintained as a single copy, so differences in mistranslation frequency between the two $tRNA^{Ser}$ variants are not due to position effects or differences in copy number. Counter to our findings, $tRNA^{Ser}_{AAC}$ V→S is expected to mistranslate more often than $tRNA^{Ser}_{AGU}$ T→S based on codon usage and the number of competing tRNAs both when examining competition only at the mRNA codon and when accounting for other codons that can be decoded through wobble (Crick 1966; reviewed in Tuorto and Lyko 2016, Supplementary table S4-2). The levels of mistranslation that we report are based on steady-state protein levels. For this reason, our estimates of mistranslation frequency are an underestimate since some mistranslation events would result in protein turnover. One might predict that the conservative serine for threonine change would be less deleterious to protein structure than the serine for valine change, thus having a greater impact on the observed frequency.

Both $tRNA^{Ser}_{AAC}$ V→S and $tRNA^{Ser}_{AGU}$ T→S replicated many of our previous results using $tRNA^{Ser}_{UGG}$ P→S (Isaacson *et al.* 2022). All three lines containing mistranslating $tRNA^{Ser}$ variants experienced extended development and increased developmental lethality. Females from the $tRNA^{Ser}_{AGU}$ T→S and $tRNA^{Ser}_{UGG}$ P→S lines also presented with significantly more deformities than males or control flies, whereas female flies containing $tRNA^{Ser}_{AAC}$ V→S presented with more deformities than female controls but not male mistranslating flies. There was a difference between $tRNA^{Ser}_{UGG}$ P→S and the two $tRNA^{Ser}$ variants tested in this study, as climbing performance was impaired in both males and females containing $tRNA^{Ser}_{UGG}$ P→S compared to control flies; flies containing $tRNA^{Ser}_{AAC}$ V→S and $tRNA^{Ser}_{AGU}$ T→S climbed as well or better than control flies at all times points measured. This difference suggests that proteins involved in neuromuscular function are more sensitive to proline substitution than valine or threonine substitution either through the prevalence of proline in essential proteins or the relatively unique properties of proline itself. Supporting this idea are our previous observations that P→S mistranslation causes dysregulation of muscle and that $tRNA^{Ala}$ variants which cause P→A mistranslation behave anomalously compared to other mistranslating $tRNA^{Ala}$ variants (Cozma *et al.* 2023). However, we note that there may be survivorship bias occurring with our mistranslating lines as the adult flies used for longevity and climbing assays necessarily escaped death during development. Use of an

inducible system to activate mistranslation only during adulthood could be used for future mistranslation studies to eliminate this potential bias.

*Differences between male and female response to mistranslation*

All three of the $tRNA^{Ser}$ variants we have tested (this study and Isaacson *et al.* 2022) induce sex-specific phenotypes, suggesting that sex-specific responses to mistranslation is a common phenomenon. Females experienced stronger positive and negative effects of mistranslation. Only females presented with more deformities compared to control flies and experienced an increase in lifespan due to mistranslating $tRNA^{Ser}$ variants. Surprisingly, females containing $tRNA^{Ser}_{AGU}$ mistranslated T→S at a significantly higher frequency than males containing the same $tRNA^{Ser}$ variant, emphasizing the importance of examining the effects of mistranslation in both sexes. The sex-specific responses to mistranslation may be caused by the increased amount of mistranslation experienced by female flies. Female susceptibility to mistranslation is likely influenced by their increased developmental requirements, as females are larger and develop faster than male flies (Bonnier 1926; Bakker 1959). Females also invest more resources into their gametes than males, and prioritize proteostasis of their gametes at the expense of somatic tissue (Fredriksson *et al.* 2012). Certain environmental conditions also extend lifespan extension primarily in one sex. For example, dietary restriction, particularly restriction of protein intake, extends lifespan of female flies more than males (Nakagawa *et al.* 2012; Regan *et al.* 2016; Garratt 2020). Specific mistranslation events may create a state physiologically similar to dietary restriction to extend female lifespan. Related to this, Lant *et al.* (2021) observed that mistranslation decreases overall protein translation in mammalian tissue culture. It would be interesting to test how mated *vs.* virgin flies respond to mistranslation, as mating status heavily impacts fly lifespan and resource allocation for both males and females (Koliada *et al.* 2020).

*Implications for eukaryotic biology*

Variant tRNA-induced mistranslation affects a wide range of physiological processes and exerts both positive and negative effects on flies. Mistranslation is most

deleterious during periods of intense growth and translational activity, including embryogenesis and pupation (Mitchell *et al.* 1977; Mitchell and Petersen 1981; Trumbly and Jarry 1983; Qin *et al.* 2007). Once flies reach the adult stage, however, mistranslation increases both fly lifespan and climbing performance. Previous studies examining mistranslation in complex eukaryotes such as mice, flies, and zebrafish identified developmental defects, organ pathologies, and neurodegeneration, but did not report any beneficial effects (Lee *et al.* 2006; Lu *et al.* 2014; Reverendo *et al.* 2014; Liu *et al.* 2014). In contrast to our results that mistranslating $tRNA^{Ser}$ variants do not affect lifespan and improve climbing performance of male flies, Lu *et al.* (2014) found that male flies constitutively expressing an editing-defective PheRS experience reduced lifespan and climbing ability. This may reflect differences in amino acid substitution or level of mistranslation. Some stress conditions, such as heat or cold shock, provide long-term resistance to future stresses after exposure, so it is possible that low levels of mistranslation may provide similar hormetic effects with physiological benefits (Hercus *et al.* 2003; Rattan 2005; Le Bourg 2007).

Translation infidelity is associated with neuropathies such as Charcot-Marie-Tooth disease and intellectual disability (Murakami *et al.* 1996; Antonellis *et al.* 2003; Abbasi-Moheb *et al.* 2012; reviewed in Kapur *et al.* 2017 and Kapur and Ackerman 2018; Zhang *et al.* 2020; Zuko *et al.* 2021). Our result that two types of mistranslation improve fly locomotion and do not cause neurodegeneration is surprising as postmitotic tissues such as neurons are expected to be especially vulnerable to translation errors (Drummond and Wilke 2008). However, other groups have observed mistranslating tRNAs being tolerated well by neuronal tissues. Hasan *et al.* (2023) tested four mistranslating tRNA variants in mouse neuroblastoma cells and found that three of the four were not cytotoxic, even in combination with the proteasomal inhibitor MG132. Lant *et al.* (2021) showed that a $tRNA^{Pro}$ variant that misincorporates alanine for proline did not cause significant cell death in mouse or human neuroblastoma cell lines (Lant *et al.* 2021). We also recognize that fruit flies and mammals have different metabolic and environmental requirements resulting in each coping with proteotoxic stress differently.

Given the potential impact of mistranslating tRNAs on disease, the prevalence of tRNA variants in humans (Parisien *et al.* 2013; Berg *et al.* 2019a), and their potential as therapeutic agents (reviewed in Anastassiadis and Köhrer 2023 and Coller and Ignatova 2023; Hou *et al.* 2024), it is important to understand both the positive and negative effects that mistranslating tRNAs have on multicellular eukaryotes and how these effects differ by sex.

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## 4.6 Supplemental Information

**Table S4-1**: *Primers used.*

| Primer name | Sequence |
|---|---|
| tSer_US | GGTATGAAGCATAGATTTCAGC |
| tSer_DS | CCCGCACGGGAAATTCCTAGG |

| | |
|---|---|
| tSerAAC_F | AGGAGATGGACTAACAATCCATTGGGTTCTAC |
| tSerAAC_R | CCCAATGGATTGTTAGTCCATGTCCTTAACCA |
| tSerAGU_F | AGGAAATGGACTAACAATCCATTGGGTTCTAC |
| tSerAGU_R | CCCAATGGATTGTTAGTCCATTTCCTTAACCA |
| pattB_tRNA_Ver_F | GGATTTCACTGGAACTAGGC |
| pattB_tRNA_Ver_R | CCTACATCGTCGACACTAGT |
| FRT-tRNA-Ver_F | GGTGGGCATAATAGTGTTGTTTAT |
| FRT-tRNA-Ver_R | CTAGAGGTACCCTCGAGCCG |

**Table S4-2**. *Calculating expected mistranslation rate at each codon.*

Cognate codons for the $tRNA^{Ser}$ variants used in this study are bolded. Number of competing tRNAs includes the single copy of variant $tRNA^{Ser}$ and were obtained from GtRNAdb (Chan and Lowe 2016).

| Codon | Codon usage (A)[a] | # of competing tRNAs (B) | Mistranslation rate (A/B, %) |
|---|---|---|---|
| **Valine** | | | |
| **GUU** | 0.18 | 17 | 1.06 |
| GUC | 0.24 | 17 | 1.41 |
| GUA | 0.11 | 17 | 0.65 |
| **Threonine** | | | |
| **ACU** | 0.17 | 29 | 0.59 |
| ACC | 0.38 | 29 | 1.31 |
| ACA | 0.19 | 29 | 0.66 |

[a]Codon usage information was obtained from https://www.genscript.com/tools/codon-frequency-table.

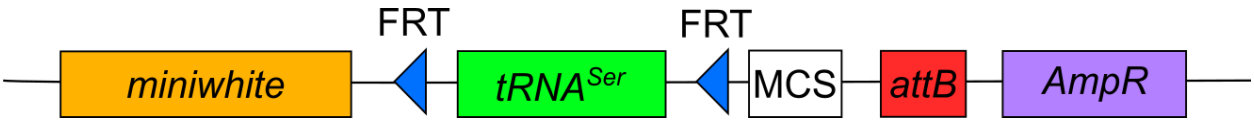


**Figure S4-1.** *Schematic of pattB-ΔNotI/pUCIDT.*

Order of key elements of the pattB-Δ*Not*I/pUCIDT plasmid used to integrate $tRNA^{Ser}$ variants into *D. melanogaster*. *miniwhite*: visible *miniwhite*$^{+}$ eye marker to identify transgenic flies. FRT: flippase recognition target. direction of triangle signals the directionality of the FRT. $tRNA^{Ser}$: $tRNA^{Ser}_{UGA}$-1-1 variant with ~300 bp of native up- and downstream sequence. MCS: multiple cloning sequence with *Not*I site removed. *attB*: *attB* component of *attB/attP* attachment sites used in ΦC31-integrase recombination. *AmpR*: ampicillin resistance gene.

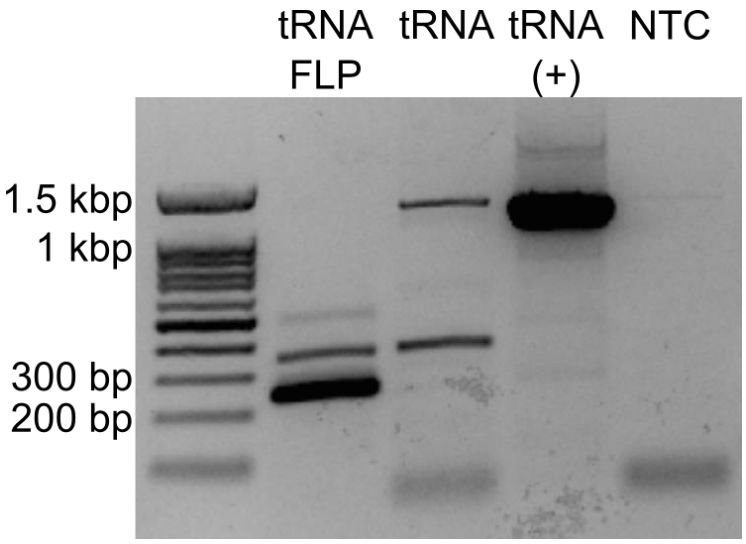


**Figure S4-2.** *PCR confirmation of tRNA removal*.

Lanes from left to right: FroggaBio 100 bp DNA ladder, $tRNA^{Ser}_{AGU}$-FLP pupal DNA, $tRNA^{Ser}_{AGU}$ pupal DNA, full-length $tRNA^{Ser}_{AGU}$ in the pattB-Δ*Not*I/pUCIDT plasmid (positive control), no template control. Full-length $tRNA^{Ser}_{AGU}$ produces a band at ~1 kbp. $tRNA^{Ser}_{AGU}$-FLP produces a band at ~240 bp after flippase-mediated removal of the tRNA. This primer set produced off-target bands at ~350 bp that are of no biological significance. PCR used primer set FRT-tRNA-VerF/R for all samples. $tRNA^{Ser}_{AGU}$-FLP bands were extracted and sequenced to confirm complete removal of tRNA.

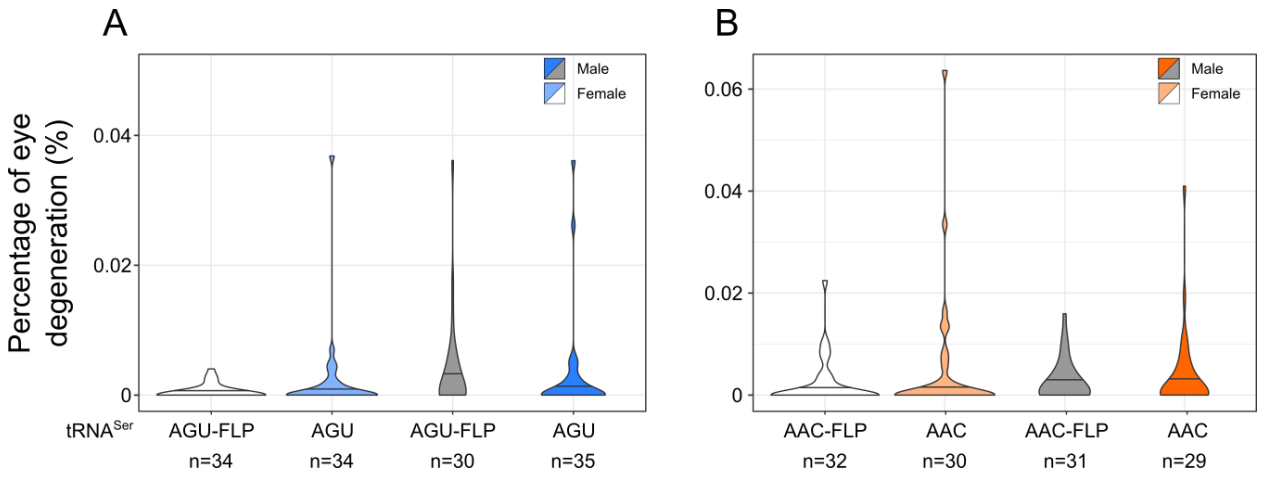


**Figure S4-3.** *Amount of eye degeneration in tRNA$^{Ser}_{AGU}$ (T→S) and tRNA$^{Ser}_{AAC}$ (V→S) compared to control flies.*

**A)** Violin plot showing the distribution of amounts of eye degeneration in a 15-ommatidia radius of the left fly eye belonging to male or female tRNA$^{Ser}_{AGU}$ (T→S) or tRNA$^{Ser}_{AGU}$-FLP. Amounts of eye degeneration were calculated as the number of pixels corresponding to degenerated areas divided by the total scored area of the eye. Numbers below the genotype label represent the sample size. **B)** Same as **A)** but for tRNA$^{Ser}_{AAC}$ (V→S) and tRNA$^{Ser}_{AAC}$-FLP flies. There were no significant differences between groups according to Wilcoxon rank-sum tests corrected using Holm-Bonferroni's method.

# Chapter 5

# 5 General discussion

## 5.1 Summary of Experimental Findings

This thesis describes my work integrating mistranslating $tRNA^{Ser}$ variants into the fruit fly *Drosophila melanogaster* to create a multicellular model of tRNA-induced mistranslation. In Chapter 2, I adapted a $tRNA^{Ser}_{UGG, G26A}$ variant identified in *Saccharomyces cerevisiae* (Hoffman *et al.* 2017) that causes proline-to-serine mistranslation for use in flies by recreating those base changes in a wild-type fly $tRNA^{Ser}_{UGA}$ and integrating it into the fly genome using ΦC31 integrase. This strategy was ideal as it incorporates a single copy of the $tRNA^{Ser}$ variant into a precise spot in the genome, thus controlling for genomic position and copy number effects (Groth *et al.* 2004; Fish *et al.* 2007). Integrating a single copy of a mistranslating tRNA variants into the fly genome is desirable as these tRNA variants are likely to arise as a single copy.

Pupae containing the mistranslating $tRNA^{Ser}_{UGG, G26A}$ variant substituted proline for serine (P→S) at levels significantly higher than control lines containing wild-type $tRNA^{Ser}_{UGA}$, demonstrating that we successfully induced mistranslation in flies. Fly lines containing $tRNA^{Ser}_{UGG, G26A}$ experienced increased development time and lethality during pupation. Upon reaching adulthood, $tRNA^{Ser}_{UGG, G26A}$ flies presented with significantly more anatomical deformities and worse climbing ability compared to control flies. Surprisingly, we found that females containing $tRNA^{Ser}_{UGG, G26A}$ were twice as likely to present with deformities than male flies. Female climbing performance also declined significantly faster than male climbing performance as flies aged, indicating that females are more susceptible to deleterious effects of the proline to serine mistranslating tRNA variants than are male flies.

In Chapter 3, I performed RNA-sequencing to investigate transcriptomic effects of P→S mistranslation and identify potential explanations for the difference in male and female response. Transcriptomic analysis identified that male and female flies containing the $tRNA^{Ser}_{UGG, G26A}$ variant downregulated genes associated with metabolism. Males also

downregulated genes associated with extracellular matrix development whereas females primarily downregulated genes involved in ATP synthesis and the electron transport chain. Few genes of known function were upregulated in male flies, though gametogenesis was upregulated in both sexes. Females upregulated cell cycle, DNA replication and repair, and mitotic processes, perhaps indicating that $tRNA^{Ser}_{UGG,\ G26A}$ causes DNA damage in females or that females are interpreting mistranslation-induced stress as DNA damage.

Previous work in the Brandl lab showed that different amino acid substitutions exert unique cellular effects (Berg *et al.* 2021b). In Chapter 4, I describe my work integrating two distinct mistranslating $tRNA^{Ser}$ variants to determine if they affect flies similarly. One variant induced valine-to-serine (V→S) mistranslation and the other induced threonine-to-serine (T→S) mistranslation. I also improved the system by which I induced mistranslation by creating control lines from a mistranslating line. This allowed me to control for genomic background effects in addition to copy number and position effects. Both mistranslating lines extended development and caused lethality, though the developmental stages affected differed between the two lines. Only the T→S mistranslating line showed a significant increase in the proportion of morphological deformities in adult flies compared to control flies, but females were again more likely to present with deformities than males. We then tested how mistranslating tRNA variants affect fly lifespan and were surprised to find that they significantly extended female lifespan without impacting male lifespan. A climbing assay provided similar results, as male and female flies from both tRNA variant lines climbed as well or better than their corresponding controls.

## 5.2 Sex-specific Impact of tRNA-induced Mistranslation

The previous chapters demonstrate that mistranslating tRNA variants affect males and females differently, as females present with more anatomical deformities but experience lifespan extension and what appear to be neuroprotective effects. Males attenuate both the positive and negative effects of these variants, as they present with neither more deformities nor increased lifespan. The increased prevalence of morphological deformities in females indicate that females are more susceptible to

mistranslating tRNA variants during development, because the deformities observed would necessarily have arisen during pupation (reviewed in Tennessen and Thummel 2011). However, it is difficult to identify from our data if other developmental processes and stages are affected in a sex-specific manner. We were unable to determine the sex of embryos in these studies, preventing us from measuring whether male or female flies died more often during certain developmental stages or if one sex developed slower than the other. Despite this issue, similar numbers of male and female adults eclosed from pupae in all three of our tRNA variant lines as determined by developmental assays (see Chapters 2 and 4), so any potential sex differences during development do not seem to affect overall survival to adult stages. This observation does not preclude sex differences during development that even out (such as increased male lethality during embryonic stages but increased female lethality during pupation), so future work should attempt to determine if any cryptic sex differences during development exist.

Given the strong downregulation of metabolic genes in both sexes, it may be that mistranslation is imposing a starvation-like state on flies during development. As discussed earlier, females are larger and develop faster (Bakker 1959; reviewed in Millington and Rideout 2018), so females require more nutrients during development as a result (Wu *et al.* 2020). Because females require additional resources during development, a starvation-like physiological state may disproportionately affect them compared to male flies which have less strict nutritional requirements. In addition, females downregulate genes associated with the electron transport chain and ATP synthesis, suggesting that their most efficient form of energy production is disrupted. A starvation-like state is supported by several of our other findings. Starvation or dietary restriction extends *D. melanogaster* larval development as they increase foraging time to acquire resources before pupation (Tennessen ands Thummel 2011; Krittika *et al.* 2019). Larval development was the only developmental stage extended in all three of the mistranslating tRNA variant lines tested, perhaps indicating that mistranslating larvae have a reduced ability to store nutrients or that they require additional resources due to the stress of mistranslation. Protein turnover and proteostasis have some of the highest metabolic costs of any cellular process, requiring between 20 and 50% of all energy produced by the cell (Buttgereit and Brand 1995; Lahtvee *et al.* 2014), so disruptions to

proteostasis due to mistranslation could have large metabolic consequences. In addition, starvation could help explain the longevity increase observed in female $tRNA^{Ser}_{AAC}$ and $tRNA^{Ser}_{AGU}$ lines, as starvation and dietary restriction extend development but increase overall adult lifespan (Nakagawa *et al.* 2012; Krittika *et al.* 2019). Interestingly, other studies report that increased lifespan is consistently associated with developmental lethality (Buck *et al.* 2000), which aligns with the results obtained for the mistranslating $tRNA^{Ser}_{AAC}$ and $tRNA^{Ser}_{AGU}$ lines.

## 5.3 Improving our Model to Induce Mistranslation

The model I created provides a straightforward way to study the effects of tRNA-induced mistranslation in *Drosophila melanogaster*. Fly lines containing new tRNA variants can be created rapidly by replacing the tRNA gene in the modified *pattB* vector and injecting fly embryos with the new vector. Control lines are also easily created from tRNA variant lines by crossing them to flippase-containing fly stocks, which completely removes the inserted tRNA from future generations when expressed in germ cells.

Altering expression of tRNA genes is difficult due to their unique structure and mechanism of transcription, and many common genomic tools used by *D. melanogaster* researchers to modify expression of target genes do not affect tRNAs. For example, RNAi-based knockdown of transcripts will not function with tRNA targets as the extensive base pairing present in tRNAs prevents shRNA or siRNA binding. However, improvements to our model can still be made that will allow for stage- or tissue-specific expression of tRNA variants. Berg *et al.* (2021a) dampened the expression of a mistranslating $tRNA^{Ser}_{UGG}$ variant in yeast cells by driving RNA polymerase II through the tRNA gene using a $Tet^{O}$-bound TetR-VP16 transcription activator. Exposing cells to doxycycline prevents TetR-VP16 from binding to $Tet^{O}$, removing $tRNA^{Ser}_{UGG}$ inhibition by RNA polymerase II and allowing the tRNA to be expressed. The doxycycline-regulated gene expression system has been used to drive expression of transgenes in *D. melanogaster* (Bieschke *et al.* 1998; Stebbins and Yin 2001), so a similar system could be used in flies to regulate expression of tRNA variants. The Tet-Off system would be particularly useful to study how mistranslation impacts adult traits (McGuire 2004), as mistranslation would be inhibited by RNA polymerase II readthrough during

development but could be activated in adult flies by feeding them doxycycline-laced food. This system also allows for the creation of fly lines containing tRNA variants that mistranslate at lethal levels as tRNA expression should only occur at high levels once flies are fed doxycycline.

Despite its advantages, relying on doxycycline makes it difficult to study the effects of mistranslation at time points where flies do not feed, such as embryogenesis and pupation. Our developmental assays revealed that these are key developmental stages to study, as $tRNA^{Ser}_{AGU}$ caused lethality during embryogenesis and all tRNA variants caused lethality during pupation. Therefore, other strategies should be considered to modulate expression of mistranslating tRNA variants. One promising solution involves the GAL4-UAS bipartite system (Duffy 2002), where GAL4 linked to a constitutively active gene such as *Act5C* would inhibit expression of a UAS-linked tRNA in the same manner as the Tet-Off system described in Berg *et al.* (2021a). A *GAL80* transgene, which represses GAL4 activity to allow expression of the variant tRNA (Ma and Ptashne 1987), can be driven in a stage- or tissue-specific manner by pairing it with an appropriate promoter. Alternatively, a temperature-sensitive GAL80 can be used to switch off tRNA expression at desired time points by transferring flies to a warm (~30°C) environment (Barwell *et al.* 2023). Mistranslation could also be completely repressed in specific tissues or life stages by using a flippase active in those tissues or timepoints, though this would be an irreversible process. These systems would allow precise control of tRNA expression and permit investigation into the effects of mistranslation on specific tissues. My lab is currently attempting to integrate $tRNA^{Ser}$ variants that lack the dampening G26A secondary mutation into *D. melanogaster*. My preliminary work found that it is possible to integrate $tRNA^{Ser}_{UGG}$ without a secondary mutation into *D. melanogaster* but stocks containing this variant cannot be fully balanced, suggesting that the $tRNA^{Ser}_{UGG}$ variant is too toxic to combine with other deleterious alleles. However, placing the $tRNA^{Ser}_{UGG}$ into one of the inducible systems described above would allow us to measure the effects of high levels of mistranslation on fly biology.

## 5.4 Implications for Genetic Code Evolution

The genetic code and current codon assignments are often thought to be unchanging—a "frozen accident" (Crick 1968). Despite the prevalence of this view, the hypothesis that codons received their current assignments simply through chance is unlikely given clear trends in the codon table, such as how amino acids with similar polar requirements tend to cluster together (Woese *et al.* 1966; Mathew and Luthey-Schulten 2008). It can be difficult to intuit an evolutionary benefit to changing codon assignments, as mutations that disrupt the code could have massive, deleterious effects on a cell's entire proteome. However, there is a large body of evidence showing that the genetic code is not static and that there are evolutionary and physiological benefits to codon reassignment and mistranslation (Gomes *et al.* 2007; Netzer *et al.* 2009; Fan *et al.* 2015; Ling *et al.* 2015; Mühlhausen *et al.* 2018; Evans *et al.* 2019; Samhita *et al.* 2020, 2021; Schuntermann *et al.* 2023).

Mistranslating tRNA variants could provide a selective advantage in some situations. Mistranslating tRNAs create a "statistical proteome" composed of many nearly-identical proteins with some amino acid substitutions due to ambiguous translation (Woese 1965, 2004). During periods of stress, some of these mistranslated proteins may confer a beneficial function to the organism, thus allowing them to better withstand the stress than non-mistranslating organisms. Mistranslation could also help cells cope with periods of starvation, as mistranslating a more abundant amino acid in place of a less abundant one would prevent ribosome stalling and may still produce functional protein, depending on the nature and location of the substitution. Ambiguous decoding by tRNA variants may facilitate genetic code evolution of the CUG codon in some yeast (Gomes *et al.* 2007; Santos *et al.* 2011; Kollmar and Mühlhausen 2017b). If a tRNA is lost from the genome, its codon becomes free to be "captured" by tRNA variants with an altered anticodon. The presence of a mistranslating tRNA variant beforehand could mitigate the deleterious effects of tRNA loss by decoding the now-unassigned codon, which would prevent ribosome stalling. Proteomic disruptions due to tRNA loss could be further reduced through wobble decoding from isoacceptors of the lost tRNA. Phylogenomic analysis of the CTG-clade and related yeast support this idea, as they contain both a wild-

type $tRNA^{Leu}_{CUG}$ and a mistranslating $tRNA^{Ser}_{CUG}$ variant and are thought to be in the final stages of codon reassignment (Krassowski *et al.* 2018).

My work has interesting evolutionary implications for mistranslation in multicellular eukaryotes. First is that mistranslating $tRNA^{Ser}$ variants extended lifespan in females without impacting male lifespan, which may provide additional mating opportunities for females and lead to increased lifetime reproductive success. Because these flies also experience less of an aging-induced decline in locomotive performance, mistranslating $tRNA^{Ser}$ variants may also provide a neuroprotective effect to both male and female flies, further enhancing potential fitness benefits. Despite these promising benefits, it is unclear if these benefits offset the increased developmental lethality and abundance of deformities caused by these $tRNA^{Ser}$ variants. These fly lines are also maintained in controlled environments with ample access to food and therefore do not need to compete for resources nor withstand significant environmental stresses, which are important aspects of evolutionary fitness. I also did not measure courtship or copulation success of flies containing mistranslating $tRNA^{Ser}$ variants, so it is possible that these flies are considered to be low-quality mates by wild-type conspecifics. However, these flies would be ideal candidates to assess the "ambivalent intermediate" hypothesis of genetic code evolution in multicellular eukaryotes. The ambivalent intermediate hypothesis proposes that an early step of codon reassignment could involve a mistranslating tRNA variant containing a dampening mutation such as G26A (Berg *et al.* 2017). As these fly lines already contain ambivalent intermediates, measuring traits such as lifetime reproductive success could identify if this theory is viable within complex eukaryotic organisms.

## 5.5 Health Implications and Future Work

The results presented in this thesis suggest interesting avenues for future research. The effects of mistranslation and cytosolic tRNA variants on human health were discussed in Chapter 1, but *D. melanogaster* is an excellent model to study how mistranslating tRNA variants contribute to disease. Fruit flies are particularly useful to study the effects of neurological disease due to well-characterized behaviours, such as negative geotaxis and courtship, as well as visible indicators of neurodegeneration like the rough-eye phenotype

(Warrick *et al.* 1999; Song *et al.* 2017; Aggarwal *et al.* 2019; reviewed in Bolus *et al.* 2020 and Nitta and Sugie 2022). Our climbing assay results indicate that mistranslating tRNA variants can have positive, negative, or neutral effects on neurobiology depending on the type of amino acid substitution and age of the fly. It would therefore be interesting to study how these tRNA variants interact with genes that cause neurodegenerative disease, such as *huntingtin* (*htt*) with an expanded polyglutamine repeat region (Chen *et al.* 2002).

Lant *et al.* (2021) studied the effects of mistranslating tRNA variants on mouse and human neuroblastoma cells containing expanded polyQ-*htt* alleles. Their work provides valuable insights into the cellular mechanisms by which mistranslation influence disease, but cancer cell lines present with some disadvantages when studying the effect of tRNA variants on complex diseases such as Alzheimer's disease or Huntington's disease. Cancer cells overexpress both cytosolic and mitochondrial tRNAs, and different cancers present with different tRNA expression profiles (Pavon-Eternod *et al.* 2009; Zhang *et al.* 2018). Most cancers are also aneuploid, further complicating the tRNA expression landscape and causing aberrant expression of non-tRNA genes (reviewed in Ben-David and Amon 2020). Unlike cancer cells, flies can provide information about how mistranslating tRNA variants affect clinically-relevant neurological phenotypes such as memory or locomotion. The fly model is also better suited to studying neurological diseases compared to yeast, as yeast are single-celled organisms and consequently neither contain neurons nor are subject to same physiological requirements as neurons. Using mice or rats to study the effects of human neurological disease provides the best basis of comparison as their nervous system closely resembles ours. However, the cost of mouse colony maintenance and time required to create transgenic lines is prohibitive. Flies represent an excellent compromise between the applicability of mouse models and the ease and speed of single-cell models. They have a differentiated nervous system with well-mapped connectomes (Scheffer *et al.* 2020; Winding *et al.* 2023), and transgenic stocks can be created and tested within weeks even by inexperienced researchers (Isaacson 2018). The fly model could therefore be a useful method to rapidly assay the contribution of mistranslating tRNA variants towards neurodegenerative diseases and narrow down candidates for further study in mammalian models.

The climbing assays performed in Chapter 2 and 4 show that the effect of tRNA variants varies by the type of amino acid substitution they cause. The $tRNA^{Ser}_{UGG}$ variant caused a decrease in both male and female climbing performance compared to control $tRNA^{Ser}_{UGA}$ flies, whereas the $tRNA^{Ser}_{AAC}$ and $tRNA^{Ser}_{AGU}$ variants have a neutral or neuroprotective effect on fly locomotion. Mistranslating $tRNA^{Ala}$ variants with proline anticodons tend to produce outlier effects in yeast compared to other mistranslating $tRNA^{Ala}$ variants (Cozma *et al.* 2023). Testing additional mistranslating $tRNA^{Ser}$ variants in flies would identify if $tRNA^{Ser}$ variants with proline anticodons have aberrant effects in flies as well. We were surprised to observe an improvement in climbing performance in $tRNA^{Ser}_{AAC}$ and $tRNA^{Ser}_{AGU}$ variant lines as a constitutively-expressed editing-deficient PheRS causes neurodegeneration, impairs climbing performance, and reduces lifespan in flies (Lu *et al.* 2014). This difference in performance is not solely due to amount of mistranslation observed, as the $tRNA^{Ser}_{AGU}$ variant showed the highest level of mistranslation but did not impair climbing performance. Learning and memory assays will be performed on these $tRNA^{Ser}$ variant lines to determine if other complex neurological traits are disrupted. Interestingly, a study in mice showed that $tRNA^{Ser}_{UGA}$ and $tRNA^{Thr}_{AGU}$ are both highly expressed in brain tissue whereas $tRNA^{Val}_{AAC}$ is expressed at lower levels (Yu *et al.* 2022). If expression patterns in flies are similar, then there may be greater competition for $tRNA^{Ser}_{AGU}$ and lower competition for $tRNA^{Ser}_{AAC}$ in neurons, thus decreasing amount of T→S and increasing amount of V→S mistranslation in neuronal tissue. However, locomotion, memory, and learning are indirect measurements of neurodegeneration so future experiments should also directly examine neuroanatomical structures such as the retina or brain to determine if neurodegeneration has occurred. Neurodegenerative disease-causing alleles such as polyQ-*htt* should also be crossed into $tRNA^{Ser}$ variant lines to determine if tRNA variants affect disease progression or severity. Some neurological diseases, such as Huntington's disease and Alzheimer's disease, also disproportionately affect one sex so the effects of mistranslation on disease progression might be sex-specific as well (Viña and Lloret 2010; Zielonka *et al.* 2013).

Our observation that both $tRNA^{Ser}_{AAC}$ and $tRNA^{Ser}_{AGU}$ variants increase female lifespan is intriguing and contrary to what we would have expected given the strongly

negative effects of mistranslation in other eukaryotes (Lee *et al.* 2006; Reverendo *et al.* 2014; Liu *et al.* 2014). Future studies should aim to identify the mechanism behind this female-specific lifespan increase. Transcriptomic and proteomic analysis of our long-lived lines at various ages could reveal gene expression and proteome shifts that implicate certain pathways in the mistranslation-induced lifespan increase. If this increase is due to a physiological state resembling dietary restriction, as our transcriptomic data in flies suggests, then we would expect to see downregulation of proteins involved in the insulin/insulin-like growth factor signalling and mTOR pathways (reviewed in Pan and Finkel 2017 and Kapahi *et al.* 2017). Developing an inducible system for tRNA expression in flies would also allow for identification of which life stages contribute to this lifespan increase.

It is important to note that all analyses in this thesis used virgin flies. Mating status has large effects on fly lifespan, behaviour, and resource allocation. Female flies experience an especially large physiological and behavioural shift after mating, as they increase egg laying rate, food intake, and even lengthen their midgut (Barnes *et al.* 2008; White *et al.* 2021). Both male and female fly lifespan decreases as number of mating partners increases, but female lifespan significantly decreases after only a single mating whereas a single mating has no effect on male lifespan (Koliada *et al.* 2020). It would be necessary to test the effect of mistranslating tRNA variants on mated flies to identify if mating status changes fly response to mistranslating tRNA variants.

Previous studies examining the effects of amber stop codon suppressor tRNAs in *D. melanogaster* found that they cause male sterility and sometimes impact female fertility (Laski *et al.* 1989; Garza *et al.* 1990). Some tRNA variants are associated with human reproductive issues as well (Mojodi *et al.* 2023) Mistranslating tRNA variants could affect various reproductive traits by impairing translation fidelity, but they could also affect reproduction through other mechanisms. There is an increasing focus on the role of tRNA-derived fragments, which are non-coding RNA molecules derived from mature tRNAs, on sperm function and reproductive health (Sharma *et al.* 2016; reviewed in Chen and Zhou 2023). Dysregulation of tRNA-derived fragments in sperm samples is associated with idiopathic infertility in humans and large offspring syndrome in cows

(Grosso *et al.* 2021; Goldkamp *et al.* 2022). Future experiments should attempt to understand how mistranslation and tRNA-derived fragment dysregulation affect various reproductive traits. Straightforward assays measuring fecundity, egg viability, courtship success, and sperm motility would provide valuable information on how tRNA variants affect reproduction and have important evolutionary implications regarding translation fidelity.

## 5.6 Conclusions

Transfer RNAs help maintain translation fidelity through their role as adaptor molecules between mRNA transcripts and the proteins they encode. Variant tRNAs that disrupt translation fidelity by inducing mistranslation negatively affect various aspects of cell biology and are implicated in disease. This thesis describes my efforts to create and characterize a model of tRNA-induced mistranslation in the fruit fly *Drosophila melanogaster*. These studies show the diverse effects mistranslating tRNA variants have on multicellular eukaryotes, ranging from physical deformities to lifespan extension. My work also cautions against generalizing the effects of tRNA variants, as each $tRNA^{Ser}$ variant I tested produced different physiological effects. Our understanding of the influence of cytosolic tRNA variants on disease is limited, but so is our understanding of their potential therapeutic role. The model I created will help uncover key insights into how organisms cope with translation errors, how mistranslation affects disease, the potential benefits tRNA variants have on eukaryotic biology, and the potential utility of tRNA-based therapeutics.

## 5.7 Literature Cited

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**Presentations**

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