Context-dependent threats to the fidelity of translation of the genetic code.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Adil Baig Moghal

Graduate Program in Biochemistry

The Ohio State University

2016

Dissertation Committee:

Dr. Michael Ibba, Advisor

Dr. Kurt Fredrick

Dr. Karin Musier-Forsyth

Dr. Karl Werbovetz
ABSTRACT

Accurate pairing of amino acids and tRNAs by aminoacyl-tRNA synthetases is a primary checkpoint in maintaining the fidelity of translation of the genetic code. Misacylated aa-tRNAs utilized by the ribosome specify insertion of amino acids into protein at positions not defined by the genetic code. As such, multiple mechanisms have evolved to limit the production of misacylated aa-tRNAs, including strict discrimination against inappropriate amino acids and tRNA that may compete for an aaRS and hydrolysis of the products of erroneous aa-tRNA synthesis. Recently, many environmental conditions that temporarily increase the frequency of aa-tRNA mischarging as well as organisms with unusually high error rates in translation have been discovered, suggesting both that the fidelity of translation of the genetic code is a fluid system and that the requirements for strict quality control in translation are more relaxed in some distinct environmental contexts than in others.

Following the observation that tyrosine starvation results in accumulation of phenylalanine at Tyr codons in protein produced Chinese hamster ovary cell culture, we examine quality control by tyrosyl-tRNA synthetase (TyrRS). We find that, under normal growth conditions, discrimination against Phe in the amino acid activation step is sufficient to limit Phe-tRNA\textsuperscript{Tyr}, but under conditions of Tyr starvation, the relative
abundance of Tyr:Phe drops significantly, resulting in accumulation of Phe-tRNA$_{\text{Tyr}}$ species and mistranslation of Tyr codons as Phe. We characterize mutant variants of CHO TyrRS and address the divergence between bacterial and eukaryotic substrate specificity determinants in TyrRS.

Bacterial phenylalanyl-tRNA synthetase (PheRS) bears a proofreading domain that catalyzes the hydrolysis of misacylated aa-tRNA$_{\text{Phe}}$ species in an active site distinct from the synthetic core. Under normal conditions, the activity of this post-transfer editing domain is dispensable. Like CHO TyrRS, bacterial PheRS displays strict discrimination against most non-cognates in the amino acid activation step. We performed extensive phenotypic and kinetic characterization of *Escherichia coli* PheRS, and find that post-transfer editing is evolutionarily conserved to protect the cell against cytotoxic mistranslation of Phe codons arising from misacylation of tRNA$_{\text{Phe}}$ species with the non-protein amino acid *meta*-tyrosine, which accumulates under oxidative stress. *m*-Tyr is one of many oxidized derivatives of the normal PheRS substrate, Phe. PheRS in *E. coli* therefore limits mistranslation by both strong discrimination against most non-cognate amino acids in the activation step, as well as post-transfer editing of a non-protein amino acid that accumulates in a specific environmental stress condition.

*Saccharomyces cerevisiae* cytoplasmic PheRS, unlike *EcPheRS*, does not discriminate against non-cognate amino acids efficiently in the activation step. Instead, it relies on post-transfer editing activity to limit production of misacylated tRNA$_{\text{Phe}}$ species with non-cognate amino acids that would otherwise not be efficiently activated by the bacterial enzyme. Given the difference between *EcPheRS* and *ScytoPheRS* in the
mechanisms that limit production of misacylated aa-tRNA$^{\text{Phe}}$ species, we performed more extensive analysis of the repertoire of non-cognate amino acids that threaten the selectivity of $SccytoPheRS$. We find that this enzyme bears at least three distinct mechanisms to limit production of misacylated aa-tRNA$^{\text{Phe}}$, particularly those species derived from non-protein products of oxidative Phe damage. Unexpectedly, defective post-transfer editing is discovered to play a non-canonical role in regulation of cell growth under conditions of amino acid stress.
DEDICATION

This work is dedicated to my families of both genetics and circumstance.
ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor Dr. Michael Ibba, who judges his personal success by the growth of those he advises. I am one of a lucky few who have the privilege of learning from mentor who I respect both as an academic giant and as a person. Secondly, I owe a debt of gratitude to Dr. Tammy Bullwinkle, Dr. Medha Raina, Andrei Rajkovic, Kyle Mohler and all of the myriad members of Mike’s lab who have taught and challenged me to be a better scientist. I am also deeply grateful for the personal interest my committee members have taken in my work, and for the personal time they have sacrificed to meet with me to discuss experimental techniques. Finally, I am grateful to The Ohio State University, The Ohio State Biochemistry Program, The Chemistry-Biology Interface training program, and the Cellular, Molecular, and Biochemical sciences program for support and training.
VITA

2011………………………B.S. Biology, West Virginia University

2011 to present……………Graduate Research Associate, Department of Microbiology,

The Ohio State University

PUBLICATIONS


FIELDS OF STUDY

Major Field: Biochemistry.

vii
### TABLE OF CONTENTS

Abstract ................................................................................................................................. ii

Dedication ............................................................................................................................... v

Acknowledgements ............................................................................................................... vi

List of Tables ......................................................................................................................... xiv

List of Figures ......................................................................................................................... xv

List of Symbols and Abbreviations ....................................................................................... xviii

Chapter 1 ............................................................................................................................... 1

1.1 Evolution of The Genetic Code ..................................................................................... 1

1.2 Aminoacyl-tRNA and the Fidelity of Translation of the Genetic Code ...................... 3

1.3 Aminoacyl-tRNA: Synthesis and Quality Control ....................................................... 4

1.3.1 Aminoacyl-tRNA Synthetases and aa-tRNA Synthesis ........................................ 4

1.3.2 aaRS Substrate Discrimination ............................................................................... 6

1.3.3 Pre-transfer Editing ................................................................................................. 7

1.3.4 Post-transfer Editing ............................................................................................... 8

1.3.5 Trans-Editing ........................................................................................................... 9

viii
1.3.6 Selective aa-tRNA Binding by Elongation Factor Tu ........................................ 9

1.3.7 Ribosomal Quality Control ................................................................................. 10

1.4 Defining Mistranslation, Deviation from the Norm, and Effects on the Cell .......... 11

1.5 Expansion of the Genetic Code ................................................................................. 16

1.6 Environmental Stress, Metabolism, and Conditional Threats to Translational
   Fidelity ......................................................................................................................... 17

1.6.1 Amino Acid Starvation and Environmental Imbalance .................................. 17

1.6.2 Oxidative Stress .................................................................................................. 21

1.7 Tools for Quantitative Analysis of Mistranslation and Amino Acid Pools ............ 23

1.8 Purpose of Study ...................................................................................................... 26

Chapter 2 ........................................................................................................................ 29

2.1 Introduction ............................................................................................................... 29

2.2 Materials and Methods .............................................................................................. 31

2.2.1 CHO phenotypic and metabolomics analysis ....................................................... 31

2.2.2 CHO TyrRS cloning and mutagenesis ................................................................. 32

2.2.3 Purification of CHO TyrRS and CHO TyrRS variants ........................................ 32

2.2.4 Construction and purification of synthetic CHO tRNA^{Tyr} ................................. 33

2.2.5 Aminoacylation assays ......................................................................................... 34

2.2.6 Kinetic characterization of amino acid activation efficiency .............................. 34
2.3 Results .................................................................................................................................................. 37

2.3.1 Tyr starvation causes a loss of productivity and viability in CHO cells expressing a monoclonal antibody. ........................................................................................................................................... 37

2.3.2 CHO TyrRS discriminates poorly against Phe at the amino acid activation step ................................................................................................................................................................. 40

2.3.3 CHO TyrRS misacylates tRNA\textsuperscript{Tyr} with Phe ........................................................................ 42

2.3.4 Loss of the CHO TyrRS C-terminal EMAP II-like domain does not affect amino acid specificity ......................................................................................................................................................... 43

2.3.5 Rational design of CHO TyrRS variants reveals substrate specificity determinants ................................................................................................................................................................................. 44

2.4 Discussion .................................................................................................................................................. 51

2.4.1 Tyr codon mistranslation in CHO cells is due to conditional amino acid imbalance ........................................................................................................................................................................... 51

2.4.2 Accumulation of Phe at Tyr codons is due to production of Phe-tRNA\textsuperscript{Tyr} ...... 53

2.4.3 Divergence in amino acid discrimination between bacterial and eukaryotic TyrRS .................................................................................................................................................................................... 53

Chapter 3 ....................................................................................................................................................... 56

3.1 Introduction ............................................................................................................................................... 56

3.2 Materials and methods ............................................................................................................................. 58

3.2.1 Strains, plasmids, and general methods ................................................................................................. 58

x
3.2.2 Construction of post-transfer editing-defective EcPheRS and SccytoPheRS strains.......................................................................................................................... 59

3.2.3 Phenotypic analysis .......................................................................................................................... 62

3.2.4 S. cerevisiae aging .......................................................................................................................... 63

3.2.5 RNA detection via Northern blotting ............................................................................................ 63

3.2.6 tRNA transcription and $^{32}$P radiolabeling ................................................................................. 64

3.2.8 Steady-state characterization of amino acid activation, as measured by ATP/PP$_i$ exchange ................................................................................................................................. 66

3.2.9 Dipeptide synthesis in vitro ........................................................................................................ 67

3.2.10 Quantification of amino acid pools from extracted soluble intracellular metabolites ............................................................................................................................... 67

3.2.11 Purification and LC-MS/MS-MRM of total soluble protein hydrolysate ...... 69

3.3 Results ............................................................................................................................................... 69

3.3.1 PheRS post-transfer editing is dispensable in E. coli and S. cerevisiae .......... 69

3.3.2 Post-transfer editing by EcPheRS confers resistance to m-Tyr ......................... 71

3.3.3 Post-transfer editing defective SccytoPheRS does not induce ER stress. ......... 77

3.3.4 EcPheRS and SccytoPheRSs have divergent substrate specificities. ............... 79

3.3.5 Phe codon mistranslation as m-Tyr in E. coli is elevated in an EcPheRS post- transfer editing defective strain. ........................................................................................................ 82
3.3.6 EcPheRS post-transfer editing is required for normal growth in oxidative stress conditions............................................................................................................. 87

3.4 Discussion .................................................................................................................. 89

3.4.1 Context-dependent threats to PheRS product specificity, and conditional requirements for post-transfer editing. .................................................................................. 89

3.4.2 Non-proteinogenic amino acids as threats to the fidelity of translation of the genetic code. .............................................................................................................. 90

3.4.3 Conditional challenges to translational quality control under oxidative stress. 92

Chapter 4 ......................................................................................................................... 94

4.1 Introduction .................................................................................................................. 94

4.2 Materials and Methods ............................................................................................. 98

4.2.1 Strains and growth conditions .............................................................................. 98

4.2.2 Quantification of Intracellular Amino Acid Abundance ...................................... 98

4.2.3 Liquid Chromatographic Purification of Amino Acids ....................................... 100

4.2.4 Wild-type and Post-Transfer Editing Defective ScytoPheRS Preparation ... 101

4.2.5 Steady-State kinetic analysis of ScytoPheRS ....................................................... 101

4.2.6 Aminoacylation of tRNA ..................................................................................... 102

4.2.7 Pre-transfer editing ............................................................................................. 102

4.2.8 Pre-Steady-State Characterization of o-Tyr Transfer ....................................... 103

4.2.9 Measurement of o-Tyr-AMP Stability ............................................................... 104
4.3 Results ............................................................................................................................................. 104

4.3.1 SccytoPheRS post-transfer editing limits the cytotoxicity of oxidative stress 104

4.3.2 Oxidized Phe derivatives accumulate under oxidative stress ......................... 105

4.3.3 SccytoPheRS Post-Transfer Editing has Amino Acid Supplement Dependent Effects on Growth ................................................................................................................. 107

4.3.4 SccytoPheRS displays poor discrimination against oxidized Phe derivatives \textit{in vitro} ........................................................................................................................................ 109

4.3.5 An Expanded form of aaRS Biological Substrate Selectivity ....................... 110

4.3.6 Aminoacylation of tRNA\textsuperscript{Phe} with o-Tyr is Inefficient ......................... 113

4.3.7 o-Tyr-tRNA\textsuperscript{Phe} Limitation is Not Due to Hydrolytic Pre-Transfer Editing.... 115

4.3.8 o-Tyr-AMP is selectively released from the SccytoPheRS active site .......... 117

4.3.9 o-Tyr-AMP release is due to slow transfer .............................................................. 117

4.4 Discussion ....................................................................................................................................... 119

4.4.1 Limitation of o-Tyr-tRNA\textsuperscript{Phe} synthesis by SccytoPheRS ................. 119

4.4.2 SccytoPheRS post-transfer editing plays a non-canonical role in tolerance to amino acid stress .............................................................................................................................. 123

Chapter 5 ............................................................................................................................................... 126

References ............................................................................................................................................ 131
LIST OF TABLES

Table 2.1 Primers used in CHO TyrRS mutagenesis .............................................36
Table 2.2 Steady-state kinetic parameters for amino acid activation, as measured by ATP-[\(^{32}\)P]PP\(_i\) exchange with CHO TyrRS and mini TyrRS ...........................................42
Table 2.3 Steady-state kinetic constants for ATP-[\(^{32}\)P]PP\(_i\) exchange for CHO cytosolic full length wild type and variant TyrRS .........................................................48
Table 3.1 Intracellular abundance of Phe, \(p\)-Tyr, and \(m\)-Tyr in wild-type and PheRS post-transfer editing-defective \(E.~coli\) in supplementation experiments ...............74
Table 4.1 Steady-state kinetic parameters for amino acid activation by \(S\)cytoPheRS...110
Table 4.2 \(S\)cytoPheRS selectivity is decreased under oxidative stress ......................112
LIST OF FIGURES

Figure 1.1 Aminoacyl-tRNA synthesis and quality control mechanisms.......................5
Figure 1.2 Lytic pre-transfer editing of a non-cognate aa-AMP ...............................8
Figure 1.3 Mistranslation and the statistical proteome..........................................14
Figure 1.4 The bacterial stringent response..........................................................18
Figure 1.5 The eukaryotic starvation response......................................................20
Figure 1.6 Oxidation of proteinogenic amino acids in conditions of oxidative stress.................................................................22
Figure 2.1 Phenotypic and productivity effects of Tyr supplementation on CHO cells expressing a monoclonal antibody.................................................................39
Figure 2.2 CHO TyrRS efficiently utilizes Phe as a substrate for activation and tRNA^{Tyr} charging with both native tRNA and synthetic tRNA^{Tyr2}.................................41
Figure 2.3 Apparent mischarging of CHO tRNA^{Tyr} with Phe is not due to contamination with *E. coli* PheRS.................................................................43
Figure 2.4 Known TyrRS active site residues that hydrogen bond with tyrosyl-adenylate..............................................................................................45
Figure 2.4 Continued..............................................................................................46
Figure 2.5 Hydrogen bond network of *G. stearothermophilus* TyrRS Asp176 (CHO Asp173) and Tyr

Figure 3.1 Post-transfer editing defective strains of *E. coli* and *S. cerevisiae*..............71

Figure 3.2 Phenotypic analysis of non-cognate amino acid supplementation in PheRS post-transfer editing defective *E. coli*.................................................................73

Figure 3.3 Effects non-cognate Tyr isomer supplementation on the growth of *SccytoPheRS* post-transfer editing-defective *S. cerevisiae*.................................77

Figure 3.4 A *SccytoPheRS* post-transfer editing defect does not induce the UPR.........78

Figure 3.5 Tyrosine isomers as substrates for tRNA$^{Phe}$ aminoacylation by PheRS variants in both *E. coli* and *S. cerevisiae*..............................................................81

Figure 3.6 Translation of m-Tyr in *E. coli*............................................................83

Figure 3.7 p-Tyr mistranslation is not elevated in *E. coli* at Phe codons in the absence of post-transfer editing by EcPheRS.................................................................86

Figure 3.8 EcTyrRS efficiently aminoacylates tRNA$^{Tyr}$ with m-Tyr.........................87

Figure 3.9 The role of EcPheRS post-transfer editing in oxidative stress conditions *in vivo*................................................................................................................88

Figure 4.1 Potential non-cognate amino acid substrates of PheRS......................96

Figure 4.2 *SccytoPheRS* post-transfer editing limits the cytotoxicity of oxidative stress....................................................................................................................105

Figure 4.3 Oxidative stress increases the abundance of non-protein amino acids........106

Figure 4.4 *SccytoPheRS* post-transfer editing activity has variable effects on growth rate dependent on supplemented amino acids............................................108
Figure 4.5 $o$-Tyr is inefficiently acylated to tRNA$^{Phe}$ ..........................................................113

Figure 4.6 Wild-type ScytoPheRS synthesizes $o$-Tyr-tRNA$^{Phe}$ as efficiently as βD243A ScytoPheRS ........................................................................................................................................114

Figure 4.7 $o$-Tyr-AMP is not efficiently hydrolyzed by ScytoPheRS .........................115

Figure 4.8 $o$-Tyr-AMP is stable in vitro .........................................................................................116

Figure 4.9 $o$-Tyr-AMP is synthesized less efficiently in the absence of tRNA$^{Phe}$ ........117

Figure 4.10 $o$-Tyr discrimination is due to inefficient transfer to tRNA$^{Phe}$ ........119
# LIST OF SYMBOLS AND ABBREVIATIONS

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<td>L-pyrrolysine</td>
</tr>
<tr>
<td>q_p</td>
<td>specific productivity</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>ScytoPheRS</td>
<td><em>Saccharomyces cerevisiae</em> cytoplasmic PheRS</td>
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<tr>
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<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SD</td>
<td>shine-dalgarno</td>
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<tr>
<td>S.D.</td>
<td>standard deviation</td>
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<td>SDC</td>
<td>synthetic defined complete media</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>Ser</td>
<td>L-serine</td>
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<td>trichloroacetic acid</td>
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<tr>
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<td>L-threonine</td>
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<tr>
<td>ThrRS</td>
<td>threonyl-tRNA synthetase</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
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<tr>
<td>Tris-HCl</td>
<td>tris-(hydroxylmethyl) aminomethane hydrochloride</td>
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<td>transfer RNA</td>
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<td>L-para-tyrosine</td>
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<td>tyrosyl-tRNA synthetase</td>
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<tr>
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<td>University of California, Los Angeles</td>
</tr>
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<td>Ura</td>
<td>uracil</td>
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<tr>
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<td>ultraviolet</td>
</tr>
<tr>
<td>Val</td>
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<td>viable cell density</td>
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<td>WT</td>
<td>wild-type</td>
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<tr>
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<td>yeast peptone dextrose agar</td>
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Chapter 1

Introduction

1.1 Evolution of The Genetic Code

Often described as the “blueprint” for the cell, the genome contains coded information specifying specific sequences of RNA, and—if the gene specifies a protein—amino acids. Over the course of genetic evolution, natural mutational variation in gene sequences, and thus differences in the sequences of RNA and proteins, has afforded nature a diverse palate of biomolecules with various effects on host organisms. While evolution is not directed toward specific “goals,” the genes most essential for survival or that confer a selective benefit to the host organism are the genes that have been retained and refined.

Whereas most modern catalytic biomolecules are derived from protein, ancient molecular biology is believed to have consisted mainly of self-replicating nucleic acids, a fraction of which bore catalytic function. Some of the most beneficial or indispensable catalytic RNAs persist today in the form of ribozymes. Perhaps the most conserved
ancient biomolecule is the ribosome, the catalytic core of which is comprised of RNA. This core, called the peptidyl transferase center, is responsible for forming peptide bonds between amino acids in the process of ribosomal protein synthesis. The ribosome does not assemble sequences of proteins randomly. Instead, information contained within a gene is first transcribed into an RNA molecule. The resulting RNA transcript can be further modified and processed to form mature messenger RNA (mRNA) prior to delivery to the ribosome.

Triplets of mRNA nucleotides—codons—specify individual and distinct amino acids, and the sequence of codons in mRNA dictates the sequence of amino acids in the corresponding protein. The ribosome processes along an mRNA molecule, adding amino acids to a growing polypeptide chain in a specific and ordered process called translation, which refers to translation between the information in sequences of nucleic acid codons to amino acid sequences of protein. The genetic code was therefore defined by comparing the amino acid sequences of proteins to the codons from which they were derived. In this manner, specific codons were defined as specifying unique amino acids.

Prior to ribosomal translation, biomolecular diversity was limited by availability of genetically-encoded monomers. The available sequence space for RNA had been limited to four nucleotides, which could be combined to sequences with $4^n$ possible combinations, where $n$ is the length of a given polymer. With 22 known proteinogenic monomer amino acids, access to genetically-encoded protein synthesis permits production of biomolecules with $22^n$ possible combinations. Moreover, some proteinogenic amino acids bear functional groups with distinct chemical activity than
those of nucleic acids, including sulfur and selenium chemistry. The drastic increase in available sequence space and chemical functional groups allowed for much greater diversity of possible binding and catalytic activities.

1.2 Aminoacyl-tRNA and the Fidelity of Translation of the Genetic Code

Central to all life is the flow of information from DNA to RNA and protein (1). However, genome replication, gene transcription, and ribosomal translation are imperfect processes. Evolution relies on replicative errors in genome duplication resulting in offspring with some infrequent genomic variation relative to their progenitors. Genomic diversity benefits species as a whole, because it prevents uniform susceptibility to infection and other environmental assaults. However, mutations may also result in defective or deleterious genes. An inaccurately transcribed DNA base can result in an mRNA codon with different identity, and inaccurate decoding of mRNA codons at the ribosome can result in inappropriate amino acid insertion in a nascent peptide. Such errors in information flow can result in truncated and/or misfolded proteins, proteins with neutral or deleterious substitutions at critical residues (2), and an overall loss in protein function at the molecular and cellular levels (3). Whereas genomic mutations do happen, the polymerases that replicate genetic material have evolved mechanisms to limit variability, such as proofreading functionality, whereby a polymerase may “check” a growing polymer and hydrolyze phosphodiester linkages between erroneously-inserted and properly-inserted nucleotides before proceeding. In Escherichia coli, spontaneous mistakes in DNA replication are limited to one mutation in ~10^10 base pairs per
generation (4). Errors in bacterial gene transcription are as low as one error in $\sim10^4$ nucleotides transcribed (5). Errors in translation are estimated to be as frequent as an order of magnitude higher—one misincorporated amino acid per $10^4$-$10^3$ codons in *E. coli* (5). Thus, the most error-prone step in protein production is translation itself.

1.3 Aminoacyl-tRNA: Synthesis and Quality Control

1.3.1 Aminoacyl-tRNA Synthetases and aa-tRNA Synthesis

The enzymes that produce the substrates for ribosomal protein synthesis are called the aminoacyl-tRNA synthetases (aaRSs). aaRSs are protein enzymes that synthesize aa-tRNAs in a two-step process (6). aaRSs first utilize the chemical potential energy stored in ATP to “activate” an amino acid, forming an aminoacyl-adenylate (aa-AMP), releasing inorganic pyrophosphate; this first step may be tRNA-dependent or tRNA-independent. In the second catalytic step, aaRSs transfer the aminoacyl moiety to a tRNA molecule, releasing adenosine monophosphate (AMP) (Fig. 1.1).
Figure 1.1. Aminoacyl-tRNA synthesis and quality control mechanisms. aa-tRNA synthesis is a two-step process, with checkpoints at each step to ensure proper product formation. An aminoacyl-adenylate formed by the activation of a noncognate amino acid may be hydrolyzed by pre-transfer editing mechanisms in the active site of the aaRS. Misactivated amino acids that escape pre-transfer editing may be acylated to tRNA, forming aa-tRNA. If an aaRS bears a post-transfer editing domain, misacylated aa-tRNA may be hydrolyzed, releasing the amino acid and tRNA.

The resulting aminoacyl-tRNA (aa-tRNA) is then released and brought to the ribosome by elongation factor Tu (EF-Tu) in bacteria, or elongation factor 1A (EF-1A) in archaea and eukarya. The anticodon of the tRNA moiety binds to a corresponding mRNA codon, which stimulates GTPase activity in aa-tRNA:EF-Tu/1A:GTP ternary complex, releasing the elongation factor bound from the ribosome. The aminoacyl moiety of the aa-tRNA is then transferred in the PTC from tRNA to the nascent polypeptide being synthesized. The tRNA is released, the ribosome proceeds forward one codon, and the cycle repeats. Most organisms contain at least 20 aaRSs, each of which is responsible for attaching a specific amino acid to one or more “isoacceptor” tRNAs with anticodons specifying the insertion of the relevant amino acid in the nascent peptide (7).

aaRSs are divided into two classes based on structural and functional features
Class I aaRSs are typically monomeric or homodimeric, and bear a Rossman nucleotide-binding motif responsible for positioning ATP for use in amino acid activation. With the exception of tyrosyl-tRNA synthetase (10), class I aaRSs bind their respective tRNAs on the minor groove, and transfer amino acids to the 2’-OH of the ribose attached to the 3’ terminal adenine (A76). Spontaneous transesterification results in movement of the aminoacyl moiety to the 3’-OH. Class II aaRSs are usually dimeric or multimeric, bearing a characteristic active site geometry consisting of seven-stranded β-sheets flanked by two α-helices (8,9). aaRSs of this class bind the major groove of their cognate tRNAs, and typically aminoacylate the 3’-OH of the A76 ribose (11). Phenylalanyl-tRNA synthetase (PheRS) is unique among the class II aaRSs in that it aminoacylates the 2’-OH of tRNA$_{Phe}$ (12), a feature typically associated with class I aaRSs.

### 1.3.2 aaRS Substrate Discrimination

For both amino acid selection and tRNA selection, a given aaRS is presented with a problem: it must select its appropriate, or “cognate” amino acid or tRNAs from a pool of mostly similar “non-cognate” substrates. The challenge of tRNA selection lies in a similar total negative charge and general tertiary structure of all tRNAs. aaRSs overcome this problem by discriminating against local structural features that differ between tRNAs. Many tRNA features can serve as “identity elements,” including nucleotides on the acceptor stem; one of the most commonly-utilized identity elements is the anticodon (13). Many aaRSs form intimate contacts with these nucleotides, rejecting non-cognate
tRNAs without the proper complementarity for anticodon binding. By changing only the CAU anticodon of *E. coli* elongator tRNA\textsuperscript{Met} to the tRNA\textsuperscript{Thr} anticodon sequence CUG, this tRNA becomes an efficiently-charged threonine acceptor, supporting the anticodon as a major identity element for both tRNA\textsuperscript{Met} and tRNA\textsuperscript{Thr} (14).

Discrimination against non-cognate amino acids is a greater challenge. These substrates are much smaller than tRNAs, and therefore have fewer functional groups available for substrate discrimination. Some natural, proteinogenic amino acids differ from one another by the presence or absence of a single oxygen atom (Phe vs. Tyr), or by replacement of a single atom with another, chemically similar atom (Ser vs. Cys). These are challenges unique to each individual aaRS, and the mechanisms of active site discrimination are diverse.

### 1.3.3 Pre-transfer Editing

Non-cognate amino acids that escape kinetic discrimination in the first catalytic step of aa-tRNA synthesis may be substrates for a quality control step called pre-transfer editing, which refers to proofreading prior to the transfer step (Fig. 1.1). Pre-transfer editing may be lytic or non-lytic, and may require the participation of tRNA (15): In lytic pre-transfer editing, an aaRS selectively catalyzes degradation of a non-cognate aa-AMP, releasing the non-cognate amino acid and AMP. Lytic pre-transfer editing can arise as a result of direct hydrolysis of the aa-AMP linkage, or from aminoester attack by a non-cognate side chain, cyclizing the amino acid and releasing AMP (Fig. 1.2). Non-lytic pre-transfer editing is the dissociation of a non-cognate aa-AMP from the synthetic active site. This
release may be selective, arising from incompatible binding geometry favoring release from the active site, or entropic, arising from eventual dissociation of a non-cognate aa-AMP that is a poor substrate for the transfer step. Often, a combination of lytic and non-lytic pre-transfer editing is observed for a non-cognate in the same aaRS.

**Figure 1.2. Lytic pre-transfer editing of a non-cognate aa-AMP.** Pre-transfer lytic editing of a non-cognate aa-AMP species may arise by either (A) hydrolysis or (B) cyclization.

### 1.3.4 Post-transfer Editing

Approximately half of aaRSs bear a catalytic domain distinct from the synthetic active site, responsible for hydrolytic cleavage of non-cognate aa-tRNAs (Fig. 1.1). Upon aminoacyl transfer from aa-AMP to tRNA, the 3’ aminoacylated A76 of aa-tRNA can be sampled by a post-transfer editing active site. For instance, *E. coli* PheRS and *Saccharomyces cerevisiae* cytoplasmic PheRS post-transfer editing recognize and
hydrolyze Tyr-tRNA$_{\text{Phe}}$ resulting from mischarging of tRNA$_{\text{Phe}}$ with tyrosine. For the same reason that amino acid discrimination is a challenge unique to a given aaRS, post-transfer editing domain active sites have binding geometry unique to specific non-cognate amino acid threats. Post-transfer editing by PheRS can act in trans, as the aaRS re-samples mischarged aa-tRNA, hydrolyzing misacylated species before they can be used as substrates in translation (16).

### 1.3.5 Trans-Editing

Though not discovered for every proteinogenic amino acid, there exist freestanding post-transfer editing proteins that monitor the cell for specific misacylated aa-tRNAs, performing the same function as aaRS post-transfer editing acting in trans (Fig. 1.1) (17,18). For example, bacterial prolyl-tRNA synthetase (ProRS) mischarges tRNA$_{\text{Pro}}$ with alanine and cysteine, and many bacterial ProRS enzymes are able to hydrolyze Ala-tRNA$_{\text{Pro}}$ using a post-transfer editing “INS” domain, but are unable to hydrolyze Cys-tRNA$_{\text{Pro}}$. The trans-editing factor YbaK is able to compensate for this deficiency, deacylating Cys-tRNA$_{\text{Pro}}$ in trans (19,20). In Caulobacter crescentus, the INS domain of ProRS is truncated and unable to hydrolyze Ala-tRNA$_{\text{Pro}}$. Instead, proline codon mistranslation in this organism is limited exclusively by YbaK, which deacylates Cys-tRNA$_{\text{Pro}}$ in trans, and a separate INS homolog ProXp-ala, which acts in trans to hydrolyze misacylated Ala-tRNA$_{\text{Pro}}$ in the absence of functional ProRS INS activity (21).

### 1.3.6 Selective aa-tRNA Binding by Elongation Factor Tu
Following release from an aaRS, aa-tRNAs bind EF-Tu to be shuttled to the ribosome for protein synthesis. EF-Tu is responsible for binding all aa-tRNA species. Interestingly, Uhlenbeck and coworkers found that misacylated aa-tRNA may bind bacterial EF-Tu either too tightly for efficient release at the ribosome, or too weakly to be efficiently shuttled to the ribosome (22,23). Thus, it seems that aa-tRNA:EF-Tu affinity is as an additional quality control step, whereby improperly acylated aa-tRNAs may be unable to bind EF-Tu in a range of affinities optimal for efficient translation.

1.3.7 Ribosomal Quality Control

Atypical of most enzymatic processes in the cell, ribosomal protein synthesis requires promiscuity in the enzymatic binding site, allowing for dozens of substrate aa-tRNAs bearing the full complement of proteinogenic amino acids to be incorporated into proteins. The nature of genetically encoded amino acid sequences necessitates specificity at the ribosome for canonical aa-tRNAs, such that for each codon, only an aa-tRNA bearing the genetically encoded amino acid can bind and participate in protein synthesis. This specificity is achieved by ribosomal quality control mechanisms that rely on codon-anticodon interactions (24,25) and discrimination against certain types of non-protein amino acids (NPAs), which can be attenuated with ribosomal mutations (26,27). However, no such quality control mechanism exists in the ribosome to exclude aa-tRNAs formed from the linkage of a standard proteinogenic amino acid to a noncognate tRNA. In such a case, codon-anticodon interactions that pass the quality control steps at the ribosome, including mismatched codon-anticodon interactions, will drive protein
synthesis forward. Ribosomal quality control may instead act retrospectively, by increasing the frequency of errors in decoding a given mRNA, facilitating premature release of mis-synthesized peptides from the ribosome (5,28,29). The phenomenon of amino acid insertion at a codon that codes for a different amino acid is termed “mistranslation,” and until recently has been thought to reflect a minor and infrequent mistake in the protein synthesis machinery.

1.4 Defining Mistranslation, Deviation from the Norm, and Effects on the Cell

In recent years, mistranslation has been viewed through a different lens than in previous decades. Mistranslation is typically limited to one erroneously inserted amino acid per $10^3$-$10^4$ translated codons (30). However, many mutations and environmental conditions are known to elevate this error rate to higher levels (31,32). These conditional threats to the fidelity of translation have revealed that standard genetically encoded proteinogenic amino acids are not the only threats to quality control in aa-tRNA synthesis. Additional "non-protein" amino acids (NPAs) with physiochemical properties similar to proteinogenic amino acids must also be discriminated against by relevant aaRSs. Post-transfer editing by E. coli leucyl-tRNA synthetase (LeuRS) has been shown to typically be dispensable, and the near-cognate proteinogenic amino acid isoleucine (Ile) is not an efficient substrate for LeuRS (33). Under conditions that favor the accumulation of certain near-cognate proteinogenic amino acids and NPAs, LeuRS post-transfer editing is critical (34,35). Norvaline is an efficient substrate for LeuRS and may represent a significant threat to quality control at leucine codons under conditions of
oxygen limitation, which induce intracellular norvaline accumulation (33,34). It seems that evolution may favor the conservation of post-transfer editing in part to protect the cell against cytotoxic mistranslation of the genetic code with NPAs, some of which are only biologically relevant threats to the cell under stress conditions.

Recent studies have uncovered differences between organisms in the requirement for quality control in protein synthesis (36-38), suggesting that perfect decoding may not be inherently ideal. Mistranslation of the genetic code in response to cellular stress has been shown in some cases to serve as a clear benefit for the cell (31). It is a misinterpretation of an ambiguous term to equate "mistranslation" with "mistakes" in all cases, as variability in decoding is sometimes evolutionarily conserved and favorable (39,40). Under conditions of oxidative stress, non-methionyl tRNAs can be methionylated by methionyl-tRNA synthetase from E. coli (39), yeast (40), and mammals (31). Because methionine may spontaneously react with reactive oxygen species (ROS) that are formed under oxidative stress, methionine residues mistranslated at non-methionyl codons may serve as ROS "sinks," to be later safely reduced by methionine sulfoxide reductases (41). Such “adaptive translation” is reviewed extensively elsewhere (42). In one notable example, the Candida albicans CUG codon is inherently decoded in an ambiguous manner, and the resulting proteomic and phenotypic diversity (43) may make this opportunistic pathogen a "moving target" for the host’s adaptive immune system (44).

Taken together, these examples illustrate the substantial difficulty in simply defining mistranslation, given the degree to which some organisms tolerate or benefit
from codon ambiguity. “Mistranslation,” “errors” in protein synthesis, and “accuracy” in translation are terms that implicitly assign a negative value to deviations from stringent definitions of codon identity. Moreover, consideration of the basal level of mistranslation on a per-protein level is too limited in scope, as the full complement of proteins in the cell has, by the statistical nature of misincorporation, a wide range of primary sequences (Fig. 1.3). This “statistical protein” model implies a frequency of misincorporation at every codon, such that any given amino acid has a certain probability of translation at a given codon. Quality control mechanisms inherent to the translation machinery limit these errors, but the system is imperfect and dynamic; perturbations in amino acid pools (45,46), modification in the copy number or modification status of tRNAs (47-49), and conditional variation in aaRSs levels (50) can all change the frequency of translated amino acids at a given codon. Expanding this picture to include every newly synthesized protein in new growth conditions and retaining the non-degraded protein from all previous growth conditions further complicates the picture of the mistranslation frequencies of protein sequences in the cell.
Figure 1.3. Mistranslation and the statistical proteome. Represented here are various copies of a single protein arising from translation in low- or high-frequency mistranslation systems. Amino acids inserted at appropriate codons are shown as blue. Inappropriately inserted amino acids are represented as green and red. Typical mistranslation is infrequent, resulting in protein populations with minor variability. In organisms that naturally mistranslate more frequently (36,38), or in conditions that promote less stringent quality control (31,39,40,46), protein populations become more diverse in their primary sequences. Proteins arising from “statistical proteomes” have various folding and binding properties, resulting in phenotypic diversity in the host organism. Expanded to include all proteins in a cell, the effects of mistranslation can be drastic at the molecular and cellular levels.

Because the primary sequence of a protein determines its fold and function, variation in the primary sequence can result in "neomorphic" proteins, which bear new and different functions in binding and catalysis than those of the parent protein. Variations in protein sequence due to genetic mutation are the basis of evolution, resulting in heritable allelic diversity. Mutation is typically deleterious or neutral at best,
but occasionally refines or gives new beneficial function to a protein. These heritable mutant proteins may grant the organism a selective advantage and thus the new sequence becomes a feature of the species.

Mistranslation in a protein population instead results in non-heritable diversity at the protein level. A population of proteins with variability in their primary sequence may have varying degrees of mistranslation with many different amino acids at many different positions, potentially yielding neomorphic individual proteins. Proteins with neomorphic moonlighting functions implicated in disease have been discussed elsewhere (51,52). Under normal conditions, the effects of low-frequency mistranslation may be minimal. In conditions that increase the frequency of these replacement events, drastically varied peptide sequences may become a double-edged sword: whereas randomness in protein populations may decrease the binding and catalytic function of a protein population as a whole and can result in aggregation and growth defects (32), individual proteins with neomorph properties may grant the cell access to new and beneficial binding partners and catalytic activities not derived from the genetically-encoded primary sequence. The cost-benefit calculus of protein quality control thus depends on environmental factors and the complement of tools for combating the negative effects of mistranslation at the disposal of the organism in question. Perhaps some NPAs conditionally charged to tRNAs will be discovered to have a role that benefits, rather than harms the cell.

Typically, UAG stop codons signal termination of ribosomal protein synthesis. In Methanosarciniaceae species, the pylT gene and its homologs encodes a tRNA with a CUA anticodon that permits of ribosomal read-through of these codons (53). The pylS
gene and its homologs encodes a class II aaRS that charges the pylT-derived tRNA with the amino acid pyrrolysine (Pyl), the most recently discovered proteinogenic amino acid. Because Pyl is an NPA in most species, and is encoded at what are typically stop codons, it is possible that Pyl is a relatively recent addition to the repertoire of proteinogenic amino acids. This would suggest that mistranslation of stop codons as Pyl conferred a selective benefit in the species that evolved to genetically encode its insertion. Pyl may be only the most recently-discovered of many extant or future natural expansions of the genetic code.

1.5 Expansion of the Genetic Code

A conditional increase in the protein alphabet implies new modes of binding and, depending on the chemical properties of the NPA, possibly unknown modes of catalysis. For this reason, NPAs have been used extensively as molecular tools. Orthogonal aminoacyl-tRNA synthetase:tRNA systems have been used to target specific codons for the incorporation of NPAs (54). The benefits of these systems to molecular research and genetic code expansion are numerous: NPAs that mimic transient post-translational modifications can be used to examine the effects of these modifications in stably “modified” forms of the protein. NPAs with novel chemical reactivity can be targeted directly in vivo for imaging (55) purposes and protein localization studies. NPAs with photocaged side chains can be specifically activated with light (56), allowing for studies of synchronous protein activity in vivo. NPAs may also be used to “improve” the activity of a given enzyme (57).
1.6 Environmental Stress, Metabolism, and Conditional Threats to Translational Fidelity

Biological systems are inherently adaptable. In the long term, populations evolve and adapt to changes in their environment. In the short term, individual cells can alter their metabolic profiles in response to rapid changes in their environment as well. Cells respond to new food sources, extracellular damage, infection, changes in temperature, and any number of conditional challenges. These responses are typically compensatory, and often alter the abundance of intracellular amino acid and tRNA pools. As a result, the selection of cognate amino acids and tRNAs can be a conditional challenge.

1.6.1 Amino Acid Starvation and Environmental Imbalance

Amino acid starvation is sensed by an overabundance of uncharged tRNAs. In bacteria, these uncharged tRNAs can bind directly at the ribosomal A site as the concentration of EF-Tu bound to aa-tRNA and GTP is decreased. This blockage causes ribosomal idling, whereby translation is slowed. Uncharged tRNA in the ribosomal A site also recruits a protein factor RelA, which then synthesizes millimolar concentrations of (p)ppGpp from ATP and either GTP or GDP (Fig. 1.4). (p)ppGpp stimulates transcription of specific biosynthetic genes, including those involved in the production of amino acids. This process, called the stringent response, is a mechanism by which bacteria simultaneously downregulate energetically costly global translation and specifically upregulate genes in response to nutrient starvation. When amino acid starvation ceases, uncharged tRNA levels decrease to normal, and cellular processes resume normally. The spoT protein is
able to rapidly degrade (p)ppGpp, and in the absence of (p)ppGpp production via RelA, the overall amount of (p)ppGpp decreases.

**Figure 1.4. The bacterial stringent response.** In bacteria, high uncharged tRNA levels resulting from amino acid starvation and limited aaRS activity will bind the ribosomal A site, recruiting the protein RelA. RelA stimulates the synthesis of (p)ppGpp, which stimulates amino acid biosynthesis to restore amino acid levels to normal.

In eukarya, amino acid starvation is also detected via uncharged tRNA; uncharged tRNAs bind to the histidyl-tRNA synthetase-like domain of protein GCN2 (Fig. 1.5). tRNA binding stimulates GCN2 kinase activity, which acts to phosphorylate serine 51 of the α-subunit of the translation initiation factor eIF2. The normal role of eIF2 is to shuttle initiator tRNA_{i}^{Met} to the ribosome for the start of translation. Upon productive binding at the ribosome, eIF2 converts GTP to GDP and dissociates from the ribosomal complex. Subsequent rounds of eIF2 activity rely on exchange of GDP for GTP, which is performed by the protein eIF2B. When eIF2 is phosphorylated, as in the case of
uncharged tRNA stimulated GCN2 activity (or during glucose starvation, high salinity, or the presence of double-stranded RNA), eIF2 binds eIF2B with higher than typical affinity, which effectively limits the available eIF2B that can perform GDP/GTP exchange with available unphosphorylated eIF2B. In this manner, phosphorylated eIF2 acts as a competitive inhibitor for its own guanine exchange factor. This effectively diminishes the global initiation of translation while elevated uncharged tRNA levels stimulate GCN2.
**Figure 1.5. The eukaryotic starvation response.** In eukaryotes, high uncharged tRNA levels resulting from low aaRS activity with limited amino acids leads to phosphorylation of eIF2 by the histidyl-tRNA synthetase-like protein GCN2. Phosphorylated eIF2 binds and sequesters its guanine nucleotide exchange factor eIF2B, preventing turnover of eIF2-GDP and limiting translation initiation. Limited initiation complex results in derepression of the GCN4 protein, a transcription factor that stimulates amino acid biosynthesis.

Phosphorylation of eIF2 also stimulates the upregulation of GCN4 by an intricate translational derepression mechanism involving re-initiation of translation at the *GCN4* gene, which is downstream of multiple upstream open reading frames in the *GCN4* mRNA. Under amino acid starvation, when eIF2:GTP:tRNA$_i$Met ternary complex is limited, typical reinitiation of translation at upstream open reading frames results in a majority of dissociated ribosomes prior to the initiation of *GCN4* translation. These upstream open reading frames represent a barrier to *GCN4* translation, but limited ternary complex limits the reinitiation at some of these upstream open reading frames, allowing for the ribosome to continue scanning and reinitiate at *GCN4*. The GCN4 protein is a central player in the upregulation of amino acid biosynthetic genes, and its starvation-dependent activity compensates for nutrient limitation.
Nutrient limitation is also linked to tight regulation of cytoplasmic tRNA in *S. cerevisiae*. Under conditions of glucose or amino acid starvation, cycling of tRNAs into and out of the nucleus is disrupted, such that tRNAs are unable to be exported to the cytoplasm (58). This limitation of available tRNAs in the cytoplasm likely affects the sensing of uncharged tRNAs by GCN2 as well as tRNA availability to aaRSs. This may alter the cognate/non-cognate tRNA availability of various aaRSs such that misacylation is more prevalent with some aaRSs relative to others.

1.6.2 Oxidative Stress

Oxidative stress is a cellular state characterized by an imbalance in pro-oxidant molecular species vs. antioxidant species that shifts the intracellular redox potential to favor oxidation of biomolecules. All biomolecules are potential targets for oxidative damage by reactive oxygen species (ROS) that may accumulate under these conditions, including proteinogenic amino acids (Fig. 1.6). This has the dual effect of decreasing the available cognate pool of a given aaRS, but also in producing a variety of multiple near-cognate derivatives. Both a loss of cognates and an accumulation of non-cognates pose a challenge to amino acid discrimination. Thus, oxidative stress poses a potential threat to the fidelity of translation.
Figure 1.6. Oxidation of proteinogenic amino acids in conditions of oxidative stress. Oxidation of phenylalanine simultaneously decreases available phenylalanine while increasing oxidation products, including the proteinogenic amino acid para-tyrosine as well as several non-protein amino acids. Shown here are only four of 20 possible hydroxylation states of 5 phenylalanine ring carbons. Oxidative damage likely produces many non-protein non-cognate amino acids that may compete with phenylalanine for the phenylalanyl-tRNA synthetase active site.

In *S. cerevisiae*, oxidative stress causes metabolic restructuring similar to that of amino acid starvation: phosphorylation of GCN2, decreased translation, and synthesis of GCN4. Hydrogen peroxide tolerance is specifically linked to GCN4 synthesis, but specific disruption of the glutathione redox buffer system with cadmium or diamide does not have the same effect (59), suggesting that the type of oxidative stress may have a greater impact on translation substrates that the mere fact of oxidative stress alone.
Oxidative stress is also associated with tRNA cleavage in eukaryotes (60). The role of resulting tRNA fragments is not yet understood, but likely plays a further role to limit translation in concert with eIF2B sequestration by phosphorylated eIF2, by limiting the available substrates for translation. The consequence of a global loss of tRNA pools is a limitation of another substrate in addition to the oxidative stress-dependent shift in cognate vs. non-cognate amino acid pools. It is not yet clear whether individual isoacceptor tRNAs are preferentially degraded, resulting in a challenge to non-cognate tRNA discrimination unique to a specific aaRS. It may also be the case that restriction of tRNAs may limit overstimulation of GCN2, or prime the cell to sense when oxidative stress has passed. Limited tRNA pools necessarily limit the available uncharged tRNA to bind GCN2 to stimulate eIF2 phosphorylation. Downstream GCN4-stimulated amino acid biosynthesis increases available amino acids, which may favor more rapid aa-tRNA synthesis. Aminoacylation of an already limited pool of available tRNAs may allow the cell to quickly shut off GCN2 via rapid limitation of uncharged tRNAs.

1.7 Tools for Quantitative Analysis of Mistranslation and Amino Acid Pools

One of the greatest challenges in studying mistranslation is quantitative measurement of amino acid substitutions, particularly low-frequency events. Traditionally, measurement of mistranslation has been carried out indirectly, by quantifying amino acid substitutions in exogenously expressed proteins, such as β-lactamase, green fluorescent protein, and others (50,61,62). In these analyses, critical residues of the reporter protein of interest are mutated such that mistranslation of the
codon of interest will restore the protein sequence and/or change the protein’s functionality. Reporter protein activity is quantified under various conditions, and residue-specific mistranslation is inferred as a result.

There are several drawbacks to this kind of analysis. Biologically relevant low-frequency amino acid substitution events may be undetectable or underrepresented in these systems (63). Perhaps most importantly, miss-sense suppression techniques are used for detection of specific amino acid substitutions at a chosen codon, limiting the scope of study to a case-by-case analysis in a specific primary sequence context. Given the anticipated variables that determine mistranslation, another drawback of these types of analyses is the assumption that they reflect mistranslation of all relevant codons. As a result, it has long been difficult to properly and sensitively quantify typical amino acid substitution rates on a per-codon basis with multiple amino acid residues, and to address global rates and effects of mistranslation.

More recently, advances in analytical mass spectrometry have provided the means for direct, highly sensitive measurement of mistranslation at each codon with multiple amino acids. In particular, liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) with or without the use of multiple reaction monitoring (MRM) mode is the new technique of choice (64-67). Proteolytically digested protein samples are separated by liquid chromatography and the eluent peptides are ionized via an electrospray source. In the case of a linear triple quadrupole setup, a target peptide of interest is mass-selected on the first quadrupole and is fragmented in a collision chamber. Resulting fragment ions are mass analyzed on a second quadrupole. MRM mode entails
analysis of a selected few fragment ions on the second quadrupole, which contrasts full scan MS/MS, in which all resulting fragment ions are quantified. MRM yields greater sensitivity than full scan MS/MS, allowing for greater detection of low-frequency mistranslation. Alternatively, fragment ions may be mass analyzed with an orbitrap type mass analyzer, which also offers a high resolution and sensitivity. These techniques allow for measurement of normal levels of mistranslation at each codon in a global fashion, and have provided the tools to examine perturbations from the norm caused by changes in the cellular environment.

Amino acid pools are quantified in a similar manner. Cells are harvested, and soluble metabolites are extracted (e.g., in cold acetonitrile and methanol). Heavy-isotope amino acid standards of known concentration are added at extraction, and samples are vacuum-dried. Metabolites are separated by high-resolution liquid chromatography, such that near-cognates with biophysical properties similar to cognates can be distinguished from one another. Amino acids of interest are counted on MS/MS. The heavy isotope amino acid standards, which are not resolved from chemically identical amino acids at the liquid chromatography step, are resolved by differences in flight on the MS/MS due to differences in mass. Amino acid signals are normalized to heavy standard signals, and compared to a standard curve to calculate the abundance of amino acids derived from biological samples. By this method, the relative abundance of cognate vs. non-cognate amino acids of interest may be quantified in various organisms, strains, and environmental conditions. Alternative approaches to quantitative analysis of amino acids in biological samples often involve derivatization of amino acid mixtures. Derivatization
poses many problems to analysis, including derivative instability and difficulties with reproducibility (68,69). Quantitation of amino acids in biological samples relative to known heavy isotope standards does not rely on chemical modification of amino acids of interest, and thus circumvents the challenges posed by derivatization.

1.8 Purpose of Study

Until recently, our knowledge of the role of the changing cellular environment on the fidelity of translation has been limited in scope. The discovery of natural diversity in the requirement for strict regulation in decoding suggested that evolution may not only favor accurate decoding of genes. Rather, it seems that specific environmental niches and cellular challenges have selected for a wide range of tolerance to mistranslation, and indeed an adaptive benefit to the loss of stringent quality control in certain species. Natural mistranslation is the norm for certain species, such as *Mycoplasma mobile* and *Candida albicans*. This implies that the view of mistranslation as a only phenomenon of “mistakes” is incorrect. The genetic code is not static; tightly-regulated codon-specific diversity means that these codons do not fit the model of a single rigid identity, but rather a flexible and stochastic set of identities.

Product synthesis for a given aaRS is dependent on the relative abundance of cognate vs. non-cognate substrates *in vivo*. Approximately half of aaRSs rely on substrate discrimination alone to maintain product specificity. In conditions that shift the abundance of cognate vs. non-cognate amino acids, the burden of overly abundant non-cognates may result in substantial increases in mistranslation at respective codons. We
previously demonstrated mistranslation of Tyr codons as Phe at levels 7-fold greater than normal in mammalian cell cultures starved for Tyr (46). Rational design of Chinese hamster tyrosyl-tRNA synthetase (CHO TyrRS) performed by a previous student in our lab (Dr. Medha Raina) conferred a substantial increase in discrimination against Phe, relative to the wild-type enzyme. Increased CHO TyrRS specificity is of interest in industrial protein production, where heterogenous protein products are unsuitable as therapeutics or molecular tools.

We have only recently developed the tools to quantitatively study the accumulation of non-cognate NPAs in various environmental contexts, due to the difficulty in resolving biophysically similar amino acids chromatographically. This study will address the evolutionary divergence between *E. coli* and *S. cerevisiae* in the requirements for translational quality control in oxidative stress conditions that challenge the product specificity of phenylalanyl-tRNA synthetase (PheRS). *E. coli* PheRS (EcPheRS) and *S. cerevisiae* cytoplasmic PheRS (SccytoPheRS) contain a post-transfer editing domain, which hydrolyzes Tyr-tRNA\textsuperscript{Phe}, but is dispensable under certain conditions. Under conditions of oxidative stress, EcPheRS post-transfer editing plays a protective role, but Tyr supplementation in otherwise normal growth conditions does not cause any apparent cytotoxicity in *E. coli*. We demonstrate variable discrimination in amino acid activation between EcPheRS and SccytoPheRS, as well as unexpected phenotypic effects upon amino acid supplementation with certain non-cognates. EcPheRS post-transfer editing activity is shown to limit mistranslation with a NPA product of Phe oxidation, *meta*-tyrosine. We show that multiple non-cognates—including at least two
non-protein oxidized Phe derivatives—threaten the product specificity of SccytoPheRS. Remarkably, SccytoPheRS employs at least three distinct mechanisms to limit production of misacylated aa-tRNA\textsubscript{Phe}. Analysis of intracellular amino acid pools with our collaborators at UCLA (Kym Faull) revealed that multiple non-protein oxidized Phe derivatives accumulate under oxidative stress, both in \textit{E. coli} and \textit{S. cerevisiae}, suggesting that diverse quality control mechanisms act to limit mistranslation of Phe codons as multiple different products of Phe oxidation appear to have evolved across species. Unexpectedly, a SccytoPheRS post-transfer editing mutant strain grew better than wild-type when challenged with supplementation of certain non-cognates and Phe itself, suggesting that post-transfer editing plays some non-canonical role in regulation of cell growth.
Chapter 2

Tyrosine starvation causes decreased tyrosyl-tRNA synthetase substrate specificity and mistranslation of Tyr codons as Phe in Chinese hamster ovary cells.

2.1 Introduction

By comparing the apparent rate of translation to the known frequency of errors in such processes as DNA replication and gene transcription, Loftfield and others predicted that aaRSs should produce misacylated aa-tRNAs at a frequency of no more than once every ~3,000 aa-tRNAs produced (30,70,71). Therefore, an aaRS with a discrimination factor against a given noncognate amino acid of significantly less than 3,000 is likely to rely on an alternative method to limit the production of aa-tRNAs with the given substrate. Bacterial TyrRS discriminates against near-cognate Phe with a specificity constant on the order of $10^5$, which would typically suggest that no alternative mechanism of discrimination against Phe is required by this enzyme (72). Indeed, TyrRS bears no post-transfer editing domain and no known pre-transfer editing activity. TyrRS product
specificity is instead maintained by strict discrimination against non-cognates in the amino acid activation step.

In Chinese hamster ovary cells used for the production and secretion of monoclonal antibody products, sequence variation has been reported at multiple codons at levels ~3% (66). Under conditions of starvation, Tyr positions are frequently occupied with Phe or His, and these sequence variants are minimized by continuous supplementation with Tyr, suggesting competition for CHO TyrRS, and subsequent mistranslation when intracellular Tyr is limited (66). We show that the discrimination for Tyr vs. Phe by CHO TyrRS is ~6,100, a value much lower than the bacterial TyrRS specificity of ~10^5. This difference in amino acid selection between prokaryotic and eukaryotic enzymes is underscored by an observed high degree of mistranslation of Tyr codons as Phe in CHO cultures starved for Tyr. Whereas a specificity constant of ~6,100 in vitro typically suggests no need for an additional quality control mechanism to limit cytotoxic Tyr codon mistranslation, Tyr starvation sharply decreases the relative abundance of Tyr and Phe to ~1:18, resulting in an effective CHO TyrRS discrimination factor of only ~350 for Tyr vs. Phe in vivo. We further characterize point-mutant variants of CHO TyrRS to define residues that confer Tyr specificity, and highlight differences between the bacterial and eukaryotic TyrRS. Human TyrRS is secreted under apoptotic conditions, whereby it is cleaved into an N-terminal fragment bearing the synthetic active site, and a C-terminal endothelial monocyte activating polypeptide-II like domain that stimulates immune cell chemotaxis (73). We show that the EMAP-II like domain does not alter the substrate specificity of CHO TyrRS.
2.2 Materials and Methods

2.2.1 CHO phenotypic and metabolomics analysis

Our collaborators at Amgen Inc. (Thousand Oaks, CA) performed a metabolic analysis of CHO cells producing a recombinant monoclonal antibody. CHO cells were grown in chemically defined media. Tyrosine (SAFC Biosciences, Lenexa, KS) was used in the supplementation study. All media and stock solutions were filter-sterilized at 0.1 µm. Cells were grown in 500-mL vented shake flasks under 36 °C, 5% CO2 and 160 rpm. The inoculation density was 1x10^6 cells/mL and the culture was grown for 16 days. Bolus media feeds were added on days 5, 7, 9, 11, and 13 at 9 % of current working volume. Tyrosine supplement was added on days 9, 11, and 13 targeting a 1 mM final concentration after addition to the culture. Glucose (Life Technology, Carlsbad, CA) was maintained in the range of 6 – 8 g/L throughout production. Viable cell density (VCD) and viability were measured using a Cedex automatic cell counter (Innovatis, Germany) and metabolites using a NOVA BioProfile automated analyzer (NOVA Biomedical, MA). Values of pH, pO2, and pCO2 were analyzed by the Bioprofile pHox (NOVA Biomedical, MA) and osmolality by the model 2020 osmometer (Advanced Instruments, Norwood, MA). Antibody titer was measured by reverse-phase HPLC (Waters, Milford, MA) using a Protein A column (Life Technologies, Carlsbad, CA). Free amino acids were measured by cation exchange HPLC (Agilent Technologies, Santa Clara, CA). HPLC-MS/MS analyses of amino acid substitutions in secreted recombinant antibodies was performed as previously described (74).
2.2.2 CHO TyrRS cloning and mutagenesis

The CHO TyrRS (EGW00102) gene, codon optimized for expression in *E. coli*, was synthesized (GenScript) and subcloned under T7 promoter control into pET33b vector at NeoI and XhoI restriction sites. The resulting plasmid pET33b-TyrRS-His<sub>6</sub> was used to transform *E. coli* BL21 (DE3) cells. CHO TyrRS mutations were constructed by PCR amplification and DpnI digestion using standard techniques. All cloning and mutagenesis were confirmed by sequencing and the resulting plasmids used to transform *E. coli* BL21 XJB (DE3).

2.2.3 Purification of CHO TyrRS and CHO TyrRS variants

Protein was produced by first growing the cells at 37 °C, shaking at 250 rpm until the cell culture reached OD<sub>600</sub>=0.6. Antibody gene expression was induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 hrs. Cells were harvested by centrifugation and resuspended in 25 mM Tris–HCl (pH 8.0), 300 mM NaCl, 10% glycerol and 5 mM imidazole. Cells were flash frozen with liquid N<sub>2</sub> and stored at −80 °C and shipped from Amgen to our lab at OSU. Cell-free extracts were produced by sonic disruption of cells in buffer A (25 mM Tris–HCl, pH 8.0, 300 mM NaCl, 5 mM imidazole and 10% glycerol) containing a protease inhibitor mixture tablet (Complete Mini, EDTA-free; Roche Applied Science) followed by centrifugation at 150,000×g for 45 min. The resulting supernatant was loaded onto a pre-equilibrated 3 ml TALON® resin metal affinity column (Clontech) followed by washing, and the protein was eluted with Buffer B (25 mM Tris–HCl, pH 8.0, 300 mM NaCl, 250 mM imidazole and 5% glycerol). Fractions
containing the protein of interest (as determined by Comassie Brilliant Blue staining after SDS–PAGE) were pooled and dialyzed twice against Buffer C (25 Mm Tris-HCl, pH 7.5, 0.1 mM EDTA, 10 mM β-mercaptoethanol and 5 % glycerol) to remove any bound tyrosyl-adenylate from TyrRS, which would confound interpretation of kinetic experiments. The enzyme was further dialyzed against two buffer changes of Buffer D (50 mM Tris-HCl, pH 7.5, 140 mM KCl, 20 mM β-mercaptoethanol, 10 mM MgCl₂ and 5 % glycerol) and finally against Buffer D with 50% glycerol and stored at -20 °C. Mini-TyrRS was produced as described for CHO TyrRS except that the region of pET33b-TyrRS-His₆ encoding the EMAP-II like domain was removed.

2.2.4 Construction and purification of synthetic CHO tRNAᵀyr

The gene for CHO tRNAᵀyrᵀ₅₆ was chosen from the various tRNAᵀyr gene sequences predicted by tRNAscans-SE analysis of the available CHO genome: (CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACTGTAGATCCTTAGGTCGCTG GTTCGATTCGGCTCGAAGGACCA). The tRNA gene was synthesized at OSU using synthetic DNA oligomers according to standard procedures (75). The 5’ nucleotide is a cytosine in CHO tRNAᵀyr, which is a poor substrate for the T7 RNA polymerase. To circumvent this problem, a hammerhead ribozyme was ligated between the T7 promoter and the tRNA sequence and cloned into pUC19 vector using BamHI and HindIII restriction sites to yield pUC19-T7 promoter- hammer head ribozyme-CHO tRNAᵀyr. This plasmid was digested with BstNI to generate 3’ CCA and used as a template for run-off transcription using T7 RNA polymerase. The tRNA transcript was purified on a
denaturing 15% polyacrylamide gel and extracted by electrodialysis in 90 mM Tris-borate/2 mM ethylenediaminetetraacetic acid (pH 8.0). The tRNA was extracted in phenol and chloroform, precipitated with ethanol and resuspended in diethylpyrocarbonate-treated ddH$_2$O.

2.2.5 Aminoacylation assays

All aminoacylation reactions were performed at 37 °C in a reaction mixture containing 144 mM Tris-HCL pH 7.78, 150 mM KCl, 10 mM MgCl$_2$, 10 mM β-mercaptoethanol, 0.1 mg/ml BSA, 5 mM ATP, either CHO total tRNA or in vitro transcribed CHO tRNA$^{\text{Tyr}}$ or *E. coli* tRNA$^{\text{Phe}}$, L-[U-$^{14}$C]-Tyr (482 mCi/mmol) or L-[U-$^{14}$C]-Phe (487 mCi/mmol) and aaRSs at concentrations indicated for specific experiments. CHO total tRNA containing native tRNA$^{\text{Tyr}}$ was prepared as previously described (76). Aliquots of reaction mixture were spotted on Whatman™ 3MM filter paper presoaked in 5% TCA (w/v) at various time points, washed in 5% TCA acid and dried. Aa-tRNA quantification was determined by scintillation counting and back-calculation based on the specific activity of the $^{14}$C-labeled amino acid used.

2.2.6 Kinetic characterization of amino acid activation efficiency

Steady-state kinetic assays were carried out at 25 °C as previously described (77,78). Reactions were carried out in buffer containing 144 mM Tris-HCl pH 7.78, 150 mM KCl, 10 mM MgCl$_2$, 10 mM β-mercaptoethanol and 2 mM PP$_i$. For ATP-PP$_i$ exchange assays to measure amino acid activation (79), concentrations of substrates were varied from 0.5
to 500 µM for Tyr, and 0.5 to 47 mM for Phe. Enzymes were added to a final concentration of 75 nM-5 µM. Kinetic parameters $k_{cat}$ and $K_M$ were calculated by fitting data to the Michaelis-Menten equation using non-linear regression (Kaleidagraph, Synergy Software), and are presented as averages from three independent reactions with the corresponding standard errors.
Table 2.1. Primers used in CHO TyrRS mutagenesis.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Set Sequences (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H77T</td>
<td>CCTGTTCGGGATCTGaacCGTTATCTGGACAAAC&lt;br&gt;GGTGTTCAAGAAGGGGAGTCCCTATGGCAACAGG</td>
</tr>
<tr>
<td>A74G</td>
<td>GATCTGCACGCTTATCTGGACGaatATGAAGCGCCGTGGGAACCTG&lt;br&gt;GATAAGCGTTGAGATCAGTCCGAGCATGACGAAAGG</td>
</tr>
<tr>
<td>N82D</td>
<td>GATCTGCACGCTTATCTGGACGaatATGAAGCGCCGTGGGAACCTG&lt;br&gt;GATAAGCGTTGAGATCAGTCCGAGCATGACGAAAGG</td>
</tr>
<tr>
<td>G120N</td>
<td>CTGAAATTATCTCAAAataCACCGACTACCGCTTCGACGAAGAAATTACGC&lt;br&gt;GACCTGCCTGACGGTGGGAACATGCGGTTTACCCG</td>
</tr>
<tr>
<td>Y123W</td>
<td>GAAATTTATCAAAGGCGACCAGACTggCAGCTGTCGATCAGCAGTTCGATGTTAGTCGGTGCCTTTGATAAATTCAG</td>
</tr>
<tr>
<td>W40C</td>
<td>GAACTGAAAGTTTATAGAGCCACCGGACCACAGGG&lt;br&gt;CCGTTAGCTGCGTGGTCCGAAGTGGATGATGCTTTCAGTTC</td>
</tr>
<tr>
<td>Y52H</td>
<td>CCGGTAACCGCATGTTGCCATTTTCTCCGATGTC&lt;br&gt;GACATCGGGACGAatGGCAACATGCGGTTTACCAG</td>
</tr>
<tr>
<td>D122N</td>
<td>GAAATTTATCAAAGGACCAGGAATCCGACTGCTGTCGATGCTGCTCTCTTGGATGACACTGC</td>
</tr>
<tr>
<td>L125W</td>
<td>CAAAGGCACCAGACTACCGGACGTGTCGATGCTGCTCTCTTGGATGACACTGC</td>
</tr>
</tbody>
</table>

All primer design and mutagenesis experiments were performed by Dr. Medha Raina.
2.3 Results

2.3.1 Tyr starvation causes a loss of productivity and viability in CHO cells expressing a monoclonal antibody.

In batch-fed monoclonal antibody production culture experiments performed at Amgen Inc. (Thousand Oaks, CA), CHO cells became limited in residual Tyr (0-500 µM), but not Phe (5-9 mM) after ~8 days (Figs. 2.1A and 2.1B). Despite intermittent nutrient supplementation, Tyr starvation was routinely observed (repeated drops to 0 µM), indicating that supplementation was insufficient for maintaining Tyr availability at levels that sustain culture needs. Though Phe remained in the mM range and steadily increased with nutrient supplementation, the antibody production, defined as specific productivity ($q_p$) declined as the CHO cells were starved for Tyr. By day 16, $q_p$ had dropped 3-fold (Fig. 2.1C). The drop in $q_p$ corresponds with a drop in titer rate, to an eventual loss of 1g/L by day 16 (Fig. 2.1D). After day 11, a loss of viability was observed (Fig. 2.1E). To address the effect of Tyr starvation, Tyr was supplemented intermittently in matched cultures in two day intervals beginning on day 9. Tyr supplementation recovered $q_p$, titer rate, and cell viability, suggesting Tyr starvation is responsible for the observed defects (Figs. 2.1C-E). Tyr starvation did not affect cell growth, as demonstrated by similar integrated viable cell density (IVCD) in unsupplemented cultures and those supplemented with Tyr (Fig. 2.1F). Metabolite analysis demonstrated that Tyr supplementation prevented Tyr depletion (Fig. 2.1A), but Phe levels were relatively unaffected by Tyr supplementation (Fig. 2.1B). Remarkably, quantitative analysis of secreted monoclonal antibodies produced by cultures starved for Tyr revealed an unusually high level of
incorporation of Phe at Tyr codons (0.7%), suggesting possible mistranslation of Tyr codons as Phe when Tyr is limited and Phe remains abundant. In CHO cultures that were not starved for Tyr, Phe was undetectable at Tyr codons (<0.01%).
Figure 2.1. Phenotypic and productivity effects of Tyr supplementation on CHO cells expressing a monoclonal antibody. (A) Tyr availability in CHO cell culture over time. (B) Available Phe in CHO cell culture over time. (C) Specific productivity $q_p$ of expressed monoclonal antibodies. (D) Titer. (E) % viable CHO cells. (F) IVCD. Dashed line (□): unsupplemented CHO cultures; solid line (■): Tyr supplemented CHO cultures. Arrows represent Tyr supplementation. CHO culture experiments were performed by our Amgen collaborators: Amanda Kano, Mathew Jerums, Paul D. Schnier, Shun Luo, Rohini Deshpande, Pavel V. Bondarenko and Henry Lin.
2.3.2 CHO TyrRS discriminates poorly against Phe at the amino acid activation step

The unusually high degree of Phe observed at Tyr codons under conditions of Tyr starvation, as well as the high level of residual Phe relative to Tyr in these conditions suggest Tyr codon mistranslation with Phe arising from an abundance of Phe-tRNA\textsubscript{Tyr} produced as a result of erroneous Phe use by CHO TyrRS. The CHO cytoplasmic TyrRS gene (EGW00102) was codon-optimized for expression in \textit{E. coli}. Purified CHO TyrRS misactivates Phe (Fig. 2.2A), and discriminates against Phe with a specificity constant of 6,100 (Table 2.2). This Tyr/Phe discrimination factor is ~25-fold lower than that of bacterial TyrRS from \textit{Geobacillus stearothermophilus} (72), suggesting differences in the requirement of these two species for TyrRS quality control. CHO TyrRS specificity is sufficient to limit misactivation of Phe when the relative abundance of Tyr:Phe is not unusually low.
Figure 2.2 CHO TyrRS efficiently utilizes Phe as a substrate for activation and tRNA$^{\text{Tyr}}$ charging with both native tRNA and synthetic tRNA$^{\text{Tyr2}}$. (A) 250 nM CHO TyrRS ATP-PP$_i$ Exchange with 5 mM Phe. (B) Aminoacylation with 4 µM tRNA$^{\text{Tyr2}}$, 100 nM CHO TyrRS, and 200 µM Phe (■) or 200 µM Tyr (○). (C) Aminoacylation with 200 µM Phe and 100 nM CHO TyrRS with 6 µM of either native tRNA$^{\text{Tyr}}$ (■) or tRNA$^{\text{Tyr2}}$ (○). Experiments performed by Dr. Medha Raina with my assistance.
Table 2.2 Steady-state kinetic parameters for amino acid activation, as measured by ATP-[\(^{32}\)P]PP\(_i\) exchange with CHO TyrRS and mini TyrRS.

<table>
<thead>
<tr>
<th></th>
<th>Tyr</th>
<th>Phe(^a)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_M) ((\mu)M)</td>
<td>(k_{cat}) (s(^{-1}))</td>
<td>(k_{cat} / K_M) (s(^{-1})/(\mu)M)</td>
</tr>
<tr>
<td>CHO TyrRS</td>
<td>15 ± 4</td>
<td>13 ± 2</td>
<td>0.85</td>
</tr>
<tr>
<td>CHO Mini TyrRS</td>
<td>16 ± 0.6</td>
<td>15 ± 3</td>
<td>0.93</td>
</tr>
</tbody>
</table>

\(^a\)\(k_{cat}/K_M\) was estimated using sub-saturating Phe concentrations from the slope of the equation, \(V = k_{cat} [E][S]/K_M\). Experiments were performed by Dr. Medha Raina and myself.

2.3.3 CHO TyrRS misacylates tRNA\(^{\text{Tyr}}\) with Phe

Whereas discrimination against Phe is poor in the activation step, there remained the possibility that an alternative quality control mechanism could limit Phe-tRNA\(^{\text{Tyr}}\) production. We therefore characterized the efficiency with which CHO TyrRS produces this species. Putative Chinese hamster tRNA\(^{\text{Tyr}}\) genes were selected using the tRNAscan-SE software package (80). Two candidate sequences were based on homology to known tRNA\(^{\text{Tyr}}\) genes: tRNA\(^{\text{Tyr1}}\) and tRNA\(^{\text{Tyr2}}\). Both genes were transcribed \textit{in vitro}, and were found to be tyrosylated by CHO TyrRS with comparable efficiencies. The \(k_{cat}\) (33±5 s\(^{-1}\)) and \(K_M\) (3±1 \(\mu\)M) of CHO TyrRS for tRNA\(^{\text{Tyr2}}\) are within the range typically observed for tRNAs transcribed \textit{in vitro}, and this tRNA was used in all aminoacylation experiments. CHO TyrRS efficiently phenylalanylates both tRNA\(^{\text{Tyr2}}\) and CHO total tRNA (Fig. 2.2B and C). Total tRNA charging excluded the possibility of misphenylalanylation resulting from the lack of native post-transcriptional modifications.
in tRNA\textsuperscript{Tyr}\textsubscript{2} transcribed \textit{in vitro}. The failure of CHO TyrRS to aminoacylate \textit{E. coli} tRNA\textsuperscript{Phe} or \textit{E. coli} PheRS to aminoacylate CHO tRNA\textsuperscript{Tyr} excluded the possibility of apparent CHO native tRNA or tRNA\textsuperscript{Tyr}\textsubscript{2} mischarging due to contamination of purified CHO TyrRS with \textit{E. coli} PheRS or \textit{E. coli} tRNA\textsuperscript{Tyr} (Figs. 2.3A and 2.3B).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_3.png}
\caption{Apparent mischarging of CHO tRNA\textsuperscript{Tyr} with Phe is not due to contamination with \textit{E. coli} PheRS. Aminoacylation of 6 µM of either \textit{E. coli} tRNA\textsuperscript{Phe} (■) or CHO tRNA\textsuperscript{Tyr} (●) in the presence of 150 µM \textsuperscript{[14]}C Phe, (A) 100 nM CHO TyrRS, or (B) 100 nM \textit{E. coli} PheRS. Experiments performed by Dr. Medha Raina.}
\end{figure}

\textbf{2.3.4 Loss of the CHO TyrRS C-terminal EMAP II-like domain does not affect amino acid specificity}

In mammals, apoptosis triggers the secretion of TyrRS, whereby it is cleaved into two fragments: an N-terminal mini-TyrRS bearing the synthetic active site, and the endothelial monocyte activating polypeptide II-like C-terminal domain, both of which are cytokines (73). However, it is not initially clear if the loss of the EMAP II-like domain impacts the substrate specificity of the N-terminal mini-TyrRS fragment. To address this, we examined the efficiency by which Tyr and Phe are utilized by mini-TyrRS, and found
that the EMAP-II fragment does not modulate amino acid discrimination by the CHO TyrRS enzyme; Mini-TyrRS activated and aminoacylated Phe and Tyr (Fig. 4A-C) with $k_{cat}$ and $K_M$ values similar to those of full-length TyrRS (Table 1). This indicates that the EMAP II-like domain likely has no role in TyrRS quality control or Tyr-tRNA$_{Tyr}^Tyr$ synthesis, suggesting that its conservation is likely due to its role as a cytokine.

2.3.5 Rational design of CHO TyrRS variants reveals substrate specificity determinants

No crystal structures are available to examine the CHO TyrRS functional groups that interact with Tyr and Phe to confer lower substrate specificity relative to the bacterial enzyme. Therefore, the known *G. stearothermophilus* (pdb 1tyd) and human (pdb 1d11) structures were examined and specific residues of interest were chosen for mutagenesis and functional analysis of substrate specificity in CHO TyrRS. *G. stearothermophilus* TyrRS is best characterized, and it is known that Tyr hydrogen-bonds with Tyr 34, Asp176, Tyr169, Asp78 and Gln173. Human and CHO TyrRS have mostly identical active site residues compared to those of *G. stearothermophilus*, with the exception of Asp78, the equivalent of which is an Asn residue in the eukaryotic enzyme (Fig. 2.4A and B).
Figure 2.4. Known TyrRS active site residues that hydrogen bond with tyrosyl-adenylate. (A) Residues known to hydrogen bond between TyrRS and substrate tyrosine. The image is a superposition of *G. stearothermophilus* and human TyrRS structures from pdb files 1tyd (chain E) and 1q11 (chain A). *G. stearothermophilus* TyrRS is shown in yellow, Human TyrRS is shown in cyan and substrate tyrosine is green. Amino acids that hydrogen bond or form hydrophobic interactions with tyrosine are denoted with Human TyrRS numbering in parentheses. (B) Hydrogen bonding interactions between tyrosyl-adenylate and TyrRS in *G. stearothermophilus* with corresponding CHO TyrRS numbering in parenthesis. MC, main chain.

(Continued)
Mutation of CHO Asn82 to the bacterial Asp had minimal effect on the $k_{\text{cat}}$ or $K_M$ of Tyr and Phe, suggesting that Asp is not a major determinant of substrate specificity (Table 2.2). Most hydrophobic-interacting residues in the CHO enzyme are conserved, with the exceptions of Ala74 and His77, which are replaced with Gly and Thr, respectively, in *G. stearothermophilus* (Fig. 2.4). Ala74Gly mutation increased the $K_M$ 7 fold and had no effect on $k_{\text{cat}}$ for Tyr. The $k_{\text{cat}}/K_M$ for Phe decreased 5-fold, the combined effect of which is a drop in Phe discrimination from 6,100 to 4,100. Mutation of His77 to the smaller
non-hydrophobic Thr increased the $K_M$ and decreased the $k_{cat}$ by 40 fold, which marginally increases specificity from 6,100 to 6,500 at the cost of efficient cognate activation (Table 2.3). Substitutions that remove hydrophobic interactions typically lead to a loss of 1-2 Kcal/mol of binding energy and an increase in the $K_M$ for cognate Tyr. Several other active site residues are not shared between *G. stearothermophilus* and human TyrRS: Cys35, His48, Thr 51 and Lys 233 in bacterial TyrRS, which interact directly with ATP during formation of the transition state (Fig 2.4) (81). The respective CHO TyrRS residues are Trp40, Tyr52, Pro55, and Ser225, none of which interact with ATP (Fig 2.4). Despite the absence of these ATP-interacting residues, the transition state stabilities for Tyr activation are nearly identical for human and bacterial TyrRS. Transition state stabilization in eukaryotic TyrRS may be achieved by interaction of a potassium ion (82). To determine if differences in ATP binding affect substrate specificity, CHO TyrRS Trp40 and Tyr52 were replaced with their equivalents in *G. stearothermophilus*: Cys and His, respectively. W40C CHO TyrRS exhibited a 30-fold decrease in $k_{cat}$ for Tyr activation and a 30-fold increase of $K_M$. The $k_{cat}/K_M$ for Phe activation decreased 2,000-fold, but no difference in the Tyr vs. Phe discrimination was observed, indicating that Trp40 is not involved in quality control in amino acid activation. The Y52H CHO TyrRS variant exhibited a 2-fold increase in $K_M$ and a 20 fold decrease in $k_{cat}$. The $k_{cat}/K_M$ for Phe decreased 280 fold, thereby increasing the specificity of the enzyme 6-fold, from 6,100 to 36,000, at the cost of efficiency in Tyr activation.
Table 2.3. Steady-state kinetic constants for ATP-[\(^{32}\)P]PP\(_i\) exchange for CHO cytosolic full length wild type and variant TyrRS.

| TyrRS variant | Disrupted contact | Tyr | Phe\(^a\) | Specificity \\
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_M (\mu M))</td>
<td>(k_{cat} (s^{-1}))</td>
<td>(k_{cat}/K_M (s^{-1}/\mu M))</td>
<td>((k_{cat}/K_M)<em>{Tyr}/(k</em>{cat}/K_M)_{Phe})</td>
</tr>
<tr>
<td>WT</td>
<td>15 (\pm) 4</td>
<td>13 (\pm) 2</td>
<td>0.85</td>
<td>(1.4 \times 10^{-4} \pm 4 \times 10^{-5})</td>
</tr>
<tr>
<td>W40C</td>
<td>500 (\pm) 200</td>
<td>0.4 (\pm) 0.1</td>
<td>7.9 (\times) 10(^{-4})</td>
<td>(7.3 \times 10^{-8} \pm 9 \times 10^{-9})</td>
</tr>
<tr>
<td>Y52H</td>
<td>36 (\pm) 4</td>
<td>0.70 (\pm) 0.04</td>
<td>0.018</td>
<td>(5 \times 10^{-7} \pm 1 \times 10^{-7})</td>
</tr>
<tr>
<td>A74G</td>
<td>Hydrophobic</td>
<td>100 (\pm) 45</td>
<td>12 (\pm) 6</td>
<td>0.11</td>
</tr>
<tr>
<td>H77T</td>
<td>Hydrophobic</td>
<td>640 (\pm) 50</td>
<td>0.30 (\pm) 0.02</td>
<td>4.7 (\times) 10(^{-4})</td>
</tr>
<tr>
<td>N82D</td>
<td>H bonding with substrate</td>
<td>40 (\pm) 15</td>
<td>5 (\pm) 2</td>
<td>0.12</td>
</tr>
<tr>
<td>G120N</td>
<td>2° H bonding</td>
<td>1100 (\pm) 50</td>
<td>1.2 (\pm) 0.2</td>
<td>1.2 (\times) 10(^{-3})</td>
</tr>
<tr>
<td>Y123W</td>
<td>2° H bonding</td>
<td>40 (\pm) 2</td>
<td>7.3 (\pm) 0.8</td>
<td>0.18</td>
</tr>
<tr>
<td>D122N</td>
<td>2° H bonding</td>
<td>15 (\pm) 2</td>
<td>9 (\pm) 0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>L125W</td>
<td>2° H bonding</td>
<td>12 (\pm) 0.4</td>
<td>8 (\pm) 1</td>
<td>0.72</td>
</tr>
</tbody>
</table>

\(^a\)\(k_{cat}/K_M\) was estimated using sub-saturating Phe concentrations from the slope of the equation \(V/[E] = (k_{cat}/K_M)[S]\). ATP-PP\(_i\) exchange experiments were performed by Medha Raina with my assistance.
In bacteria, TyrRS residues Asp176 and Tyr34 form hydrogen bonds with the hydroxyl group of Tyr, and confer amino acid specificity to the enzyme (72,83). These residues also hydrogen bond other active site residues, which surround the residues that directly interact with tyrosyl-adenylate. Trp126 and Asn123 both hydrogen bond with Asp176 (Fig 2.5). However, Trp126 and Asn123 are not conserved across the three domains of life; the equivalent residues in eukaryotic TyrRS are Tyr and Gly, respectively (Fig 2.5). A comparison of the G. stearothermophilus TyrRS and human TyrRS crystal structures revealed that CHO TyrRS Gly120 corresponds to bacterial TyrRS Asn123, and does not hydrogen bond with any residue, and could potentially interact with the OH group of substrate Tyr (Fig 2.5).
Figure 2.5 Hydrogen bond network of *G. stearothermophilus* TyrRS Asp176 (CHO Asp173) and Tyr. The image shows the bacterial TyRS hydrogen bond network formed by two residues that interact directly with the substrate tyrosine hydroxyl via hydrogen bonding: Asp-176 and Tyr-34 (CHO numbering in parentheses). This image is a superposition of *G. stearothermophilus* and human TyrRS crystal structures (pdb 1tyd, chain E and 1q11, chain A). *G. stearothermophilus* TyrRS is shown in yellow. Human TyrRS is shown in cyan, and substrate tyrosine is green. Analysis performed by Dr. Medha Raina.
Gly120Asn CHO TyrRS exhibited a 70-fold increase in $K_M$, an 11-fold decrease in $k_{cat}$ for Tyr, and a 280-fold decrease in $k_{cat}/K_M$ for Phe, resulting in a substrate specificity of 2,400. Replacement of Tyr123 (equivalent to bacterial Trp126) with Trp had minimal effects. Remarkably, Asp122Asn CHO TyrRS exhibits a 40-fold decrease in $k_{cat}/K_M$ for Phe but has no effect on the $K_M$ and $k_{cat}$ of Tyr activation, leading to 3-fold increases in the specificity of the CHO enzyme from 6,100 to 20,000 with no loss of efficiency in cognate activation. These changes indicate that Asp122 is involved in the discrimination of cognate over non-cognate amino acid, and raise the question as to why CHO TyrRS has conserved Asp122 rather than Asn at this position, which is appears to have no negative consequences for cognate activation, but which would confer an expected 3-fold lower rate of Tyr codon mistranslation with Phe.

2.4 Discussion

2.4.1 Tyr codon mistranslation in CHO cells is due to conditional amino acid imbalance.

AaRSs limit mistranslation in a variety of ways, from strict discrimination against non-cognate substrates to entropic and catalytic quality control mechanisms that may act on non-cognate products. Mistranslation can occur at a rate of $4 \times 10^{-4}$ to $5 \times 10^{-5}$ misincorporation events per codon or 0.005–0.04% per site under normal growth conditions (84,85). Moreover, we now know that error rates in protein synthesis can increase under a variety of situations, such as oxidative stress, (31,50,86), changes in codon bias (65,87), genetic heterogeneity (88), heterologous overproduction (89) and
amino acid starvation (85). To determine the rate and extent of mistranslation under different conditions, it is necessary to directly quantify amino acid misincorporation in protein (90,91). Typical predictive analysis of mistranslation relies on characterization of a given aaRS and putative non-cognate substrates in vitro. However, these analyses do not account for the context-dependent availability of cognate and non-cognate substrates, and are thus insufficient without direct analysis of protein and amino acid pools in vivo. Measurement of mistranslation is further complicated by the likelihood that misincorporation can lead to protein mis-folding, aggregation, and degradation by the cellular protein quality control machinery. Analysis of an excreted monoclonal antibody revealed mistranslation of Tyr codons as Phe at a frequency of approximately 0.7% during Tyr starvation. This substantial rate of mistranslation was prevented by Tyr supplementation, suggesting that the increase in error rate was due to an imbalance in cellular Tyr:Phe levels. Amino acid stress of this nature is encountered by mammalian cells under various conditions, including bacterial infections that trigger acute intracellular amino acid starvation due to host membrane damage (92), or tumor microenvironments that become nutritionally deprived due to rapid cell proliferation. Our findings suggest that under such growth conditions, the frequency of mistranslation may also rise substantially. Whether specific conditional increases in mistranslation would be expected to have beneficial or detrimental effects on the cell is unclear, and likely depends on the specific species, condition, and codon of interest (91).
2.4.2 Accumulation of Phe at Tyr codons is due to production of Phe-tRNA\textsuperscript{Tyr}

The specificity of bacterial TyrRS for Tyr vs. Phe in the activation step is 10\textsuperscript{5} (72), and the Phe:Tyr ratio in a dividing bacterial cell is typically around 1.9:1 (93). This kinetic discrimination is sufficient to maintain quality control at Tyr codons without the need for an additional quality control mechanism. Conversely, CHO TyrRS specificity in amino acid activation is 6,100 for Tyr vs. Phe, in contrast to a reported specificity of 150,000 for bacterial TyrRS. During Tyr starvation the relative concentration of Phe vs. Tyr was as high as 18:1 thereby decreasing the selectivity for cognate over non-cognate amino acid to around 350 \textit{in vivo}. Under normal conditions, CHO TyrRS specificity is sufficient to limit mistranslation of Tyr codons as Phe; however, amino acid selectivity is significantly lowered during Tyr limitation, and results in substantial mistranslation. This conditional susceptibility to mistranslation observed in mammalian tissue is in contrast to strict limitation of mistranslation in bacteria, which suggests that quality control in protein synthesis has evolved with different constraints in each kingdom.

2.4.3 Divergence in amino acid discrimination between bacterial and eukaryotic TyrRS

Bacterial TyrRS displays high specificity in activating Tyr, but not Phe (72). Our results demonstrate that eukaryotic TyrRS has evolved to have lower discrimination against Phe than its bacterial counterparts (Table 1). This may suggest that bacterial species may be threatened with drastic shifts in available Tyr vs. Phe, and that higher eukaryotes may not be subject to drastic shifts at a local cellular level. Although the active site residues in
G. stearothermophilus and human TyrRS are largely conserved, several key differences exist, including residues that modulate substrate specificity. Bacterial TyrRS Asp78 mediates an important H bonding interaction, which is missing in eukaryotic TyrRS, and is located in a loop region between helix α4 and helix α5. This loop is located at the entrance to the active site, and undergoes a substantial conformational change upon Tyr binding. The loop region of the bacterial enzyme is also more hydrophilic than the eukaryotic counterpart. In eukaryotic TyrRS, this loop provides a hydrophobic lid over the tyrosine-binding pocket, and the conformational change is thought to sequester tyrosyl-adenylate from water during the catalytic reaction, effectively preventing spontaneous hydrolysis (94). The active site of the eukaryotic TyrRS also has two residues, His77 and Ala74, which form hydrophobic interactions, and which are missing in bacterial TyrRS. His77 is important for substrate binding (Table 2), suggesting that the eukaryotic enzyme has evolved in a way that, counterintuitively, may improve binding of Phe in the active site of CHO TyrRS. In addition to differences in direct interactions with substrate, changes in the hydrogen-bonding network are also evident in CHO TyrRS. A secondary interaction between the catalytically important Asp173 and residue Asp122 in CHO TyrRS was found to contribute substrate specificity. This residue is located on helix η6 and the loop connecting helix η2 and α6, and may influence specificity by, for example, inducing conformational changes in the loop or the protein backbone, as shown recently for aspartate aminotransferase (95). Mutation of Tyr52, which is located in the ATP binding region, removes a potential interaction with the catalytically important potassium ion, and is associated with a 19-fold decrease in $k_{cat}$. However, this mutation
decreases the catalytic efficiency of Phe activation more than that of Tyr, resulting in a 6-fold higher specificity for Tyr vs. Phe. Rational design of aaRS with enhanced substrate specificity will likely be of great interest to industrial protein manufacturers.

In a broader context, our data demonstrate differences between eukaryotic and bacterial TyrRS in the requirements for strict quality control in aa-tRNA synthesis. The substrate specificity of 6,100 for activation of Tyr vs. Phe is sufficient under normal growth conditions, but in conditions of nutrient deprivation, changes in available aaRS cognate availability overwhelm CHO TyrRS, and decrease this substrate selectivity to 350. In this manner, starvation directly impacts the fidelity with which the genetic code is translated.
Bacterial phenylalanyl-tRNA synthetase post-transfer editing limits mistranslation of Phe codons with the non-protein Phe oxidation product meta-tyrosine.

3.1 Introduction

Unlike TyrRS, which relies on strict discrimination against non-cognate amino acids in the amino acid activation step (Chapter 2), *E. coli* PheRS and *S. cerevisiae* cytoplasmic PheRS rely instead on hydrolytic editing of mischarged aa-tRNA\textsubscript{Phe} at a hydrolytic editing site distinct from the synthetic active site (96,97). PheRS post-transfer editing is an important step in limiting mistranslation of Phe codons; one substrate for post-transfer editing by PheRS, Tyr-tRNA\textsubscript{Phe}, is readily delivered to the ribosome by elongation factor Tu in the absence of editing, whereby Phe codons are erroneously decoded as Tyr in the nascent protein (16,98).

aaRS editing mechanisms are not conserved, and aaRS quality control has varying effects on the cell (32,37,99,100). *Mycoplasma mobile*, for example, tolerates an unusually high degree of mistranslation at Phe codons, and its PheRS lacks a functional
post-transfer editing domain, as do other aaRSs in this organism (36,101). *S. cerevisiae* cytoplasmic PheRS exhibits poor discrimination against Tyr in the amino acid activation step, and is capable of post-editing, but the yeast mitochondrial enzyme has no post-transfer editing domain, and instead relies entirely on discrimination against non-cognate amino acids in the activation step. *E. coli* PheRS has retained both strict discrimination against non-cognate Tyr as well as post-transfer editing (102). The diversity of PheRS variants alone underscores the different evolutionary constraints various species have placed on the requirements for translational quality control (101). Remarkably, post-transfer editing activity by PheRS is dispensable in *E. coli*, and it is not immediately obvious why the catalytically-active post-transfer editing domain has been conserved (93,102).

Whereas quality control mechanisms limit non-cognate proteinogenic amino acid use by aaRSs, similar non-protein amino acids may also be subject to these mechanisms. Many aaRSs bear hydrolytic editing mechanisms to limit the production of aa-tRNAs with non-protein amino acids, such as homocysteine, norleucine, α-aminobutyrate and meta-tyrosine, although exact cellular contexts in which these mechanisms are required are not fully understood [reviewed in (103)]. *E. coli* and *Thermus thermophilus* PheRS are both capable of post-transfer editing against *m*-Tyr, which may be formed as a result of oxidative damage of Phe (104-106). Some fescue grasses produce *m*-Tyr as a chemical terraforming mechanism, which poisons the proteomes of neighboring plants, and the accumulation of *m*-Tyr in CHO cells is suspected to play a cytotoxic role in translation (107,108). Taken together, these findings suggest that oxidative stress could result in
reactive oxygen species that damage Phe pools and cause the accumulation of m-Tyr that can threaten the fidelity of translation of the genetic code and poison cells. Under oxidative stress conditions that favor the accumulation of intracellular m-Tyr, the post-transfer editing activity of PheRS against m-Tyr-tRNA\textsuperscript{Phe} would be essential to maintain cellular viability. Here we show that the bacterial PheRS post-transfer editing domain does efficiently hydrolyze m-Tyr-tRNA\textsuperscript{Phe}, and that this editing activity is essential for typical growth and viability under both oxidative stress conditions and supplementation of m-Tyr exogenously. We also show that PheRS post-transfer editing in \textit{S. cerevisiae} provides only limited protection from m-Tyr, but unlike the \textit{E. coli} enzyme, post-transfer editing is critical for limitation of p-Tyr-tRNA\textsuperscript{Phe}.

3.2 Materials and methods

3.2.1 Strains, plasmids, and general methods

Proteins and tRNAs were prepared as previously described (109). Mutation of the \textit{EcPheRS} gene in the pQE31-EcFRS expression plasmid was performed with standard PCR-based site-directed mutagenesis (98). Purification of His-tagged EcPheRS variants included an additional dialysis step in 25 mM Tris-HCl pH 7.5, 100 mM KCl, 0.1 mM sodium pyrophosphate, 3 mM 2-mercaptoethanol, and 10% glycerol, in order to release any enzyme-bound adenylate via competition with inorganic pyrophosphate. EcPheRS variants were then dialyzed against 25 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM MgCl\textsubscript{2}, 3 mM 2-mercaptoethanol, and 10% glycerol, followed by dialysis against this same buffer with 50% glycerol, flash freezing, and storage at -80°C. Active enzyme
concentrations were determined by active site titration as previously described (110). Phe, p-Tyr, DL-o-Tyr, and DL-m-Tyr were purchased from Sigma Aldrich.

3.2.2 Construction of post-transfer editing-defective EcPheRS and ScytoPheRS strains

The PheRS editing-defective strain of E. coli, pheT (G318W), was constructed using established recombineering methods involving the λ-red/gam pKD46 plasmid (111). The pheS ts E. coli strain NP37, which contains a G98D mutation (112) was used as the parental strain to allowed for selection of recombination events within the region of the neighboring pheS and pheT genes. Site directed mutagenesis of the pQE31-EcFRS wild-type plasmid (98) was used to construct pQE31-EcFRSG318W/V364V. Linear PCR products were amplified from this plasmid and introduced to the pKD46 containing NP37 parent strain, via electroporation. PCR primers were as follows: p14 EcFRS: 5’-AACCATGTCACATCTCGC and P16AS EcFRS: 5’-CGTTGGTGATATCAATTACCG. This linear DNA contains the wild type pheS gene to allow for colony selection at 42°C, the pheT gene containing a G318W mutation, and a silent V364V mutation that introduces a BamHI site for screening of colonies. Recombinant strains were confirmed via sequencing. A wild type pheS/pheT strain was also constructed in the same manner, but without changing G318. The λ-red recombineering system was used to introduce the pheT (G318W) mutation into the E. coli MG1655 background. Competent cells were prepared as previously described (113) of an MG1655 derivative containing pSIM6, a
plasmid that carries the λ-red system (114). These cells were transformed with a 70-mer oligonucleotide (5’-CACAACAAGGCCTGGCGATGGGAAGAAATATTTTGGGGAGAGCATTCAGGGTGAATGACGAAACACAAAG) that has several wobble mutations (underlined) on either side of the pheT(G318W) mutation. The wobble mutations overwhelm the mismatch repair system (115). Positive clones were identified by colony PCR, with a primer that recognized the mutated sequence (5’-AGGAATATTTTGGGGAGAGCATTC) and a reverse primer 500-bp distant (5’-CCGATCAGGCGATCCAGTTTG), and subsequent DNA sequencing. One clone was chosen to serve as the intermediate strain and was subjected to a second round of recombineering, as indicated above, with an oligo (5’CACAACAAGGCCTGGCGATGGGCGGCATCTTCTGGGCGAACACTCTGGCGTGAATGACGAAACACAAAG) to remove the wobble mutations and leave solely the pheT (G318W) mutation. The intermediate strain was also transformed with an oligo (5’-CACAACAAGGCCTGGCGATGGGCGGCATCTTCTGGGCGAACACTCTGCGTGAATGACGAAACACAAA) that would revert the strain back to the wild type pheT sequence. This strain served as the wild-type control strain in studies with the pheT (G318W) derivative of E. coli MG1655. Positive clones were again screened by colony PCR (primer 5’-CGGCATCTTCTGGGCGAACACTCTGGCGTGAATGACGAAACACACT for pheT (G318W) and primer 5’-CGGAGAGCATCTTCTGGCGAACACTCT for wild-type, both with the reverse primer indicated above) and DNA sequencing.

Strains derived from S. cerevisiae W303 (MATa/MATα, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100) were used to construct chromosomal mutants of FRS1.
A 2084 bp fragment of *frs1-1*, obtained through PCR of the plasmid pFL36-*frs1-1* (93), was inserted into the integrative plasmid YIP5 (116) at the EcoRI and NruI restriction sites by In-Fusion cloning (Clontech), resulting in the plasmid YIP5-*frs1-1*. W303 (*MATa/MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*) was transformed with YIP5-*frs1-1* and insertion of the plasmid was selected for by growth on complete supplement media without uracil (CSM -Ura; Sunrise Science Products). Recombinant strains were grown in YPDA at 30 °C, shaking at 300 rpm, for 24 hours, and plated on YPDA rich media. Crossovers were selected for by replica plating onto media containing 5-flouroorotic acid. TRP1 prototroph strains were created through the PCR amplification of the *TRP1* locus from *S. cerevisiae* strain BY4743 (*MATa/MATa, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0*) and the linear product used to transform the W303 (*MATa/MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, FRS1/frs1-1,*) strain. TRP1 recombinants were selected on synthetic complete without tryptophan media. Haploids were obtained by sporulation, dissection onto YPDA, replica plated onto complete supplement media minus tryptophan, and tryptophan prototroph colonies selected. Haploids were screened for the presence of the *frs1-1* mutation, resulting in the strains NR1 (*MATa, ade2-1, his3-11,15, leu2-3,112, ura3-1, can1-100*) and NR2 (*MATa, ade2-1, his3-11,15, leu2-3,112, ura3-1, can1-100, frs1-1*).
3.2.3 Phenotypic analysis

Single colonies of *E. coli*, (wild-type *pheT* or *pheT* (G318W)), were picked from LB plates, resuspended in sterile water and used to inoculate liquid culture at an initial OD$_{600}$=0.04. Cultures were grown in M9 media supplemented with glucose (2 g/L), thiamine (1 mg/L), MgSO$_4$ (1 mM), CaCl$_2$ (0.1 mM), and varying amounts of amino acids. Cultures were grown at 37°C in 250 µL volumes using 96-well plates for ease of titrating several amino acid concentrations. Phe was kept constant at 3 µM and *p*-Tyr or DL-*m*-Tyr was varied from 3 µM to 3 mM. OD$_{600}$ values were read using an xMark Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories) after 12-18 hours of growth. Growth curve analyses were performed in supplemented M9 media containing 0 or 0.5 mM DL-*m*-Tyr, and 100 mL cultures were grown at shaking at 37°C. Growth experiments in the presence of oxidative stress agents were also set up in 96-well plates in M9 minimal media containing 0.5 mM Phe, 0.1 mM FeSO$_4$, and 2-4 mM H$_2$O$_2$. For all growth assays of the *S. cerevisiae* strains NR1 and NR2, cells were streaked on YPDA and incubated at 30 °C. After approximately 72-hour single colonies were picked, resuspended in sterile water and used to inoculate liquid cultures to an initial OD$_{600}$ of 0.01. Microtiter growth assays were completed by inoculating 150 µL of MM (Difco™ yeast nitrogen base without amino acids, 0.002% adenine, 0.002% uracil, 0.002% L-histidine, 0.01% L-leucine, and 2% glucose) + Phe:Tyr (where Phe was kept constant at 3 µM and Tyr was varied from 3 µM to 1.2 mM) in a 96 well microtiter plate. Plates were incubated at 30 °C and growth was measured after 16 h by OD$_{600}$. 

62
3.2.4 *S. cerevisiae* aging

*S. cerevisiae* aging assays were completed using the methods of Fabrizio and Longo (117). The *S. cerevisiae* strains NR1 and NR2 were streaked on YPDA and incubated at 30 °C. After approximately 72 hours, several colonies were picked and resuspended in sterile water and used to inoculate 50 mL of synthetic complete medium with variable Phe:Tyr ratios in a 250 mL flask to an initial OD$_{600}$=0.1. Cultures were grown in SDC + Phe:Tyr 1:1 where Phe was at a concentration of 3 µM. SDC media consisted of 0.18 % yeast nitrogen base without amino acids and ammonium sulfate, 0.5 % ammonium sulfate, 0.14 % NaH$_2$PO$_4$, 80 mg/L adenine, 80 mg/L uracil, 80 mg/L tryptophan, 80 mg/mL histidine-HCl, 40 mg/L arginine-HCl, 80 mg/L methionine, 1200 mg/L leucine, 60 mg/L isoleucine, 60 mg/L lysine-HCl, 100 mg/L glutamic acid, 100 mg/L aspartic acid, 150 mg/L valine, 200 mg/L threonine, and 400 mg/L serine. Cultures were shaken at 220 rpm at 30 °C for 72 hours, after which samples of 1 mL were taken every 48 h, serial dilutions performed in sterile water, and plated on YPDA. Plates were allowed to grow for 2-3 days at 30 °C, colonies counted, and CFU/mL calculated for each culture. Assays were performed in triplicate.

3.2.5 RNA detection via Northern blotting

Strains NR1 and NR2 were inoculated into 50 mL liquid YPDA, minimal media (MM) + Phe:Tyr 1:1 (3 µM Phe, 3 µM Tyr), or minimal media (MM) + Phe:Tyr 1:50 (3 µM Phe, 0.15 mM Tyr) and grown to an OD$_{600}$ of 0.8. Cells were harvested by centrifugation, washed with 50 mL dH$_2$O, frozen, and stored at -80°C overnight for RNA extraction.
The UPR was induced by treating cultures at an OD$_{600}$=0.4 with Tunicamycin (10 µg/mL), grown to OD$_{600}$=0.8 and processed as before. Total cellular RNA was extracted using hot phenol. Northern blot analysis was carried out using the NorthernMax –Gly Kit (Ambion) according the manufacturer’s instructions. Agarose gels were loaded with 5 µg of total RNA for each sample. A single stranded DNA probe (5’-CAACAAATTGTGTGTTGTTGT CTACGGCAGGTAG-3’) which binds to base pairs 529-559 of the HAC1 transcript was radiolabeled with [γ-$^{32}$P]-ATP by T4 polynucleotide kinase and utilized in hybridization.

3.2.6 tRNA transcription and $^{32}$P radiolabeling

Purified native E. coli tRNA$^{\text{Phe}}$ was purchased from Chemical Block, Moscow. S. cerevisiae cytoplasmic and mitochondrial tRNA$^{\text{Phe}}$ were made from T7 runoff transcription in vitro, as previously described (109,118). DNA template for tRNA transcription was generated from plasmids carrying tRNA genes (75) by PCR amplification and extended only to C75 to allow for $^{32}$P labeling of A76. After ethanol precipitation, tRNA transcripts were purified on denaturing 12% polyacrylamide gel and extracted by electrodialysis in 90 mM Tris-borate/2 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0). The tRNA was extracted in phenol and chloroform, precipitated in ethanol, dried, and resuspended in DEPC-treated ddH$_2$O. tRNA transcript refolding was carried out by heating the tRNA at 70°C for 2 min, followed by the addition of 2 mM MgCl$_2$ and slow-cooling to room temperature. tRNAs were $^{32}$P-labeled at A76 as described previously (109). For E. coli tRNA$^{\text{Phe}}$, the CCA-3’-end was removed prior to
labeling by treatment of 20 µM tRNA transcript with 100 µg/ml *Crotalus atrox* venom (Sigma) in a buffer containing 50 mM Na-Gly (pH 9.0) and 10 mM magnesium acetate. The reaction was incubated for 40 min at 21°C and extracted in phenol and chloroform, ethanol precipitated, and desalted by gel filtration through a Sephadex G-25 column (Amersham Biosciences). The CCA-3’-end of the tRNA was reconstituted and radiolabeled using *E. coli* tRNA terminal nucleotidyltransferase and [α-32P] ATP as described (109). *S. cerevisiae* cytoplasmic and mitochondrial tRNA_{Phe} C75 transcripts were labeled the same way, however CTP was excluded from the reaction mix. Samples were treated with one volume of phenol, and the tRNA was extracted in phenol and chloroform, then gel filtered twice through a G-25 column.

### 3.2.7 Aminoacylations and post-transfer editing

Aminoacylation reactions were performed at 37 °C in aminoacylation buffer (100mM Na-Hepes (pH 7.2), 30 mM KCl, 10 mM MgCl₂, 10 mM DTT) with 8 mM ATP, 60 (E. coli) or 100 µM (S. cerevisiae) cold amino acid, 0.5 µM ³²P-tRNA. PheRS (100 nM) was added to initiate the reactions. Aliquots were removed at chosen time points, treated with an equal volume of 0.5 M sodium acetate (pH 4.2) and incubated for 30 min at room temperature with S1 RNase (Promega). Free [α-³²P]AMP and aminoacyl-[α-³²P]AMP were separated by thin layer chromatography on polyethyleneimine cellulose (Sigma Aldrich) pre-ran with water, in 100 mM ammonium acetate, 5% acetic acid, and visualized as described previously (119). Mischarging of *E. coli* tRNA_{Phe} was performed at 37°C for 20 min in aminoacylation buffer with 8 mM ATP, 100 µM cold with p-Tyr or
DL-\textit{m}-Tyr, 4 µM $^{32}$P-tRNA and 1 µM $\alpha$A294G/$\beta$G318W PheRS (96). Reactions were stopped by the addition of 1 volume of phenol pH 4.5, and the aminoacylated tRNA was extracted in phenol and chloroform, then gel filtered twice through a G-25 column pre-equilibrated with 5 mM sodium acetate pH 4.2. Editing assays were performed in aminoacylation buffer and contained 0.1 µM Tyr-$[^{32}$P] tRNA$^{\text{Phe}}$, and 10 nM G318W EcPheRS. Reactions were arrested at various time points and analyzed by TLC as described for the aminoacylation reactions (see above). Post-transfer editing assays of the cell-free extracts were performed similarly, but mischarged $[^{14}$C]Tyr-tRNA$^{\text{Phe}}$ was formed (109), and 1 µM was used in reactions containing aminoacylation buffer, 2 mM ATP, and cell-free extract that was normalized for aminoacylation activity.

### 3.2.8 Steady-state characterization of amino acid activation, as measured by ATP/PP$_i$ exchange

ATP/PP$_i$ exchange assays were performed according to standard methods as previously described (96,109). Reactions were carried out at 37 °C in 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 10 mM MgCl$_2$, 2 mM NaF, 2 mM ATP, 2 mM $[^{32}$P]PP$_i$ (2 cpm/pmol), varying amounts of Phe (1-200 µM) and DL-\textit{m}-Tyr (20 to 2000 µM ), and 40 nM $E$. coli PheRS, 100-150 nM yeast cytosolic enzyme. After 1–1.3 min, 25 µl of the reaction were quenched in a solution containing 1% charcoal, 5.6% HClO$_4$, and 75 mM PP$_i$. The charcoal-bound ATP was filtered through a 3 MM Whatman filter discs under vacuum and washed three times with 5 mL of water and once with 5 mL EtOH. The filters were dried, and the radioactivity content was determined by liquid scintillation counting. We
previously reported the activation specificity of Phe versus p-Tyr to be 7800 (93), however this discrepancy appears to be due to differences in enzyme-bound aminoacyl adenylate during protein purification affecting the measured active enzyme concentration. This problem was resolved here through extensive dialysis against PPi, which competes away bound amino acids.

### 3.2.9 Dipeptide synthesis in vitro

Initiation complexes were formed using tight-coupled 70S ribosomes, [35S]fMet-tRNA\(^{\text{fMet}}\), Met-Phe coding mRNA, and initiation factors essentially as described (120). Ternary complexes were formed using aminoacylated tRNA\(^{\text{Phe}}\) and activated EF-Tu (120). Reactions were initiated by mixing 1 µM ternary complex with 0.1 µM 70S IC and incubated for 1 min at 21°C before quenching with 1/5\(^{\text{th}}\) volume of 2 M KOH and 1 M H\(_2\)O\(_2\). Quenched reactions were then incubated at 37 °C for 20 minutes to deacylate tRNA\(^{\text{Phe}}\), and [35S]fMet-Phe dipeptides were separated from [35S]fMet by TLC on silica plates in buffer containing 1-butanol:acetic acid:H\(_2\)O (4:1:1). TLC plates were then exposed and quantified by phosphor imaging.

### 3.2.10 Quantification of amino acid pools from extracted soluble intracellular metabolites

Cultures were grown to late log phase in M9 media supplemented with or without 0.5 mM Tyr in 5 ml volumes, and harvested by vacuum filtration over a nylon filter, followed by washing cells three times with 1 mL H\(_2\)O. Cells and filters were then placed
upside down in 0.5 ml extraction buffer (40% acetonitrile, 40% methanol) containing internal standards (100 pmol [U^{13}C]Phe and 100 pmol [U^{13}C]Tyr) at -20°C for 15 minutes. Metabolites were extracted as described (121) and vacuumed dried. Samples were re-dissolved in water (50 ul), centrifuged (16,000 x g, 5 min) and the supernatant transferred to LC injector vials. Aliquots of the supernatant (typically 5 µl) were injected onto a reverse phase HPLC column (Phenomenex Kinetex XB-C18, 2.1 x 100 mm, 1.7 µm particle size, 100 Å pore size) equilibrated in solvent A (water/formic acid, 100/0.1, v/v) and eluted (100 µl/min) with an increasing concentration of solvent B (acetonitrile/formic acid, 100/0.1, v/v; min/%B, 0/1, 5/1, 26/70, 27/1, and 35/1). The effluent from the column was directly connected to an electrospray ion source (Agilent Jet Stream) attached to a triple quadrupole mass spectrometer (Agilent 6460) scanning in the multiple reaction monitoring mode with standard resolution settings (FWHM 0.7) using previously optimized conditions for the following transitions: Tyr, 182→136; U^{13}C-Tyr, 191→144; Phe, 166→120; U^{13}C-Phe, 175→128. With each batch of samples a series of standards was prepared with the same amount of internal standards and increasing amounts of Tyr and Phe (0, 0.1, 1, 10 and 100 pmol in 50 ul of water, in duplicate). Typical retention times for p-Tyr, m-Tyr, o-Tyr and Phe were 4.8, 6.6, 8.6 and 9.7 min, respectively. Peak areas were measured using instrument manufacturer supplied software (Agilent MassHunter). The amount of each analyte in each sample was determined by interpolation from the curves constructed from the standard samples (peak area Tyr or Phe/peak area U^{13}C-Tyr or –Phe against amount of Tyr or Phe).
3.2.11 Purification and LC-MS/MS-MRM of total soluble protein hydrolysate

*E. coli* cultures (100 mL), prepared in duplicate, were grown in M9 minimal media with or without 0.5 mM *m*-Tyr to exponential phase and harvested by centrifugation (6,000xg, 10 minutes). Cell pellets were washed twice, resuspended in water, and lysed by sonication. To precipitate ribosomes and nucleic acids, streptomycin sulfate was added to a final concentration of 8 mg/mL (122). Samples were incubated at 4°C for one hour, then centrifuged at 11,000xg for 5 minutes. Supernatants were collected and brought to 55% acetone (v/v) at 4°C for 1 hour. Precipitated material was pelleted at 11,000 g for 5 minutes. Supernatants were discarded, and pellets were washed twice with 60% acetone (ice cold). The pellets were then subjected to two methanol/chloroform extractions, vacuumed dried, and weighed. One set of samples was used for measurement of protein content (bicinchoninic assay, Thermo Scientific). After resuspending in water and addition of internal standards (U^{13}C-Tyr and U^{13}C-Phe, 100 pmol each), the other set of samples was subjected to acid hydrolysis (6 M HCl for 24 hrs at 110°C). LC-MS/MS-MRM was performed on the hydrolysate as described above.

3.3 Results

3.3.1 PheRS post-transfer editing is dispensable in *E. coli* and *S. cerevisiae*

To investigate the role of *EcPheRS* editing *in vivo*, we constructed an *E. coli* strain with a point-mutation (G318W) within *pheT*, which encodes the post-transfer editing β-subunit of PheRS. Replacement of Gly318 with Trp occludes the post-transfer editing site, and limits *EcPheRS* post-transfer editing activity by more than 70-fold *in vitro* (96,123).
coli strain NP37, which encodes a temperature-sensitive pheS allele, facilitated selection of mutants (124). Lysates from NP37-derived strains with wild-type pheT and pheT (G318W) alleles were prepared and their PheRS activities compared to confirm a loss of post-transfer editing activity in the mutant strain. Only the wild-type PheRS strain retained p-Tyr-tRNA\textsuperscript{Phe} hydrolase activity (Fig. 3.1A). Wild-type and G318W strains exhibited identical levels of aminoacylation efficiency, and growth at 37 °C, suggesting that post-transfer editing is not required for growth. A cytoplasmic PheRS post-transfer editing defective strain of \textit{S. cerevisiae} was constructed by mutation of the chromosomal \textit{FRS1} gene, which encodes the post-transfer editing β-subunit of the enzyme. Replacement of a catalytically important Asp with Ala in \textit{FRS1} (D243A) eliminated p-Tyr-tRNA\textsuperscript{Phe} editing by cell-free extract [Fig. 3.1B, (93)], but did not alter growth relative to wild-type \textit{S. cerevisiae}. 
Figure 3.1  **Post-transfer editing defective strains of E. coli and S. cerevisiae.** (A) Post-transfer editing against 1µM [14C]- Tyr-tRNA\textsuperscript{Phe} by lysate extracted from wild-type (●) or pheT (G318W) (■) E. coli (140mg/ml protein) or buffer (▲) at 37°C. (B) Post-transfer hydrolysis by βD243A ScytoPheRS lysate. Yeast extract reactions were performed at 37 °C with 2 µM Tyr-tRNA\textsuperscript{Phe} and wild-type S. cerevisiae or βD243A frs1-1 cell-free extracts, normalized to aminoacylation efficiency (93). Data represent the mean of three replicates, with error representing ±S.D. Experiments were performed by Dr. Tammy Bullwinkle.

3.3.2 **Post-transfer editing by EcPheRS confers resistance to m-Tyr**

Following the seemingly paradoxical observations that post-transfer editing by EcPheRS is both evolutionarily conserved and dispensable under normal growth conditions, we tested various atypical growth conditions in order to determine what benefit post-transfer editing by EcPheRS may confer to E. coli. We compared the growth of G318W E. coli to that of wild-type in 1920 growth conditions in a phenotypic microarray format (Biolog). Remarkably, no significant changes were observed between strains. Further experiments included a range of alternative growth conditions, including heat shock, cold shock, pH.
extremes, and aging, but none resulted in any growth defects in post-transfer editing
defective *E. coli*. Building on the observation that cognate starvation causes
mistranslation of Tyr codons in CHO cells, despite high discrimination against its non-
cognate in the amino acid activation step (Chapter 2), we tested the limits of G318W *E.
coli* by direct supplementation with proteinogenic *para*-tyrosine. Remarkably, no
difference in growth was observed between strains (Fig. 3.2A).
Figure 3.2 Phenotypic analysis of non-cognate amino acid supplementation in PheRS post-transfer editing defective E. coli. Optical density readings at 600 nm of E. coli post-transfer editing defective (G318W) strain (grey bars) relative to wild-type (black bars) at various concentrations of p-Tyr (A) or D,L-m-Tyr (B) relative to supplemented Phe. Cultures were grown in minimal media supplemented with amino acids denoted, expressed as a ratio of supplemented [Phe]:[Tyr]. “1” corresponds to 3 µM of a given amino acid. (C) Growth of PheRS editing-defective E. coli in an MG1655 the presence of m-Tyr at 37°C. Data are presented as the mean of three biological replicates ± SD. Experiments performed by Dr. Tammy Bullwinkle.
Analysis of extracted soluble metabolites showed that *E. coli* *pheT* (G318W) contained similar intracellular abundance of *p*-Tyr and Phe, relative to wild-type, suggesting that the post-transfer editing mutation does not drastically disrupt amino acid import/export and metabolism (Table 1). With no amino acids supplemented, the relative intracellular abundance of Phe:*p*-Tyr was 1:1, but rose to 1:9 upon addition of *p*-Tyr, which confirmed import of supplemented amino acids in *pheT* (G318W).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Supplement</th>
<th><em>m</em>-Tyr (µM)¹</th>
<th><em>p</em>-Tyr (µM)</th>
<th>Phe (µM)</th>
<th><em>p</em>-Tyr/Phe</th>
<th><em>m</em>-Tyr/Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>+ <em>m</em>-Tyr</td>
<td>2.9±0.06</td>
<td>0.56±0.1</td>
<td>0.63±0.2</td>
<td>0.9±0.0</td>
<td>5±1</td>
</tr>
<tr>
<td><em>pheT</em>(G318W)</td>
<td>+ <em>m</em>-Tyr</td>
<td>2.7±0.5</td>
<td>0.46±0.02</td>
<td>0.90±0.2</td>
<td>0.9±0.2</td>
<td>6±1</td>
</tr>
<tr>
<td>Wild type</td>
<td>+ <em>p</em>-Tyr</td>
<td>ND</td>
<td>11±4</td>
<td>0.91±0.1</td>
<td>12±4</td>
<td>ND²</td>
</tr>
<tr>
<td><em>pheT</em>(G318W)</td>
<td>+ <em>p</em>-Tyr</td>
<td>ND</td>
<td>8.9±0.4</td>
<td>0.93±0.1</td>
<td>9.7±1</td>
<td>ND</td>
</tr>
</tbody>
</table>

¹Concentrations of intracellular Phe, *p*-Tyr, and *m*-Tyr in wild type or *pheT*(G318W) post-transfer editing defective *E. coli* strains grown in M9 minimal media supplemented with *m*-Tyr or *p*-Tyr.

²ND indicates concentrations were below the detectable limit (0.01 µM).

Experiment performed by Dr. Tammy Bullwinkle.

We then investigated the growth of *E. coli* *pheT* (G318W) upon media supplementation with *m*-Tyr, a non-proteinogenic amino acid previously shown to be an efficiently-activated near-cognate substrate for bacterial PheRS (104). Relative to wild-type, growth of *E. coli* strain *pheT* (G318W) was drastically limited when cultures were supplemented *m*-Tyr, suggesting PheRS post-transfer editing activity acts to hydrolyze
m-Tyr-tRNA_{Phe} in a manner that limits cytotoxicity *in vivo* (Table 3.1 and Fig. 3.2B). aa-tRNA_{Phe} hydrolase assays performed *in vitro* confirmed that, as with p-Tyr, post-transfer editing by EcPheRS against m-Tyr-tRNA_{Phe} by PheRS is ablated by the βG318W editing domain mutation (Fig. 3.2). The cytotoxic effect of m-Tyr supplementation on pheT (G318W) growth in the absence of post-transfer editing was also observed in *E. coli* mutants derived from strain MG1655 which, unlike the NP37 background, encodes an intact stringent response (Fig. 2.2C). This confirmed that the m-Tyr supplementation cytotoxicity phenotype was not unique to strains lacking the stringent response, wherein *E. coli* is unable to sense and respond to amino acid starvation (as described in Chapter 1). Growth of *E. coli* pheT (G318W) was also examined in supplementation experiments with alternative Phe and p-Tyr hydroxylation derivatives ortho-tyrosine and 3,4-dihydroxy-L-phenylalanine (125). Neither o-Tyr, nor L-DOPA inhibited growth of wild type or the pheT (G318W) *E. coli*, in contrast to similar experiments in yeast (Chapter 4) (Fig. 3.2). This suggests that, like p-Tyr, L-DOPA and o-Tyr are not threats to PheRS specificity in *E. coli*.

Post-transfer editing-defective yeast strain frs1-1 (βD243A) displayed no difference relative to wild-type under either heat shock or ethanol stress, but exhibited a marked defect in resistance to p-Tyr, consistent with a ~22-fold lower discrimination against p-Tyr in the amino acid activation step relative to EcPheRS (Fig. 3.3A, Table 3.2). Growth of both strains was inhibited by m-Tyr supplementation, though frs1-1 (β243A) did grow more slowly than wild-type (Fig. 3.3B); these results are consistent with only moderate *S. cerevisiae* PheRS post-transfer editing activity against m-Tyr-tRNA_{Phe}
The *frs1* (βD243A) strain also exhibited a marked aging defect relative to wild-type. Wild-type *S. cerevisiae* entered stationary phase earlier than *frs1* (βD243A) and remained in this phase for several days longer (Fig. 3.3C). By day 19, the *frs1* (D243A) *S. cerevisiae* exhibited ~100-fold lower survival rate than matched wild-type cultures. The significant decrease in lifespan of *frs1* (D243A) cells compared to wild-type suggests that ScytoPheRS post-transfer editing targeting p-Tyr-tRNA^{Phe} is critical for maintenance of cell survival during stationary phase.
3.3.3 Post-transfer editing defective SccytoPheRS does not induce ER stress.

The growth defect observed in SccytoPheRS editing-defective S. cerevisiae grown with supplemented p-Tyr suggested that Phe codon mistranslation might lead to upregulation of the unfolded protein response. To determine if the UPR is induced in frs1-1 (βD243A),
strains were grown in either minimal media + Phe:p-Tyr 1:1 or MM + Phe:p-Tyr 1:50, and UPR stimulation was determined by established methods. Briefly, UPR upregulation is readily demonstrated indirectly by quantifying splice variants of the UPR transcription activator, $HAC1$ (126). Splice variants were only detected in cultures grown in the presence of positive control tunicamycin, which induces the UPR via inhibition of glycosylation of newly synthesized proteins. In minimal media, $HAC1$ splicing is not induced in the $frs1-1$ (D243A) strain regardless of [Phe]/[p-Tyr] levels (Fig. 3.4). This suggests that mistranslation of Phe codons as Tyr and growth defects observed under amino acid stress are not accompanied by mis-folded protein accumulation in the ER.

Figure 3.4. A ScytoPheRS post-transfer editing defect does not induce the UPR. Wild-type and $frs1-1$ (βD243A) cells were grown in rich media, minimal media + 0.003 mM Phe, 3 µM p-Tyr (1:1), or minimal media + 3 µM Phe, 0.15 mM p-Tyr (1:50). 5 µg of total RNA was loaded onto a 1% agarose gel. As a positive control, the UPR was induced with 10 µg/mL tunicamycin (Tm). (A) RNA detection of $HAC1$ mRNA splicing. Blot was probed for $HAC1$ mRNA. Uninduced $HAC1^u$ and induced $HAC1^i$ splice variants of $HAC1$ mRNA are indicated. (B) Agarose electrophoretic separation of total extracted RNA, with 28S and 18S rRNA indicated. Experiment performed by Dr. Noah Reynolds.
3.3.4 EcPheRS and SccytoPheRSs have divergent substrate specificities.

EcPheRS post-transfer hydrolase activity efficiently edits \(m\)-Tyr-tRNA\(^\text{Phe}\) (104), and the loss of this activity in the G318W variant demonstrates that this catalysis occurs at the site previously described for hydrolytic editing of \(p\)-Tyr-tRNA\(^\text{Phe}\) (123) (Fig. 3.2). Wild-type EcPheRS does not produce tRNA\(^\text{Phe}\) charged with either \(m\)- or \(p\)-Tyr; however, G318W aminoacylates tRNA\(^\text{Phe}\) with either isomer, with \(m\)-Tyr being a more efficient substrate (Figs. 3.5A and 3.5B), suggesting that post-transfer editing activity by this enzyme is a critical checkpoint in quality control that limits production of misacylated tRNA\(^\text{Phe}\) species with either \(m\)-Tyr or \(p\)-Tyr. G318W EcPheRS is unable to aminoacylate tRNA\(^\text{Phe}\) with \(o\)-Tyr or L-DOPA, consistent with the absence of any differences in growth between wild-type \(E. coli\) and the \(pheT\) (G318W) strain in the presence of these tyrosine isomers (Fig. 3.2). Direct examination of steady-state amino acid activation revealed that discrimination against \(m\)-Tyr by EcPheRS is only 35-fold, an exceptionally poor value, given the specificity constant in activating Phe vs. \(p\)-Tyr of \(\sim3,000\) (Table 2). Taken together, these observations suggest that the mechanism of quality control by EcPheRS depends on the non-cognate: \(p\)-Tyr is poorly activated and requires no further quality control mechanism; discrimination against \(m\)-Tyr in the amino acid activation step is poor, and requires post-transfer editing of \(m\)-Tyr-tRNA\(^\text{Phe}\).

In contrast to the \(E. coli\) enzyme, wild-type SccytoPheRS discriminates poorly against both \(p\)-Tyr and \(m\)-Tyr in the amino acid activation step, and readily mischarges tRNA\(^\text{Phe}\) with either substrate. Charging of tRNA\(^\text{Phe}\) with \(m\)-Tyr was observed at concentrations where \(p\)-Tyr-tRNA\(^\text{Phe}\) synthesis was not detected (Fig. 3.5C, Table 3.2). In
contrast to EcPheRS, $p$-Tyr-tRNA$^{\text{Phe}}$ is a better substrate for post-transfer editing by ScytoPheRS, relative to $m$-Tyr-tRNA$^{\text{Phe}}$ (Fig. 3.6). Poor post-transfer editing of $m$-Tyr-tRNA$^{\text{Phe}}$ may explain the cytotoxicity of $m$-Tyr to wild-type $S. \text{cerevisiae}$, though the moderate post-transfer editing clearly plays some protective role in the presence of high levels of $m$-Tyr (Fig 3.3B).
Figure 3.5. Tyrosine isomers as substrates for tRNA\textsuperscript{Phe} aminoacylation by PheRS variants in both \textit{E. coli} and \textit{S. cerevisiae}. tRNA\textsuperscript{Phe} aminoacylation by (A) wild-type or (B) G318W \textit{E. coli} PheRS with 60 µM Phe, \textit{p}-Tyr, or \textit{m}-Tyr. Aminoacylation of (C) wild-type \textit{S. cerevisiae} cytoplasmic PheRS and (D) wild-type mitochondrial \textit{S. cerevisiae} PheRS with 100 µM Phe, \textit{p}-Tyr, or \textit{m}-Tyr. Data are presented as the mean of three technical replicates, with errors bars representing ± S.D. Experiments performed by Drs. Tammy Bullwinkle and Noah Reynolds.
ScmitoPheRS, which lacks \( p\)-Tyr-tRNA\(^{\text{Phe}} \) post-transfer editing activity (109), was also found to synthesize \( m\)-Tyr-tRNA\(^{\text{Phe}} \) more efficiently than \( p\)-Tyr-tRNA\(^{\text{Phe}} \) at similar tyrosine isomer concentrations (Fig. 3.5D). In \( \text{frs1-1} \) (βD243A), the absence of quality control mechanisms in either the SccytoPheRS or ScmitoPheRS suggests that \( m\)-Tyr toxicity results from the accumulation of mischarged tRNA\(^{\text{Phe}} \) in both cellular compartments. Notably, there is only one tRNA\(^{\text{Phe}} \) isoacceptor in \( S. \text{cerevisiae} \), and this same tRNA is recognized by PheRS in both compartments.

### 3.3.5 Phe codon mistranslation as \( m\)-Tyr in \( E. \text{coli} \) is elevated in an \( Ec\text{PheRS} \) post-transfer editing defective strain.

The cytotoxicity of \( m\)-Tyr in post-transfer editing-defective \( E. \text{coli} \), poor discrimination against \( m\)-Tyr in the activation step, and efficient post-transfer editing against \( m\)-Tyr-tRNA\(^{\text{Phe}} \) by wild-type \( Ec\text{PheRS} \) strongly suggest mistranslation of Phe codons as \( m\)-Tyr in the absence of post-transfer editing activity by this enzyme. To confirm that no ribosomal mechanism prevents translation of Phe codons as \( m\)-Tyr, we tested synthesis of dipeptides \textit{in vitro} using \( m\)-Tyr-tRNA\(^{\text{Phe}} \):EF-Tu:GTP as a substrate for decoding of a ribosomal A site Phe (UUC) codon. Under these conditions, similar levels of fMet-\( m\)-Tyr and fMet-Phe were synthesized, indicating no apparent discrimination against \( m\)-Tyr at the A-site of \( E. \text{coli} \) ribosomes (Fig. 3.6A).
Figure 3.6 Translation of $m$-Tyr in *E. coli*. (A) 70S ribosomal dipeptide synthesis with either Phe-$tRNA^{Phe}$ or $m$-Tyr-$tRNA^{Phe}$ *in vitro* (B) LC-MS/MS-MRM quantification of $m$-Tyr and Phe in protein hydrolysate isolated from *E. coli*, expressed as molar ratio of $[m$-Tyr]/[Phe]. Wild-type and *pheT* (G318W) *E. coli* grown in M9 minimal media with or without supplementation with $m$-Tyr. Data are presented as the mean of 3 replicates ± S.E.M. Dipeptide analysis was performed by Dr. Tammy Bullwinkle. Global mistranslation analysis was performed by and Dr. Kym Faull and myself.
The effect of \( m \)-Tyr on global protein synthesis \textit{in vivo} was investigated by analyzing the accumulation of \( m \)-Tyr in the soluble protein of wild-type and \( E. \ coli \) \( pheT \) (G318W) cells. Cytosolic protein samples were isolated from \( m \)-Tyr treated \( E. \ coli \) cells and samples subjected to 0.1 N HCl acid hydrolysis to generate individual amino acids. The resulting amino acid hydrolysate was resolved by reverse-phase liquid chromatography-tandem mass spectrometry with multiple reaction monitoring. To validate peak assignments of the Tyr isomers, co-chromatography was performed with synthetic \( m \)-Tyr or \( o \)-Tyr standards added to proteome samples. Only one peak for each of the isomers was observed, validating assignments. Some level of \( m \)-Tyr was found to be present in the proteomes of both wild type and \( phe \) (G318W) strains, indicating incorporation could be occurring through more than one route. Because total soluble protein was isolated by 55% acetone precipitation, the mechanism of which involves inversion of soluble protein structures and aggregation of exposed hydrophobic residues, it is likely that a significant portion of \( m \)-Tyr is precipitated along with protein samples. Nevertheless, comparison of proteome total amino acid levels between wild type and \( pheT \) (G318W) strains indicated a level of misincorporation of 1 % \( m \)-Tyr at Phe codons in the absence of \( Ec \)PheRS editing (Fig. 3.6B). In wild-type protein, \([m \text{-Tyr}]/[\text{Phe}]\) is 0.015, increasing to 0.025 in samples isolated from the \( pheT \) (G318W) strain grown in the same conditions. This suggests that post-transfer editing by PheRS provides protection of the \( E. \ coli \) proteome from misincorporation of \( m \)-Tyr at Phe codons under conditions where intracellular \( m \)-Tyr is abundant. \( p \)-Tyr does not change between the wild-type and \( pheT \) (G318W) \( E. \ coli \), indicating that this proteinogenic amino acid is not
significantly misincorporated at Phe codons, even in the absence of PheRS editing (Fig. 3.7). These analyses show a ratio of \( p\text{-Tyr}/\text{Phe} \) of 0.6, which correlates with previous estimates of amino acid usage in \( E.\ coli \) (127).
Figure 3.7 p-Tyr mistranslation is not elevated in *E. coli* at Phe codons in the absence of post-transfer editing by *EcPheRS*. LC-MS/MS quantification of p-Tyr and Phe in protein hydrolysate from *E. coli*, expressed as molar ratio of \([p\text{-Tyr}]/[\text{Phe}]\). Wild-type and *pheT* (G318W) strains grown in in M9 minimal media alone, or supplemented with *p*-Tyr are shown. Data represent the average of 3 replicates ± S.E.M. Experiment performed by Dr. Tammy Bullwinkle and Dr. Kym Faull.

Because *m*-Tyr is detected in wild-type *E. coli* protein supplemented with *m*-Tyr, but minimal *m*-Tyr is detected in either strain without supplementation, it is likely that either this non-proteinogenic amino acid escapes PheRS editing, infiltrates the proteome by means other than misincorporation at Phe codons, such as direct oxidation of protein Phe, or is carried over during cytosolic protein preparation. To control for carry-over in supplementation experiments, wild-type *E. coli* was grown in the presence of 0.5 mM *o*-Tyr, which is not a substrate for EcPheRS, and total protein samples were subjected to acid hydrolysis and LC-MS/MS-MRM, as before. Trace *o*-Tyr was detected, suggesting that free amino acid carry-over may contribute to *m*-Tyr detected in the samples from the wild-type strain in *m*-Tyr supplementation experiments. Whether the *m*-Tyr seen in the wild-type *E. coli* is formed post-translationally or is incorporated during protein synthesis
via another promiscuous tRNA synthetase in *E. coli* is unclear. Aminoacylation of tRNA$^{\text{Tyr}}$ with *m*-Tyr by *EcTyrRS* was detected *in vitro*, suggesting this synthetase may provide a route of *m*-Tyr incorporation, even when PheRS editing is not disrupted (Fig. 3.8).

![Figure 3.8](image)

**Figure 3.8** *EcTyrRS* efficiently aminoacylates tRNA$^{\text{Tyr}}$ with *m*-Tyr. Aminoacylation of 0.5 µM *E. coli* $[^{35}\text{P}]-$tRNA$^{\text{Tyr}}$ with 1 mM *m*-Tyr by 50 nM *EcTyrRS* at 25 °C. Experiment performed with Dr. Tammy Bullwinkle.

### 3.3.6 *EcPheRS* post-transfer editing is required for normal growth in oxidative stress conditions

Reactive oxygen species generated under oxidative stress can directly damage intracellular Phe, forming isomers of tyrosine, such as *m*-Tyr, which could potentially threaten the fidelity of protein synthesis in the absence of post-transfer editing (106,125). To directly investigate if oxidative stress can generate potentially toxic levels of *m*-Tyr *in vivo*, wild-type and post-transfer editing-defective *EcPheRS* strains were grown in the
presence of H$_2$O$_2$ and FeSO$_4$. LC-MS/MS-MRM analyses indicated that m-Tyr was elevated in extracted intracellular metabolites extracted from *E. coli* grown in oxidative stress conditions (Fig. 3.9A). In addition to m-Tyr, significant *de novo* o-Tyr accumulation was also observed following ROS treatment, however the poor efficiency by which o-Tyr is utilized by EcPheRS suggests that o-Tyr is likely not a threat to quality control in Phe codon translation.

**Figure 3.9** The role of EcPheRS post-transfer editing in oxidative stress conditions *in vivo*. (A) LC-MS/MS-MRM chromatograms for p-, m- and o-Tyr (m/z 182→136 transition) extracted from cells grown in the absence (left) or presence (right) of H$_2$O$_2$ and FeSO$_4$. (B) Growth of *E. coli* pheT(G318W) strain relative to wild-type in M9 minimal media supplemented with 0.1 mM FeSO$_4$ and various concentrations of H$_2$O$_2$. Data represent the mean of three biological replicates ± S.D.

*E. coli* lacking PheRS post-transfer editing activity displayed a reduction in growth relative to wild-type *E. coli*, when grown in media where ROS exposure increased, consistent with the accumulation of free m-Tyr and its subsequent utilization by EcPheRS and mistranslation of Phe codons as m-Tyr (Fig. 3.9B). Taken together, our data indicate that EcPheRS post-transfer editing activity protects *E. coli* against the co-
translational insertion of $m$-Tyr that accumulates during oxidative stress. Attempts to identify $m$-Tyr in the total protein hydrolysis samples under oxidative stress conditions revealed the presence of $m$-Tyr and $o$-Tyr in both the wild type and pheT(G318W) strains, despite efficient discrimination by this enzyme against $o$-Tyr, suggesting that there may be a degree of carry-over in supplemented amino acids in protein purification. Additionally, post-translational oxidation of Phe in protein under H$_2$O$_2$ treatment is likely at least partially responsible for the accumulation of hydroxylated Phe residues.

3.4 Discussion

3.4.1 Context-dependent threats to PheRS product specificity, and conditional requirements for post-transfer editing.

EcPheRS limits misacylation of tRNA$^{Phe}$ species with both $p$-Tyr and $m$-Tyr via distinct mechanisms, suggesting both that multiple non-cognate amino acids threaten the fidelity of Phe codon translation in a cytotoxic manner in the absence of these quality control steps, and that $meta$- and $para$-substituted Phe derivatives pose distinct challenges to PheRS quality control. In eukaryotes, cytoplasmic PheRS post-transfer editing is necessary to protect the proteome from $p$-Tyr misincorporation, as discrimination against non-cognate amino acids is poor in the activation step (93). It is unclear to what degree protection from $m$-Tyr incorporation is achieved through post-transfer editing, as the S. cerevisiae strain encoding wild-type ScytoPheRS is sensitive to high concentrations of $m$-Tyr. ScmitoPheRS efficiently synthesizes $m$-Tyr-tRNA$^{Phe}$, and other eukaryotic proteomes are vulnerable to this non-protein amino acid (107). Taken together, these
findings suggest that either $m$-Tyr accumulation is not a substantial threat in eukaryotes, or that low-level mistranslation with $m$-Tyr in certain proteomes confers some as yet unknown evolutionary benefit.

**3.4.2 Non-proteinogenic amino acids as threats to the fidelity of translation of the genetic code.**

Naturally-occurring non-protein amino acids are widespread in nature, and are well-characterized by-products and/or intermediates of biosynthetic pathways (128). Due to only recent advances in mass spectrometry that allow for high-resolution chromatographic separation of non-protein amino acids with biophysical properties similar to proteinogenic amino acids, it is likely that a significant amount of mistranslation with proteinogenic amino acids reported is actually mistranslation with non-protein amino acids that were previously indistinguishable from similar proteinogenic amino acids. The actual threats that non-protein amino acids pose to protein synthesis and cell viability are unknown, as are the mechanisms of aaRS quality control that protect the proteome from such amino acids. The non-proteinogenic amino acid $m$-Tyr has been detected in several eukaryotic proteomes and is one of the products of proteinogenic amino acid oxidation (129,130). The presence of hydroxylated forms of Phe and Tyr in proteomes have previously been attributed to post-translational damage to proteins by ROS, and have been used as markers for tissue damage corresponding to the oxidative conditions of aging and disease. $m$-Tyr and other Tyr analogues—for example, L-DOPA, are substrates for translation in some organisms, and may be incorporated
directly during protein synthesis (104,107,131-133). We show that post-transfer editing by EcPheRS prevents the use of \( m \)-Tyr during protein synthesis, demonstrating the threat amino acid oxidation poses to the proper functioning of the bacterial translation machinery.

Translation of Phe codons as \( m \)-Tyr in \textit{E. coli} is toxic, and in the absence of PheRS post-transfer editing, this non-proteinogenic amino acid is as an efficient substrate for translation. Other non-proteinogenic amino acids have also been shown to be potential threats to translation, including as \( \alpha \)-aminobutyrate, which, in the absence of ValRS post-transfer editing is toxic at high concentrations (134). The robust editing activity maintained by \textit{EcPheRS} to protect the proteome from \( m \)-Tyr under conditions of oxidative stress demonstrates the significant threat non-protein amino acids pose when misincorporated at specific near-cognate positions. In contrast, the presence of \( m \)-Tyr in the proteome of wild type \textit{E. coli} suggests misincorporation also occurs at Tyr codons but without cytotoxic sequelae, indicating that the effects of incorporation of this non-proteinogenic amino acid are codon-dependent. The replacement of \( p \)-Tyr with \( m \)-Tyr amino acid appears undistruptive to cell growth, as seen in the wild-type strain, whereas \textit{EcPheRS} editing is crucial for preventing the toxic effects of \( m \)-Tyr translated at Phe codons. No \textit{E. coli} codons specify \( m \)-Tyr, so any advantage or disadvantage this amino acid might provide to the proteome cannot easily be selected for, or against, at the level of the genetic code. In \textit{E. coli}, global misincorporation of \( m \)-Tyr at Phe codons in the absence of PheRS quality control occurred at a frequency of 1% and had a significant negative impact on cellular viability. This contrasts with the effects of misincorporation
of certain proteinogenic amino acids, which have been shown to be tolerated at rates of up to 10% without inhibiting growth (135). Taken together with earlier studies, our findings show that the misincorporation of non-proteinogenic amino acids can be a substantial challenge for protein synthesis quality control in specific and unique environmental contexts. This in turn suggests that many apparently “dispensable” editing functions, both in aaRSs and trans-editing factors, may in fact be essential for growth under conditions that lead to the accumulation of potentially toxic levels of non-proteinogenic amino acids. Moreover, controlled laboratory environments do not necessarily represent the myriad environmental stresses and changes in nutrient availability that wild strains encounter.

3.4.3 Conditional challenges to translational quality control under oxidative stress.

Oxidative damage of amino acids caused by reactive oxygen species such as hydroxyl radical and superoxide anions results in limited alteration of amino acid structure, such as the addition of a hydroxyl group. Such modification can create potential substrates for tRNA misacylation in vivo, while simultaneously limiting the availability of cognate substrates. Damaged amino acids can challenge the protein synthesis machinery; L-DOPA and leucine hydroxide have been shown to be incorporated into murine protein, and o-Tyr and m-Tyr have been observed in CHO protein (131,132,136). Aerobic respiration results in elevated endogenous H$_2$O$_2$, but bacterial cells are also exposed to ROS present in their environment. Uncharged H$_2$O$_2$ penetrates the cell membrane and accumulates intracellularly when H$_2$O$_2$ is present in the extracellular microenvironment.
At physiological pH, H\textsubscript{2}O\textsubscript{2} quickly oxidizes ferrous iron and generates a hydroxyl radical via the Fenton reaction. The resulting radical can react with nearby cellular targets as diverse as lipids, nucleic acids, or protein (137). Accumulation of toxic levels of m-Tyr in \textit{E. coli} is readily observed under experimental conditions that promote the formation of hydroxyl radicals, but m-Tyr is not the only byproduct of Phe oxidation. o-Tyr is the more abundant hydroxylation product under ROS-generating conditions used here, which may represent preferential oxidation or metabolic inter-conversion (Fig. 3.9). However, there is no observed o-Tyr aminoacylation of tRNA\textsuperscript{Phe} by wild type EcPheRS \textit{in vitro}, or inhibition of cell growth, in the presence of supplemented o-Tyr. These, and the corresponding biochemical data, indicate how EcPheRS has evolved to effectively discriminate against different Tyr isomers with a combination of substrate specificity in the activation step (o-Tyr, p-Tyr) and post-transfer editing activity (m-Tyr, p-Tyr). In contrast, ScytoPheRS has mainly evolved specifically to discriminate for p-Tyr by editing, reflecting differences in the evolution of aaRS quality control.
Chapter 4

Multiple quality control pathways limit non-protein amino acid use by yeast cytoplasmic phenylalanyl-tRNA synthetase.

4.1 Introduction

The units of genetic information contained in mRNA codons need to be precisely translated at the ribosome into corresponding amino acid sequences in proteins. Because protein function is dictated by structure, and thus amino acid sequence, mistakes in translation of the genetic code can yield drastic alterations in the properties of products of mistranslation. Quality control mechanisms exist that help maintain translation fidelity, limiting mistranslation to one mistake in $10^3$-$10^4$ codons under normal growth conditions (30). The aminoacyl-tRNA synthetase (aaRS) enzymes, which pair amino acids and tRNAs for participation as substrates in protein synthesis, are among the primary determinants of translational fidelity. In the event that a non-cognate amino acid or tRNA is utilized as an aaRS substrate, the resulting misacylated aminoacyl-tRNA (aa-tRNA) species may lead to an error in decoding at the ribosome, resulting in mistranslation of
the genetic code. To limit these errors, aaRS enzymes have evolved strict quality control mechanisms, which typically confer high substrate specificity and contribute to an estimated rate of misacylated aa-tRNA production limited to roughly 1 per 3,000 aa-tRNAs produced (70), though deviations exist between species and specific aaRSs (30,36,138). Some aaRS enzymes hydrolyze or selectively release (139) aminoacyl-adenylate (aa-AMP) species resulting from non-cognate amino acid activation, in a quality control step prior to transfer of the aminoacyl moiety to tRNA. This "pre-transfer" editing can be tRNA-dependent (140) or tRNA-independent (15). Approximately half of aaRS enzymes bear a "post-transfer" editing domain distinct from the synthetic domain (96). The active sites of these editing domains serve to recognize misacylated tRNA species and hydrolyze misacylated aa-tRNA ester linkages. AaRSs also rely heavily on substrate discrimination in the synthetic active site. Amino acids or tRNAs with incompatible biophysical geometry in an aaRS active site may bind weakly, if at all. As a result, poor substrates for aaRS catalysis are limited in their participation in aa-tRNA synthesis, and thus ribosomal translation.

Translational fidelity mediated by aaRS enzymes can be a context-dependent phenomenon. In particular, a shift in the abundance of an aaRS cognate or non-cognate substrate may result in unusually high misacylation of tRNAs. Illustrative of this effect is the observation that Chinese hamster ovary cells starved for tyrosine exhibit a high degree of mistranslation of Tyr codons as Phe (46). Rather than relying on editing activity, tyrosyl-tRNA synthetase exhibits high kinetic discrimination between Tyr and Phe at the amino acid activation step. In an environment depleted for its cognate Tyr, an unusually high degree of near-cognate Phe utilization by TyrRS and mistranslation of Tyr
codons as Phe are observed (Chapter 2). Oxidative stress also plays an important role in non-cognate aa-tRNA synthesis, either by generating non-protein amino acids which must then be proofread (Chapter 3) (45), or by direct inactivation of such proofreading (31,50). Oxidative stress effects an increase in reactive oxygen species, which may directly damage amino acids. For example, Phe may be oxidatively converted to one of several derivatives. Among these are the proteinogenic amino acid p-Tyr, and the non-protein amino acids 3,4-dihydroxy-L-phenylalanine (L-DOPA), ortho-tyrosine (o-Tyr), and meta-tyrosine (m-Tyr) (Fig. 4.1).

![Chemical structures](image)

**orth**-tyrosine (o-Tyr)  
**meta**-tyrosine (m-Tyr)  
**para**-tyrosine (p-Tyr)  
3,4-dihydroxyphenylalanine (L-DOPA)

**Figure 4.1. Potential non-cognate amino acid substrates of PheRS.** Shown are hydroxylated phenylalanine derivatives that may accumulate as the result of oxidative damage, metabolic conversion, or import.

Of these, only m-Tyr is a threat to translational fidelity in an *Escherichia coli* (*E. coli*) phenylalanyl-tRNA synthetase (EcPheRS) mutant strain defective in post-transfer editing activity (45). The post-transfer editing activity of the wild-type enzyme can
hydrolyze $m$-Tyr-tRNA$^{\text{Phe}}$ species synthesized as $m$-Tyr accumulates under conditions of oxidative stress. $p$-Tyr, which is present at levels necessary for typical protein synthesis under normal conditions, is the most obvious candidate as a non-cognate threat to PheRS product specificity; surprisingly, $p$-Tyr presents no such challenge to EcPheRS. Without wild-type EcPheRS post-transfer editing activity, *E. coli* grows as well as wild-type, even when challenged with an excess of $p$-Tyr. Kinetic analysis revealed a high degree of EcPheRS discrimination against $p$-Tyr (but not $m$-Tyr) in the amino acid activation step. Taken together, these observations show that normal EcPheRS editing activity is critical, not under typical growth conditions, but to protect *E. coli* from cytotoxic mistranslation of Phe codons in conditions that favor intracellular accumulation of $m$-Tyr (45).

In contrast, *Saccharomyces cerevisiae* cytoplasmic PheRS (Sc$\text{cytoPheRS}$) exhibits poor discrimination against $p$-Tyr in the amino acid activation step (~20-fold lower than EcPheRS), but $p$-Tyr-tRNA$^{\text{Phe}}$ production is limited by post-transfer editing (45). As with EcPheRS, Sc$\text{cytoPheRS}$ post-transfer editing of $m$-Tyr-tRNA$^{\text{Phe}}$ limits cytotoxicity of $m$-Tyr. Here, we show unexpected phenotypic effects of direct media supplementation of *S. cerevisiae* with cognate Phe, proteinogenic non-cognate Ala, and non-protein oxidized Phe derivatives in a Sc$\text{cytoPheRS}$ post-transfer quality control mutant. We highlight striking differences in the promiscuity of the yeast and bacterial enzymes for non-protein amino acids, and discuss multiple strategies employed by Sc$\text{cytoPheRS}$ to maintain product specificity in the face of conditional threats to translational quality control in a biological context.
4.2 Materials and Methods

4.2.1 Strains and growth conditions

We have previously constructed a βD243A ScytoPheRS strain with defective post-transfer editing activity (*frs1-1*), and a wild-type strain constructed from this background, which we refer to as wild-type throughout this manuscript (141). Wild-type and *frs1-1* S. *cerevisiae* were grown in the presence of varying levels of supplemented amino acids as indicated at 30°C in a 96-well microplate format (xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad Laboratories, Inc.), and optical density at 600 nm was measured at 30 minute intervals following brief agitation. In order to characterize growth defects under oxidative stress, wild type and *frs1-1* S. *cerevisiae* strains were grown in minimal media with or without the oxidative stressor methyl viologen (paraquat) (Sigma-Aldrich). 50 mL cultures were prepared in triplicate for each condition, and shaken at 240 rpm, 30°C. Optical density at 600 nm was measured at various time points. Oxidative stress cultures contained 2.0 mM paraquat. 10 mL samples of exponentially-growing culture were passed over Whatman® Protran® BA85 nitrocellulose filter membranes, washed with 10 mL water, and stored at -80°C for processing and analysis of intracellular amino acid abundance.

4.2.2 Quantification of Intracellular Amino Acid Abundance

Frozen cell pellets were processed for analysis as previously described (45). Briefly, a mixture of 13C internal standards for Phe, *p*-Tyr, and L-DOPA were added to each cell pellet prior to extraction in -20°C extraction buffer (40% methanol, 40% acetonitrile, 20% water). Solutions were clarified by centrifugation at 16,000 x g for 5 minutes at 4°C.
Supernatants were transferred to clean tubes and vacuum dried. Dried samples were re-dissolved in solvent A (water/formic acid, 100/0.1, v/v; 50 ul), centrifuged (16,000 x g, 5 min) and the supernatant transferred to LC injector vials. Aliquots of the supernatant (typically 4 ul) were injected onto a reverse phase HPLC column (Phenomenex Kinetex C18, 2.1 x 150 mm, 1.7 μm particle size, 100 Å pore size) equilibrated in solvent A and isocratically eluted (100 μl/m). The effluent from the column was directly connected to an electrospray ion source (Agilent Jet Stream) attached to a triple quadrupole mass spectrometer (Agilent 6460) scanning in the positive multiple reaction monitoring mode with standard resolution settings (FWHM 0.7) using previously optimized conditions for the following mass transitions: L-DOPA, 198→181, 198→152; U^{13}C_6-L-DOPA, 204→187, 204→152; p-Tyr, 182→136; U^{13}C_9-p-Tyr, 191→144; Phe, 166→120; U^{13}C_9-Phe, 175→128. With each batch of samples a series of standards was simultaneously prepared with the same amount of internal standards and increasing amounts of L-DOPA, m-Tyr and o-Tyr (0, 10, 25, 50 and 100 pmol), p-Tyr (0, 0.5, 1.25, 2.5 and 5 nmol) and Phe (0, 1.5, 3.75, 7.5, and 15 nmol), in duplicate. Typical retention times for L-DOPA, p-Tyr, m-Tyr, o-Tyr and Phe were 7.3, 10.8, 13.5, 18.1 and 20.6 min, respectively. Peak areas were measured using instrument manufacturer supplied software (Agilent MassHunter). The amount of each analyte in each sample was determined by interpolation from the curves constructed from the standard samples (peak area L-DOPA, p-Tyr or Phe/peak area U^{13}C_6-L-DOPA, U^{13}C_9-p-Tyr or –Phe against amount of L-DOPA, p-Tyr or Phe in each standard sample).
4.2.3 Liquid Chromatographic Purification of Amino Acids

The accuracy of kinetic analyses of non-cognate amino acid substrates for enzyme specificity determination depend on the degree of cognate amino acid contamination(33). Therefore, we purified \( p \)-Tyr, DL-\( o \)-Tyr, and L-DOPA prior to their use for kinetic analyses. \( p \)-Tyr (Regis Chemical company) was purified by recrystallization from a minimal volume of boiling water as previously described(142). DL-\( o \)-Tyrosine (\( o \)-Tyr, Fluka, >96.0% NT) was dissolved in 0.1% trifluoroacetic acid and injected (250 uL aliquots) onto a thoroughly washed semi-preparative reverse phase HPLC column (Keystone Scientific Aquasil C18, 250 x 10 mm) equilibrated and eluted (3 ml/min) with water. Thirty second fractions were collected, and aliquots (8 uL) of individual fractions across the peak of 280 nm absorbance eluting between 12.5 and 15 min were screened by LC/MS with 8 uL injections onto an Intakt Scherzo SS C18 column (100 x 2 mm) equilibrated in eluent A (water/acetonitrile/formic acid, 97/3/0.1) and eluted with an increasing concentration of eluent B (45 mM ammonium formate/acetonitrile, 65/35: min/% B; 0/0, 10/0, 40/20, 42/0, 55/0) with the mass spectrometer (Agilent 6460) scanning in the positive ion mode from m/z 100 to 300. Fractions 27, 28 and 29 each had a signal for \( o \)-Tyr (retention time (rt) 24.5 min; 3, 4 and 1 x 10\(^8\) area counts in the m/z 182 extracted ion chromatograms (EIC), respectively) with no detectable signals for either \( p \)-Tyr (rt 16.7 min; m/z EIC 182), \( m \)-Tyr (rt 18.4 min; m/z EIC 182) or Phe (rt 24.2 min; m/z EIC 166). With limits of detection for \( p \)-Tyr, \( m \)-Tyr and Phe estimated to be below 3.6, 4.5 and 1.4 x 10\(^4\) area counts, respectively, fractions 27-29 appeared to contain less than 1 part in 10\(^4\) of either \( p \)-Tyr, \( m \)-Tyr or Phe. Fractions 27-29 were pooled, lyophilized to dryness, weighed (4 mg) and used for the experiments described below.
3,4-dihydroxy-L-phenylalanine (L-DOPA, Sigma-Aldrich) was purified in an identical manner using the same semi-preparative column after it had been thoroughly washed to remove traces of residual analytes. Fractions containing the 280 nm peak of absorption eluting between 11 and 14 minutes were screened by LC/MS as described above. Fractions 23-28 contained a large peak for L-DOPA with no detectable amount of p-Tyr, m-Tyr, o-Tyr or Phe. These fractions were pooled, lyophilized to dryness (7.2 mg) and used for the experiments described below.

4.2.4 Wild-type and Post-Transfer Editing Defective SccytoPheRS Preparation

His$_6$-tagged wild-type and post-transfer editing defective SccytoPheRS were purified from previously constructed strains (78), and dialyzed against PP$_i$ to clear the active site of pre-bound Phe, as before (45). Active enzyme stock concentrations were determined as previously described (143).

4.2.5 Steady-State kinetic analysis of SccytoPheRS

SccytoPheRS kinetic parameters for amino acid activation were determined by measurement of time-course ATP/PP$_i$ exchange using standard protocols (45,46,78,96). Wild-type SccytoPheRS (5 nM for Phe and DL-\(\alpha\)-Tyr, 500 nM for L-DOPA) catalyzed the activation of a given amino acid substrate (25-1000 \(\mu\)M Phe, 100-3000 \(\mu\)M DL-\(\alpha\)-Tyr, or 200-1000 \(\mu\)M L-DOPA) in a reaction mix containing 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 10 mM MgCl$_2$, 2 mM NaF, 10 mM \(\beta\)-mercaptoethanol, 2 mM ATP, 2 mM \[^{32}\text{P}]\text{PP}_i\) (~1-2 cpm/pmole). The reaction was quenched in a solution containing 1% activated charcoal in 5.6% HClO$_4$ and 75 mM PP$_i$, which was then filtered through 3 MM
Whatman filter discs and washed with water. Negative control values (no enzyme present) were subtracted to account for retained PP_i. The velocity of [^{32}P]ATP formation as a function of substrate concentration was fitted to the Michaelis-Menten equation by non-linear regression. As a practical consideration, L-DOPA V_{max} was not observable. k_{cat} and K_M are thus not reported individually for L-DOPA. Δ(v/E)/Δ[L-DOPA] in a linear range was used as a measure of k_{cat}/K_M with L-DOPA.

4.2.6 Aminoacylation of tRNA

Transcription of tRNA^{Phe}ΔA76 in vitro, [^{32}P]tRNA^{Phe} radiolabeling with tRNA nucleotidyltransferase, and analysis of the relative efficiency of aminoacylation of tRNA^{Phe} by PheRS were carried out as reported previously(45). The ratio of aa-[^{32}P]-AMP and [^{32}P]-AMP accumulation over time was used as a measure for the relative efficiency in SccyoPheRS aminoacylation of tRNA^{Phe} with a given amino acid.

4.2.7 Pre-transfer editing

Analysis of pre-transfer editing was carried out as previously described(15). Wild-type SccytoPheRS-catalyzed rates of [α-^{32}P]-AMP and aa-[α-^{32}P]-AMP synthesis were compared. Reactions contained 1 µM SccytoPheRS, 2 mM DL-α-Tyr, 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 10 mM MgCl_2, 2 mM NaF, 10 mM β-mercaptoethanol, 50 µM ATP, 10 µM natively purified tRNA^{Phe} (Sigma-Aldrich), and 0.5 µM [α-^{32}P]-ATP. Matched no-enzyme control values were subtracted to account for spontaneous hydrolysis of [α-^{32}P]-ATP. 1 µL samples of the reaction mix were quenched at various time points with 1.5 µL 0.5 mM NaOAc (pH 4.2). 1.5 µL of quenched reactions at
various time points were spotted onto polyethyleneimine cellulose (Sigma-Aldrich) pre-equilibrated with water. Products were resolved in a mobile phase consisting of 100 mM ammonium acetate and 5% acetic acid. Spots corresponding to \(\text{o-Tyr-}[\alpha-^{32}\text{P}]-\text{AMP}\) and free \([\alpha-^{32}\text{P}]-\text{AMP}\) were visualized as described previously (45,119).

4.2.8 Pre-Steady-State Characterization of \(\text{o-Tyr}\) Transfer

The rate of \(\text{o-Tyr}\) transfer from \(\text{o-Tyr-AMP}\) to \(\text{tRNA}^{\text{Phe}}\) was determined with a quench flow RQF-3 KinTek instrument as before (101), with modifications based on unavailability of \(^{14}\text{C-o-Tyr}\). Because we expected \(\text{o-Tyr-AMP}:\text{PheRS}\) complex instability, \(\text{o-Tyr}\) activation was carried out \textit{in situ}. Syringe A contained 4 mM ATP, 5 \(\mu\text{M}\) post-transfer editing ablated SccytoPhers, 10 mM dithiothreitol, 2 U/mL inorganic pyrophosphatase, 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 10 mM MgCl\(_2\), and either 2 mM DL-o-Tyr or 1 mM Phe. Syringe B contained 1.5 \(\mu\text{M}\) \([^{32}\text{P}]\)-\(\text{tRNA}^{\text{Phe}}\), 10 mM dithiothreitol, 100 mM Na-Hepes (pH 7.2), 30 mM KCl, and 10 mM MgCl\(_2\). Reactions were carried out at 30°C and quenched with 0.5 M NaOAc. An equal volume of quenched reaction mix and 1:10 diluted S1 were mixed, and the S1 nuclease reaction was carried out at 37°C for 30 minutes. 1.5 \(\mu\text{L}\) of each time point was spotted on polyethyleneimine cellulose and developed as detailed above. \(\text{aa-tRNA}\) formation over time was fitted to the single-exponential equation \([\text{aa-tRNA}] = C + A (1-e^{kt_{\text{trans}}})\), where \(A\) is the amplitude, and \(C\) is the \(y\)-intercept.
4.2.9 Measurement of \( o \)-Tyr-AMP Stability

\( o \)-Tyr-AMP was synthesized under aminoacylation reaction conditions described above in the absence of tRNA\(^{\text{Phe}}\), using \([\alpha^{\text{32P}}]\text{-ATP}\). \( o \)-Tyr-AMP was then purified after 15 minutes via standard acidic phenol-chloroform extraction techniques. The stability of \( \approx 14 \) \( \mu \text{M} \) \( o \)-Tyr-AMP under reaction conditions in the absence of enzyme was observed by TLC separation, as above.

4.3 Results

4.3.1 ScytoPheRS post-transfer editing limits the cytotoxicity of oxidative stress

Following our observations that PheRS post-transfer editing activity is required to protect \( E. \coli \) from \( m \)-Tyr, we examined the role that post-transfer editing activity plays in a eukaryotic species, \( S. \cerevisiae \). Having previously demonstrated that \( p \)-Tyr and \( m \)-Tyr are more toxic to a cytosolic ScytoPheRS post-transfer editing mutant strain (\( \text{frs}1-1 \)) (45), we examined a possible protective effect of editing in the presence of oxidative stress. Methyl viologen (paraquat) is used as a surrogate for oxidative stress, as it exacerbates electron transport leakage from the mitochondrion and single-hydrogen reduction of molecular oxygen. We found that ScytoPheRS post-transfer editing activity limited the toxicity of paraquat (Fig. 4.2), suggesting a similar protective role of PheRS post-transfer editing in both bacteria and eukarya under oxidative stress.
4.3.2 Oxidized Phe derivatives accumulate under oxidative stress

To test if the protective role that SccytoPheRS post-transfer editing plays in \textit{S. cerevisiae} is due to a decrease in the availability of intracellular Phe, accumulation of non-cognate PheRS substrates, or some indirect effect, we extracted soluble intracellular metabolites from exponentially-growing \textit{S. cerevisiae} grown in minimal media, and examined by LC-MS/MS the relative molar abundance of putative SccytoPheRS substrates (Fig. 4.3).
Figure 4.3. **Oxidative stress increases the abundance of non-protein amino acids.** Abundance of extracted intracellular amino acids from 10 mL samples of exponentially-growing wild-type or frs1-1 grown in minimal media with or without 2.0 mM paraquat. Values are normalized to the optical density at 600 nm of parent cultures at the time of harvest. Samples were taken from triplicate cultures. Error bars represent ± SEM. *p<0.05, unpaired t-test. One of the three biological samples taken had a total metabolite mass ~2.5-2.8 times that of the other two replicates, resulting in sufficient variability to not meet the standard conventions of statistical significance for the comparison between unstressed and stressed wild-type cultures for p-Tyr (p=0.0581), m-Tyr (p=0.1455), and L-DOPA (p=0.1127). There was also no significant difference between the amount of m-Tyr in frs1-1 with or without paraquat (p=0.1867).

There are 20 possible hydroxylation states of the phenyl ring of Phe; here, we show the detectable intracellular accumulation of five of them: Phe, p-Tyr, o-Tyr, m-Tyr, and L-DOPA. 2.0 mM paraquat induced the accumulation of not only oxidized Phe
derivatives, but also a two-fold increase in intracellular Phe. This may represent a sensing mechanism, whereby \textit{S. cerevisiae} compensates for oxidation of its Phe pool by overproducing Phe. Proteinogenic \textit{p}-Tyr accumulates 5- to 6-fold under oxidative stress, and may do so as a result of a combination of Phe oxidation and \textit{p}-Tyr biosynthesis. Notably, there is no known hydroxylase in \textit{S. cerevisiae} that can convert Phe to \textit{p}-Tyr. \textit{o}-Tyr accumulates 6- to 7-fold under oxidative stress, and \textit{m}-Tyr increases by 2- to 3-fold. L-DOPA, which is undetectable in unstressed \textit{S. cerevisiae}, accumulates to 1-2 orders of magnitude greater than \textit{o}-Tyr or \textit{m}-Tyr, which may represent oxidation of intracellular \textit{p}-Tyr as well as Phe. We also observed that unstressed \textit{S. cerevisiae} grown in rich media contain levels of L-DOPA similar to those in stressed cultures (not shown). This suggests a degree of L-DOPA contamination in rich media components, and subsequent cellular uptake. Because commercial yeast extracts and peptones are undefined and may become oxidized over time, the degree of non-protein amino acid contamination is a challenge to interpretation of results with such undefined media components.

\textbf{4.3.3 SccytoPheRS Post-Transfer Editing has Amino Acid Supplement Dependent Effects on Growth}

To further investigate the possible effects of non-protein aromatic amino acid accumulation, editing defective SccytoPheRS and wild-type \textit{S. cerevisiae} were grown with \textit{o}-Tyr and L-DOPA (Figs. 4.4 \textit{o}-Tyr and L-DOPA).
Figure 4.4. *SccytoPheRS* post-transfer editing activity has variable effects on growth rate dependent on supplemented amino acids. Exponential phase growth rate of wild-type (dark gray) and frs1-1 (light gray) yeast in minimal media supplemented with indicated amino acids. Data represent the average of measurements from three cultures. Error bars represent ± SEM. *p<0.05, unpaired t-test.

Given that *SccytoPheRS* poorly discriminates against p-Tyr relative to the *E. coli* enzyme, it was not surprising that L-DOPA is more toxic to *S. cerevisiae* when post-transfer editing activity is ablated. *E. coli* does not show increased sensitivity to L-DOPA in the absence of *EcPheRS* post-transfer editing, underscoring an evolutionary divergence in PheRS substrate specificity. Less expected was the complete phenotypic reversal with o-Tyr supplementation. The post-transfer editing ablated *SccytoPheRS* strain tolerates o-Tyr better than wild-type *S. cerevisiae*. When supplemented with either cognate Phe, or
non-cognate Ala (Figs. 4.4 Phe and Ala), the post-transfer editing mutant also grew better than wild-type. The degree of metabolic conversion of o-Tyr or Ala to other substrates is unclear, but the cognate Phe supplementation experiment suggests that the phenotypic effect of post-transfer editing in this condition is independent of its canonical aa-tRNA\textsuperscript{Phe} proofreading activity.

4.3.4 SccytoPheRS displays poor discrimination against oxidized Phe derivatives \textit{in vitro}

To examine the substrate specificity of SccytoPheRS, we compared steady-state activation of Phe, o-Tyr, m-Tyr, p-Tyr, L-DOPA, and Ala. No detectable activity was found for 1 mM Ala at 500 nM SccytoPheRS (data not shown), but all oxidized Phe derivatives tested are poorly discriminated against by SccytoPheRS \textit{in vitro} (Table 4.1). Values for p-Tyr and m-Tyr are adapted from our previous work(45). o-Tyr, in particular, is only an order of magnitude less efficiently activated than Phe, suggesting a substantial threat to SccytoPheRS product specificity and probable Phe codon mistranslation without an o-Tyr-specific quality control mechanism.
Table 4.1. Steady-state kinetic parameters for amino acid activation by ScytoPhers. Data are the average of 3 replicates ± SEM. †m-Tyr and p-Tyr values adapted from Bullwinkle et al. (2014).

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_M$ (μM)</th>
<th>$k_{\text{cat}}/K_M$ (s$^{-1}$μM$^{-1}$)</th>
<th>Specificity (Phe/x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe</td>
<td>58 ± 5.8</td>
<td>42 ± 7.0</td>
<td>1.4</td>
<td>-</td>
</tr>
<tr>
<td>o-Tyr</td>
<td>39 ± 0.9</td>
<td>310 ± 48</td>
<td>0.13</td>
<td>11</td>
</tr>
<tr>
<td>m-Tyr</td>
<td>26 ± 4.0†</td>
<td>1200 ± 230†</td>
<td>0.023†</td>
<td>71†</td>
</tr>
<tr>
<td>p-Tyr</td>
<td>-</td>
<td>-</td>
<td>0.014†</td>
<td>120†</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>-</td>
<td>-</td>
<td>7.5x10$^{-4}$±4.4x10$^{-5}$</td>
<td>1800</td>
</tr>
</tbody>
</table>

4.3.5 An Expanded form of aaRS Biological Substrate Selectivity

Enzyme specificity for a given substrate is calculated by a comparison of reaction rates:

$$\text{General Enzyme Specificity}_{A/B} = \frac{v_A}{v_B} = \frac{k_{\text{cat}}}{K_M}_A[A]$$

For aaRS amino acid specificity calculations, a comparison between two amino acid substrates is typically sufficient, where A is the cognate amino acid and B is a given near-cognate or non-cognate. In this simple two-substrate comparison, the preference for one substrate vs. the other is typically expressed as a direct ratio of catalytic efficiency constants, where [A]=[B]:

$$\text{aaRS Specificity}_{\text{two substrates,where}} \left( \frac{[\text{cognate}]}{[\text{noncognate}]} \right) = \frac{k_{\text{cat}}}{K_M}_{\text{cognate}}$$

However, relative reaction rates can change in vivo, as substrate concentrations are often in flux. Proper estimation of cognate specificity relies on measurement of the
intracellular concentrations of the relevant substrates, which in turn allows expression of aaRS substrate preference as selectivity in a biological context as follows:

\[
aaRS \text{ Selectivity (one noncognate)} = \left( \frac{k_{\text{cat}}}{K_M} \right)_{\text{cognate}} \frac{[\text{cognate}]}{\left( \frac{k_{\text{cat}}}{K_M} \right)_{\text{noncognate}} [\text{noncognate}]}
\]

Multiple non-cognate amino acids may simultaneously compete for access to the active site of a given aaRS (45), rendering one-to-one reaction rate comparisons insufficient in a biological context. Given the poor individual discrimination that SccytoPheRS exhibits against o-Tyr, m-Tyr, p-Tyr, and L-DOPA, one-to-one comparisons do not adequately describe aaRS cognate selectivity in vivo. Instead, an expanded form is appropriate:

\[
aaRS \text{ Selectivity (all noncognates)} = \frac{v_{\text{cognate}}}{\sum_{i=1}^{n} (v_{\text{noncognate}_i})} = \frac{\left( \frac{k_{\text{cat}}}{K_M} \right)_{\text{cognate}} [\text{cognate}]}{\sum_{i=1}^{n} \left( \frac{k_{\text{cat}}}{K_M} \right)_{\text{noncognate}_i} [\text{noncognate}_i]}
\]

Because it is effectively impossible to identify every potential substrate for aaRS activity that may exist, it is practical to simply include terms for known non-cognates and express biological selectivity as less than or equal to the calculated value. For SccytoPheRS, we now include terms for o-Tyr, m-Tyr, p-Tyr, and L-DOPA (Table 4.2), where SccytoPheRS selectivity under each condition is calculated as follows:

\[
\left( \frac{k_{\text{cat}}}{K_M} \right)_{\text{Phe}} [\text{Phe}] = \left( \frac{k_{\text{cat}}}{K_M} \right)_{o-Tyr} [o-Tyr] + \left( \frac{k_{\text{cat}}}{K_M} \right)_{m-Tyr} [m-Tyr] + \left( \frac{k_{\text{cat}}}{K_M} \right)_{p-Tyr} [p-Tyr] + \left( \frac{k_{\text{cat}}}{K_M} \right)_{L-DOPA} [L-DOPA]
\]
Table 4.2. ScytoPheRS selectivity is decreased under oxidative stress.

<table>
<thead>
<tr>
<th></th>
<th>wild-type</th>
<th>frsI-1</th>
<th>wild-type, 2.0 mM paraquat</th>
<th>frsI-1, 2.0 mM paraquat</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 127 ± 1</td>
<td>≤ 124 ± 2</td>
<td>≤ 42 ± 7</td>
<td>≤ 61 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

The calculated upper limits of wild-type and post-transfer editing defective ScytoPheRS biological selectivity are shown from strains grown with and without 2.0 mM paraquat. Data are the average of three replicates ± SEM. Wild-type ScytoPheRS selectivity is decreased 3-fold under oxidative stress (p=0.0003). Post-transfer editing ablated ScytoPheRS selectivity is decreased 2-fold under oxidative stress (p<0.0001). No statistically significant difference in selectivity exists between strains either without paraquat (p=0.2668) or with paraquat (p=0.0584).

Of the 19 possible ring-hydroxylated non-cognate Phe derivatives, only four were tested here. Moreover, Phe modification need not be limited to hydroxylation of the phenyl ring alone. Phe hydroperoxides, nitrosylated derivatives, or any number of alternatively modified Phe derivatives may be substrates for this enzyme. The intracellular abundance of multiple oxidized Phe derivatives increased under the oxidative stress condition chosen (Fig. 4.3). In this case, selectivity is driven primarily by Phe and p-Tyr abundance. When expanded to include all untested ScytoPheRS substrates and variable environmental stresses that cause non-protein amino acids to accumulate, the combined effect of many non-cognates represents a substantial conditional burden to the fidelity of Phe codon translation. The known promiscuity of ScytoPheRS implies multiple quality control mechanisms address the threat of various non-cognate amino acids that may be misactivated to a greater degree in various environmental contexts.
4.3.6 Aminoacylation of tRNA$^{\text{Phe}}$ with $o$-Tyr is Inefficient

The observed poor discrimination against $o$-Tyr during amino acid activation suggested that a later quality control mechanism may prevent substantial $o$-Tyr-tRNA$^{\text{Phe}}$ production. The efficiency of $o$-Tyr-tRNA$^{\text{Phe}}$ production was tested *in vitro* using purified post-transfer editing ablated ScytoPheRS. $o$-Tyr-tRNA$^{\text{Phe}}$ was synthesized to a level of ~10% of chargeable tRNA$^{\text{Phe}}$, which is substantially lower than the ~60% charging observed with $p$-Tyr (Fig. 4.5).

Figure 4.5. *o*-Tyr is inefficiently acylated to tRNA$^{\text{Phe}}$. Aminoacylation of tRNA$^{\text{Phe}}$ by post-transfer editing-defective ScytoPheRS with 75 μM Phe, 75 μM $p$-Tyr, and 150 μM DL-$o$-Tyr, expressed as fraction chargeable tRNA$^{\text{Phe}}$ ± SEM (n=3). tRNA$^{\text{Phe}}$ was transcribed *in vitro* without A76 prior to addition of A76 with tRNA nucleotidyltransferase, using α-$[\text{32P}]$ATP.
Because the post-transfer editing-defective ScytoPheRS enzyme was used for this aminoacylation, o-Tyr-tRNA$^{\text{Phe}}$ limitation is not due to post-transfer editing, but must come at a step after activation, given the ~10-fold superior efficiency in o-Tyr activation when compared to p-Tyr (Table 4.1). No difference in aminoacylation efficiency is observed between wild-type and βD243A ScytoPheRS for either Phe or o-Tyr (Fig. 4.6).

Figure 4.6. Wild-type ScytoPheRS synthesizes o-Tyr-tRNA$^{\text{Phe}}$ as efficiently as βD243A ScytoPheRS. Aminoacylation of tRNA$^{\text{Phe}}$ with wild-type or βD243A ScytoPheRS. Phenylanylation with wild-type (closed circles) or βD243A ScytoPheRS (open circles), para-tyrosylation with wild-type (closed squares) or βD243A ScytoPheRS (open squares), and ortho-tyrosylation with wild-type (closed triangles) or βD243A ScytoPheRS (open triangles) are shown. No difference was observed between enzymes in aminoacylation with Phe or o-Tyr (n=2). Error bars represent ± SEM.
4.3.7 $o$-Tyr-tRNA$^{\text{Phe}}$ Limitation is Not Due to Hydrolytic Pre-Transfer Editing

Selective hydrolysis of the products of amino acid misactivation is an established mechanism of aminoacyl-tRNA synthetase quality control. To test if $o$-Tyr-AMP is selectively hydrolyzed following activation, we observed $o$-Tyr-AMP and AMP accumulation in vitro. If $o$-Tyr-AMP were efficiently hydrolyzed following activation, we would see an accumulation of one of the products of this hydrolysis, AMP, faster than the accumulation of $o$-Tyr-AMP. However, we see that the steady-state rate of $o$-Tyr-AMP accumulation is 1-2 µM/min, whereas AMP does not accumulate appreciably at all by 2 min (Fig. 4.7), indicating that $o$-Tyr activation is faster than $o$-Tyr-AMP hydrolysis. Therefore, rapid pre-transfer hydrolysis of $o$-Tyr-AMP does not provide a likely mechanism to limit $o$-Tyr-tRNA$^{\text{Phe}}$ synthesis by ScytoPheRS.

![Figure 4.7](image)

**Figure 4.7.** $o$-Tyr-AMP is not efficiently hydrolyzed by ScytoPheRS. $o$-Tyr-AMP accumulates to a concentration at least 6-fold greater than wild-type (Panel A) or βD243A (Panel B) ScytoPheRS by 2 minutes. AMP is not appreciably formed. Data are presented as the average of three replicates ± SEM.

We also find that $o$-Tyr-AMP is stable in the absence of ScytoPheRS under reaction conditions (Fig. 4.8). This likely does not reflect the stability of $o$-Tyr-AMP produced in vivo, but controls for misinterpretation of kinetic analysis. If $o$-Tyr-AMP
were unstable in these reaction conditions, the observed rate of o-Tyr-AMP accumulation would be an underestimate, as it would be the combined effect of o-Tyr-AMP synthesis and degradation.

Figure 4.8. o-Tyr-AMP is stable in vitro. Shown is the stability of ~14 µM o-Tyr-AMP in reaction conditions over the course of 12 minutes. Data are presented as the average of three replicates ± SEM.
4.3.8 *o*-Tyr-AMP is selectively released from the ScytoPheRS active site

To test if *o*-Tyr-tRNA<sup>Phe</sup> limitation is due to selective release of *o*-Tyr-AMP from the ScytoPheRS active site, we observed the accumulation of *o*-Tyr-AMP relative to the concentration of ScytoPheRS *in vitro*. *o*-Tyr-AMP accumulates to a concentration at least 6-fold greater than the concentration of post-transfer editing defective ScytoPheRS by 2 minutes (Fig. 4.7), suggesting multiple turnover events in *o*-Tyr activation, as well as release of *o*-Tyr-AMP from the enzyme active site. Activation of *o*-Tyr is less efficient in the absence of tRNA<sup>Phe</sup> (Fig. 4.9), which may reflect a more stable activation complex when tRNA<sup>Phe</sup> is present, or direct interaction of tRNA<sup>Phe</sup> in activation chemistry.

![Graph A](image1.png)

**Figure 4.9.** *o*-Tyr-AMP is synthesized less efficiently in the absence of tRNA<sup>Phe</sup>. Pre-transfer analysis with 1 µM of wild-type or βD243A ScytoPheRS in the absence of tRNA<sup>Phe</sup>.*o*-Tyr-AMP accumulates to a concentration 5-fold greater than wild-type (Panel A) or βD243A (Panel B) ScytoPheRS by 2 minutes. AMP is not appreciably formed. Data are presented as the average of three replicates ± SEM.

4.3.9 *o*-Tyr-AMP release is due to slow transfer

Whereas *o*-Tyr-AMP has now been shown to be released from the ScytoPheRS active site prior to transfer, it was unclear if this release is competitive with the rate of *o*-Tyr transfer from *o*-Tyr-AMP to tRNA<sup>Phe</sup>, or if there is kinetic discrimination arising from
slow o-Tyr transfer. We addressed this ambiguity by comparing the rate of pre-steady-state transfer for cognate Phe and non-cognate p-Tyr to that of o-Tyr. The rate of Phe transfer from Phe-AMP to tRNA$^{\text{Phe}}$ was $2.3 \pm 0.3 \text{ s}^{-1}$, and the rate of p-Tyr transfer was $2.1 \pm 0.2 \text{ s}^{-1}$ (Fig. 4.10). o-Tyr transfer from o-Tyr-AMP to tRNA$^{\text{Phe}}$ was undetectable over the same time scale, indicating strong kinetic discrimination against o-Tyr in the transfer step. Notably, Phe and p-Tyr transfer are effectively identical, indicating kinetic discrimination at the transfer step is a mechanism specific to o-Tyr, and not non-cognate p-Tyr.
**Figure 4.10.** *o*-Tyr discrimination is due to inefficient transfer to tRNA<sup>Phe</sup>. Single-turnover aminoacyl-tRNA<sup>Phe</sup> synthesis with *o*-Tyr, *p*-Tyr, and Phe by post-transfer editing defective *S*cytoPheRS is shown. *p*-Tyr is transferred to tRNA<sup>Phe</sup> as efficiently as Phe. *o*-Tyr transfer is undetectable by 2 seconds. Data are presented as the average of three replicates ± SEM.

### 4.4 Discussion

#### 4.4.1 Limitation of *o*-Tyr-tRNA<sup>Phe</sup> synthesis by *S*cytoPheRS

Whereas kinetic discrimination against *o*-Tyr is poor at the activation step (Table 4.1), *o*-Tyr-tRNA<sup>Phe</sup> is not produced efficiently, independent of post-transfer editing activity (Fig. 4.5). Despite a 10-fold higher activity in activation when compared to *p*-Tyr, *o*-Tyr is much less efficiently acylated to tRNA<sup>Phe</sup>. These observations suggest a quality control mechanism distinct from established post-transfer editing activity, which acts to limit *o*-Tyr-tRNA<sup>Phe</sup> production. Analysis of the products of *o*-Tyr-stimulated ATPase activity revealed rapid *o*-Tyr-AMP synthesis (Fig. 4.7), consistent with activation data (Table...
AMP accumulation is minimal when compared to \( o\)-Tyr-AMP synthesis, suggesting that if hydrolysis of \( o\)-Tyr-AMP does contribute to quality control, it is outweighed by the specific rejection of \( o\)-Tyr-AMP as the primary effector of pre-transfer quality control.

\( o\)-Tyr-AMP release may be due to either poor substrate selection in the transfer step resulting in entropic dissociation, or to selective adenylate release in competition with an otherwise rapid transfer step. To determine if selective \( o\)-Tyr-AMP release is due to inefficiency in the transfer step, we measured the rate of \( o\)-Tyr transfer under single-turnover conditions, and found \( o\)-Tyr transfer to be much slower than cognate Phe transfer. Taken together, our data demonstrate that \( o\)-Tyr is efficiently activated, but the resulting \( o\)-Tyr-AMP is a poor substrate for transfer to tRNA\(^{\text{Phe}}\). Much of the \( o\)-Tyr-AMP produced is released from the \( S\)cyt\( o\)PheRS synthetic active site, though low-level transfer does contribute to a minimal degree of aminoacylation of tRNA\(^{\text{Phe}}\) with \( o\)-Tyr (Fig. 4.5). This mechanism for preventing a non-protein amino acid from threatening the product specificity of \( S\)cyt\( o\)PheRS is in addition to the high substrate discrimination against most amino acids in the activation step, as well as the established post-transfer editing activity that limits \( p\)-Tyr-tRNA\(^{\text{Phe}}\) and \( m\)-Tyr-tRNA\(^{\text{Phe}}\) release. Simultaneous pre- and post-transfer editing activity is not unique to \( S\)cyt\( o\)PheRS (144,145). Human cytoplasmic LeuRS, like \( S\)cyt\( o\)PheRS, is threatened with multiple non-protein amino acids, requiring distinct mechanisms of quality control by the same enzyme; whereas norvaline is a target for post-transfer editing activity, \( \alpha\)-aminobutyrate is primarily a substrate for pre-transfer editing by this enzyme (145).
Our findings show that SceytoPheRS bears not only post-transfer editing activity against proteinogenic and non-protein amino acids, but both kinetic discrimination against noncognate amino acids at the activation step and kinetic discrimination against a noncognate aminoacyl-adenylate at the transfer step—specifically one derived from a non-protein amino acid. Non-protein amino acids are an under-examined threat to translational fidelity, due primarily to the only recent advances in chromatographic separation and quantitation of them in the face of large amounts of proteinogenic amino acids with nearly identical biophysical properties in biological samples.

Environmental conditions may drastically alter the intracellular concentration of a given amino acid, skewing the selectivity of the relevant aaRS in vivo. Theoretically, the catalytic efficiency of a given aaRS for any of its substrates may also be context-dependent. However, direct measurement of $k_{cat}/K_M$ for every potential substrate in vivo is problematic, particularly when the added dimension of environmentally dependent catalytic efficiency is included. Accounting for conditional substrate abundance, discrimination against $m$-Tyr is ~240,000 under typical growth conditions, and ~120,000 in the presence of 2.0 mM paraquat, which would normally indicate no further requirement for quality control. However, SceytoPheRS does bear moderate post-transfer editing activity against $m$-Tyr-tRNA$^{Phe}$, suggesting that the requirements for SceytoPheRS product specificity may be higher than the aggregate expected rate of one misacylated aminoacyl-tRNA species in 3,000 aa-tRNAs produced (70). Given that the discrimination against $o$-Tyr is only ~24,000 under typical growth conditions, and ~6,900 in the presence of 2.0 mM paraquat (much lower discrimination than against $m$-Tyr), kinetic discrimination against $o$-Tyr-AMP by SceytoPheRS may play a role in Phe codon
quality control at biologically relevant levels, particularly in conditions that favor oxidation of intracellular Phe. Alternatively, the import of exogenous o-Tyr is a plausible scenario, as structurally similar m-Tyr has been shown to be produced and exported by certain plant species in order to shape the ecology of their environments and inhibit the growth of competitor species (108). Production and export of non-protein amino acids is an effective strategy for chemically shaping a competitive environmental niche, provided the producer has the means to prevent the cytotoxic incorporation of these species (reviewed previously (146)).

Extended exposure of S. cerevisiae to low levels of hydrogen peroxide increases the level of proteins containing o-Tyr and m-Tyr in mutants defective in glutathione metabolism (147), though it is unclear whether this reflects oxidation of protein post-translationally, or oxidation of intracellular Phe and subsequent mistranslation. Recently, monoclonal antibody production in Chinese hamster ovary cells limited in intracellular Phe has been shown to yield protein products with m-Tyr and o-Tyr at Phe codons (136). Interestingly, p-Tyr is not detected at these codons, suggesting that non-protein amino acid derivatives are a unique threat to the mammalian system. This finding is a surprise, because the post-transfer editing activity that limits p-Tyr-tRNA\textsuperscript{Phe} production is apparently insufficient to prevent m-Tyr misincorporation, despite m-Tyr-tRNA\textsuperscript{Phe} editing activity in SccytoPheRS. Further testing with mammalian cytoplasmic PheRS is needed to address this apparent discrepancy.

o-Tyr presents only a minor threat at Phe codons throughout the lifespan of a typical yeast cell, but in long-lived cell-types, such as terminally differentiated neuronal cells, the cumulative effect of protracted low-level o-Tyr incorporation may become a
problem. o-Tyr and m-Tyr have long been used as biomarkers of age and oxidative damage in proteins, and oxidative damage of neuronal protein correlates with neurodegeneration, but it is not yet clear if a causal relationship exists, or what role persistent low-level mistranslation with oxidized amino acids may play in disease states in aging and neurodegenerative disease. *S. cerevisiae* has evolved at least three distinct mechanisms in part to prevent oxidized Phe derivatives being incorporated at Phe codons, despite an apparent minimal need for such stringent quality control under normal conditions. The cumulative effects of long-term low-level mistranslation and oxidative damage merit further study.

### 4.4.2 ScytoPheRS post-transfer editing plays a non-canonical role in tolerance to amino acid stress

In *E. coli*, amino acid starvation is sensed generally by accumulation of uncharged tRNAs that interact at the ribosomal A site, recruiting RelA. Phe starvation can also be detected by sensing elevated uncharged tRNA\(^{Phe}\), a surrogate for low Phe-tRNA\(^{Phe}\) levels. In this manner, uncharged tRNA\(^{Phe}\) can directly upregulate Phe biosynthesis. Without PheRS post-transfer editing, mischarged tRNA\(^{Phe}\) species, including m-Tyr-tRNA\(^{Phe}\) accumulate, masking Phe-tRNA\(^{Phe}\) limitation, and preventing uncharged tRNA\(^{Phe}\) sensing (148). This, in turn, fails to upregulate Phe biosynthesis, further compounding Phe limitation relative to EcPheRS non-cognates such as m-Tyr. The yeast amino acid starvation sensing mechanism relies on general uncharged tRNA sensing via GCN2. GCN2, upon binding uncharged tRNA, phosphorylates eIF2, which causes downstream upregulation of amino acid biosynthesis genes and simultaneous restriction of general
protein synthesis. It is plausible that, as with EcPheRS, a loss of SccytoPheRS editing prevents sensing of Phe starvation due to accumulation of mischarged tRNA\textsuperscript{Phe} species. \textit{S. cerevisiae} rRNAs and tRNAs are cleaved in a conserved response to oxidative stress. The role of tRNA cleavage products is not well understood, but preliminary work suggests tRNA cleavage as an additional mechanism to limit translation (60,149). Further limitation of uncharged tRNA pools under oxidative stress highlights a challenge to \textit{S. cerevisiae} at Phe codons. Not only are Phe pools oxidized, forming additional non-cognates that compete for ScytoPheRS, but a decrease in global tRNA limits the tRNA\textsuperscript{Phe} available for aminoacylation. This may suggest a need for the decreased levels of available tRNA\textsuperscript{Phe} to be charged accurately in this condition of tightly-regulated translation, which underscores the importance for ScytoPheRS product specificity mechanisms under oxidative stress.

A loss of post-transfer editing activity is toxic when yeast are exposed to oxidative stress (Fig. 4.2), but supplementation with specific PheRS non-cognates yields variable toxic effects (Fig. 4.4). Post-transfer editing defective \textit{S. cerevisiae} does not tolerate m-Tyr, p-Tyr, or L-DOPA as well as wild-type, but a reversal of these phenotypes is observed upon supplementation with o-Tyr, cognate Phe, or Ala. If post-transfer editing were only responsible for preventing the supplemented amino acids from misacylation of tRNA\textsuperscript{Phe} and cytotoxic insertion at Phe codons, supplementation with cognate Phe should have no effect, particularly because there is no known phenylalanine hydroxylase in \textit{S. cerevisiae} to convert Phe to non-cognate p-Tyr. Ala, which is not a substrate for PheRS activation, should play no direct role in tRNA\textsuperscript{Phe} charging at all, though the level of metabolic conversion to alternative ScytoPheRS substrates is
unclear. These data suggest an additional role in yeast for post-transfer editing in nutrient sensing, analogous to that observed in *E. coli*. As a result *o*-Tyr, Phe, and Ala addition must have some indirect metabolic effects that alter typical growth.

Given the limited insight that can be gained by phenotypic analysis alone, it is not yet clear what role post-transfer editing is playing in regulation of cellular growth, and the simple answer of conditional mistranslation may not tell the complete story. Particularly, the negative effect cognate Phe and Ala have on wild-type growth rate suggest that mistranslation plays only a minor role here. Future work will focus on the metabolic profile of post-transfer editing defective *ScytoPheRS* yeast to address the role of post-transfer editing in growth under oxidative stress and growth limitation when intracellular amino acid ratios are atypical. In particular, Phe and *p*-Tyr levels, which seem to be the primary drivers of substrate specificity (Fig. 4.3) likely shift in abundance when *S. cerevisiae* is supplemented with various amino acids. Oxidative stress causes accumulation of noncognate species, but the degree to which those species are metabolically converted to alternative *ScytoPheRS* substrates, or to which they interact with alternative metabolic processes, is unclear. Direct measurement of the levels of mischarged and uncharged aminoacyl-tRNA species will also be a critical step in understanding the role of context-dependent tRNA misacylation in the cell. No matter what the mechanism of growth rate feedback is that describes these phenotypic effects, it is clear that the quality control mechanisms in aminoacyl-tRNA synthesis do not have a role in limiting mistranslation alone.
Chapter 5.

Outlook

The substrate specificity of a given enzyme is a mathematical characterization of the relative efficiency in catalyzing a chemical reaction with a given substrate relative to one or more alternative substrates. High substrate specificity, as is typical for CHO TyrRS (Chapter 2) under normal growth conditions, is characterized by the strict limitation of catalysis to one or more substrates. High substrate specificity is achieved by high discrimination against non-cognate substrates in binding, catalysis, or both. In contrast, product specificity refers to the degree of limitation of the products of enzyme catalysis: aaRS product specificity combines substrate specificity in both amino acid and tRNA selection, as well as pre- and post-transfer editing mechanisms. Because aaRSs catalyze two distinct synthetic steps, mechanisms that limit production to a single aminoacyl-adenylate, and a single aa-tRNA species all contribute to overall aaRS product specificity, and an aaRS that readily produces non-cognate aminoacyl-adenylate or aa-tRNA species can be described as having low product specificity. aaRSs have developed multiple mechanisms to limit their product specificities, particularly under conditions
where multiple non-cognate substrates may make strict substrate specificity unusually challenging. *E. coli* PheRS, for example, relies both on discrimination against the proteinogenic amino acid p-Tyr and non-protein amino acids o-Tyr and L-DOPA in the amino acid activation step, as well as post-transfer hydrolytic editing of tRNA<sub>Phe</sub> misacylated with m-Tyr under conditions that favor an abundance of intracellular m-Tyr (Chapter 3). Remarkably, substrate specificity by *S. cerevisiae* PheRS is so poor that o-, m-, and p-Tyr are poorly discriminated against in the amino acid activation step (Chapter 4), and L-DOPA discrimination is moderate. However, these substrates, while biophysically similar, are unique challenges to *S. cerevisiae* PheRS product specificity: post-transfer editing activity is efficient against p-Tyr-tRNA<sub>Phe</sub>, but only moderately so against m-Tyr-tRNA<sub>Phe</sub>. o-Tyr-tRNA<sub>Phe</sub> limitation is uniquely accomplished by discrimination against o-Tyr-AMP in the transfer step. Taken together, at least three mechanisms exist in *S. cerevisiae* PheRS to limit product specificity, particularly against non-protein amino acids that threaten Phe codons under conditions of oxidative stress.

Recent advances in the chromatographic separation of non-protein amino acids and quantitation of intracellular metabolites are promising first steps toward understanding the mechanisms by which aaRSs have evolved mechanisms to limit product specificity. There are likely many non-protein amino acids that threaten the product specificity of several aaRSs in various species and cell types. Moreover, the conditional flux of these and proteinogenic amino acids underscore the unique nature of each aaRS and the challenges a given organism faces in resisting genetic code degeneracy. There are, of course, many more non-protein amino acids than the 22 known proteinogenic amino acids, from the myriad potential oxidized derivatives of
proteinogenic amino acids to intermediates in amino acid metabolism. The mechanisms to limit production of misacylated tRNAs with these non-cognates will certainly vary from species to species. Differences between species in amino acid use by specific aaRSs can be and are being exploited in applications ranging from basic research to antimicrobial therapeutics. Regarding the latter point, it is feasible that a non-protein oxidized Phe derivative would be a relatively harmless additive to bacterial biofuel reactors, which could act to limit yeast contamination by specifically targeting the eukaryotic PheRS. Bacterial PheRS efficiently discriminates against these substrates, but the eukaryotic enzyme must rely on alternative mechanisms to limit product specificity, and indeed is poisoned by them (Chapters 3 and 4).

Given the discovery of multiple non-cognate amino acids that compete for the same aaRS and the diverse mechanisms that limit product specificity, it is no longer sufficient to reduce the frequency of aaRS errors to simple 1:1 cognate:non-cognate substrate specificity comparisons. Loftfield, Fersht, and others calculated the expected frequency of misacylated aa-tRNAs produced to be less than or equal to one in 3,000 aa-tRNAs produced (30,70,71), which is a product specificity claim, and not a substrate specificity claim as it is so often misinterpreted. The expanded form of product specificity that includes terms for multiple non-cognates presented in Chapter 4 is a good place to start, but is also limited, in that it can only be used to predict quality control mechanisms distinct from discrimination against non-cognates in the amino acid activation step.

The predictive value of specificity constants is only as useful as the expected value of product specificity. Unfortunately, the value of one erroneously produced aa-
tRNA per 3,000 aa-tRNAs produced is based on rudimentary measurements of amino acid misincorporation as old as over five decades ago. We now know that the comparison of *C. albicans* CUG codon mistranslation or *M. mobile* mistranslation at Phe and other codons will result in natural, evolutionarily conserved variation that predicts aaRS “errors” at a frequency much higher than one in 3,000 aa-tRNAs produced (36, 38). Moreover, *E. coli* Thr codons are known to be mistranslated more frequently under oxidative stress conditions, due to direct inactivation of a catalytic Cys in its post-transfer editing domain (50). Globally, multiple non-methionyl tRNAs are mismethionylated under oxidative stress in both bacteria and eukarya (39, 40). Nutritional limitation directly impacts the frequency of mistranslation at Tyr codons in mammals (Chapter 2). We now have the means to examine frequencies of mistranslation in a codon-specific manner in multiple cell types, under various environmental conditions, and there will likely be some surprises in the degree of proteinogenic and non-protein amino acid mistranslation in various cell types and environmental conditions.

The unexpected phenotypes we observed in amino acid supplementation experiments suggest an additional non-canonical role for post-transfer editing by *SccytoPhe*RS in the regulation of cell growth (Chapter 4). The observation that cognate Phe and noncognate Ala both confer an apparent advantage in growth in the absence of *SccytoPhe*RS editing is particularly puzzling, and will require further investigation. Ongoing experiments address the intracellular abundance of Phe and noncognates under amino acid stress, as the degree of metabolism of supplemented amino acids to other potential substrates or differences in the import and export of these substrates are unclear. Additionally, the frequency of tRNA$^{\text{Phe}}$ mischarging is an active area of investigation in
our lab, as is the degree to which GCN4-stimulated amino acid biosynthesis is altered under amino acid stress. We also have some preliminary data to suggest that our post-transfer editing-defective ScytoPheRS strain is more mutagenic than the wild-type strain, which may account for variability in tolerance to specific amino acids. We are currently characterizing the mutation rate of these strains. Additional experiments may address the levels of amino acid biosynthesis transcripts under various amino acid supplementation conditions, as well as the long-term viability of these strains after amino acid stress. Future work should expand this analysis to other aaRS and trans-editing factor mutant strains in various species, so that the role of aa-tRNA quality control on the regulation of cell growth under stress may be tested.
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136


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